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REVIEWS AND CASE REPORTS

RESEARCH ARTICLES

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Editors Communique

Stay Protected, Stay Safe in the Cradle of Nature

On behalf of Bioscience Biotechnology Research Communications we falter at words to express our deep sense of solitude and grief on the catastrophic events of the world wide pandemic, spanning over a year now, with no signs of relief. We pray to Almighty to give us the strength to bear this universal calamity and come up with long lasting fortitude to eradicate it soon.

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On behalf of Biosc. Biotech. Res.Comm. its my privilege to thank its reverend readers, contributors, reviewers and well-wishers who have helped it to achieve the distinction of entering the 14th year of successful publication, carving a niche of its own.

Quality publication is one of the ways to keep science alive, and good journals have a leading role to play in shaping science for humanity! As teachers, we have great responsibilities, we have to advocate our students to accomplish and show them the path to test their mettle in hard times to excel, especially in the post COVID 19 era. Science and its advocates will rise to the occasion and will soon provide succor to the already grief stricken humanity.

We have to fuel our science students with a never say die attitude to let humanity survive!

Amicably yours

Sharique A. Ali, PhD
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Managing Atrophic Maxilla Using Ridge-Split Technique: A Review Based Analysis with two Case Reports

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ABSTRACT

Ridge split is one of the techniques utilized to increase bone width before or at the time of implant placement. The paper reviewed the ridge splitting technique since its start, its indications and drawbacks, and the instruments utilized to perform it. The technique can be done in one or two stages depending on initial bone width and cross-sectional form. The aim of this paper is to review the current knowledge about ridge split different techniques with report of 2 cases utilizing those techniques in atrophic maxilla. Two patients with edentulous atrophic maxillae are reported. Ridge split technique was chosen as the treatment modality for dental implant placement. One patient was treated with 2-step ridge splitting approach while the other with simultaneous ridge splitting with implant placement. Patients treated with two-stage and one-stage ridge splitting had their prosthetic delivery after six months of temporization. The survival at that time was 100%. Different techniques of preparing bone for dental implants are well-known. The combination of knowledge and clinical skills are important in deciding the best technique in each clinical scenario. Ridge splitting is one of those techniques that can be used in specific type of clinical presentations.

KEY WORDS: ATROPHIC MAXILLA, BONE GRAFT, DENTAL IMPLANT, PIEZO SURGERY, RIDGE SPLIT.

INTRODUCTION

Jaw atrophy involves a reduction of alveolar height and width together with bone remodeling that affects the external shape and internal bone structure. It occurs chronically and irreversibly following tooth extraction, trauma, infection, pneumatization of the maxillary sinus, or ablative tumor surgery (Ishak and Kadir, 2013). However, the pattern of alveolar ridge atrophy varies between the maxilla and the mandible– the maxilla exhibits centripetal resorption, while the mandible shows centrifugal resorption (Berger et al., 2019).The bearing area available following atrophic maxilla may be inadequate, and this can lead to a lack of prosthesis retention, causing both functional and physiological problems for a patient (Dohiem et al., 2015).Oral rehabilitation in areas where bone width is insufficient is complex.

Insufficient bone width is common in edentulous patients, especially when alveolar fracturing occurs during dental extraction. When the bone loss results from a maxillofacial trauma, vertical dental root fracture, or from extensive periodontal/endodontic diseases, the effects are even more severe. Bone loss might result in insufficient vertical and horizontal support to install dental implants and may impair, or even limit, the options available for prosthetic rehabilitation (Waechter et al., 2017). These problems can be treated for patient satisfaction with an implant-supported fixed or removable complete or partial denture. Atrophic edentulous jaws can represent a significant challenge to the successful use of endosseous implants for prosthetic reconstruction of the edentulous mandible (Eufinger et al., 1997, Tolstunov et al., 2019).

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Several methods have been employed to augment the alveolar crest; for example, guided bone regeneration, bone block grafting, ridge splitting for bone expansion, and distraction osteogenesis. Guided bone regeneration (GBR) using resorbable membranes in combination with particulate autologous bone or a mixture of autologous bone chips and xenogenic bone material, autogenous block onlay grafts harvested intraorally or from the hip, or distraction osteogenesis have been suggested for alveolar ridge augmentation. These treatment options increase the treatment time and costs, have a conspicuous risk of dehiscence and infections and negatively affect patients' morbidity (Bassetti et al., 2016, Starch-Jensen and Becktor, 2019).

Splitting and expanding the edentulous ridge for bone augmentation and implant placement is considered to represent an innovative technique because it avoids the need for a second surgical site, which further reduces the ailment of the patient (Kumar et al., 2016). In 1986, Nentwig reported a bone crest division technique that simultaneously allowed the expansion of the alveolar crest and implant insertion (Nentwig, 1986, Li et al., 2020). Later in the early nineties, Simion et al. aimed to create a ''self-space making defect'' by splitting the atrophic crests into two parts with a longitudinal greenstick fracture displacing the vestibular cortical bone both in the maxilla or mandible to create a gap into which the implants were subsequently inserted (Simion et al., 1992, Li et al., 2020).

The split ridge technique (SRT) is recommended when the ridge width is insufficient, but the alveolar height is acceptable. However, in ridges with low elasticity, trabecular bone volume is compromised, and bone expansion will be less predictable. This can undermine the success of the technique (Mechery et al., 2015, Waechter et al., 2017). A recent systematic review suggested several anatomical requirements are necessary for SRT: 2-3 mm minimally of ridge width, minimum bone height of 10 mm, presence of type III or IV of bone, absence of concavities in bone profile, and 1 mm between adjacent teeth in case of partial edentulism (Bassetti et al., 2016). Ridge width is an essential factor in planning a suitable approach for bone augmentation in isolation or in combination with dental implant placement. An updated decision tree on horizontal bone augmentation suggested the use of the ridge split option when the width of the ridge was a minimum of 3.5mm (Mechery et al., 2015).

This amount of bone is essential to allow the splitting of alveolar bone into three layers of bone: Two cortical plates (buccal and palatal/lingual plates) and one layer of cancellous bone to allow ridge expansion (Tolstunov and Hicke, 2013). However, the bone morphology can have a direct impact on the suitability of the ridge split technique within a given case; the presence of bone concavities, a narrow base of less than 3 mm, and hour-glass shape ridges are factors that should be carefully examined before attempting ridge splitting (Elnayef et al., 2015, Tolstunov et al., 2019). The technique is considered relatively fast as healing occurs in the same way as that observed with bone fractures; by rapid vascularization and remodeling of bone (Kumar et al., 2016, Tolstunov et al., 2019).

To avoid major complications, including plate fracture, several factors must be carefully assessed before planning a ridge split. Cortical plate fracture results from poor case selection in the presence of a thick cortex (Li et al., 2017). In the case of a plate fracture, it is important to avoid dislodgment of the fragmented bone as this might result in bone necrosis and, subsequently, more severe ridge defects due to problems in perfusion and remodeling resorption (Teng et al., 2014, Dohiem et al., 2015, Li et al., 2017). Another issue concerns the risk of buccal exposure of dental implant or osseointegration (Teng et al., 2014, Berger et al., 2019), which can be avoided by maintaining at least 1.5 mm of bone buccally (Spray et al., 2000, Teng et al., 2014, Berger et al., 2019).

If the procedure fails, a massive bone loss will occur and complicate the treatment (Arora and Kumar, 2015) which make it often perceived to be inferior to other augmentation techniques (Kaneko et al., 2013). In some cases, implant stability might be low; however, choosing a tapered implant will increase the primary stability and also decrease the incidence of fracture (Elnayef et al., 2015, Yao et al., 2018). However, implant stability is considered to represent a superior approach as new bone is formed between the two cortical plates (Arora and Kumar, 2015, Berger et al., 2019).The ridge splitting technique is considered an advantageous procedure that eliminates the need for further surgery as it allows simultaneous implant placement. As such, it reduces treatment time and morbidity (Arora and Kumar, 2015, Anitua and Alkhraisat, 2016, Bassetti et al., 2016, Yao et al., 2018, Li et al., 2020). Furthermore, postoperative, swelling and pain are lower with this approach than it is with alternative augmentation techniques (Kumar et al., 2016, Altiparmak et al., 2017).

Ridge Split Technique: The ridge split technique was initially described as a one-stage ridge split in which implants are placed, followed immediately by ridge splitting (Nentwig, 1986). It offers lower morbidity, cost, and time of treatment and is advantageous in terms of bone healing (Bassetti et al., 2016, Li et al., 2020). However, it is important to carefully evaluate the density and width of the bone to achieve an acceptable implant primary stability (30Ncm or more) during one-stage ridge splitting (Demetriades et al., 2011, Zhang and Huang, 2020). In 2013, the Osborn technique was introduced, which involves performing the ridge splitting process over two stages. During the first stage, the inter-cortical area is filled with autogenous bone or bone substitutes, while the implants are placed eight-to-twelve weeks later in a second procedure (Gonza 'lez-Garc 'ıa et al., 2011, Li et al., 2020).

A two-stage approach may be used when the ridge is narrower than 3 mm, as this is associated with an

increased risk of buccal plate fracture, or when the implant stability is questionable (Anitua and Alkhraisat, 2016, Kumar et al., 2016). The presence of a bone graft increases the vascularization during implant bed preparation and protects against compromised implant placement angulations (Cha et al., 2014, Arora and Kumar, 2015). Demetriades and his group analyzed the difference between the two approaches and found that osseointegration did not vary between the one- and two-stage processes; however, there were fewer postoperative complications in patients who underwent the two-stage ridge splitting process (Demetriades et al., 2011, Li et al., 2020).

Traditionally, flap reflection in ridge splitting was advocated to be a full-thickness flap as excessive bleeding can be avoided, making handling and visualization more straightforward (Agrawal et al., 2014, Tolstunov et al., 2019). A partial-thickness flap was suggested to preserve blood supply, which helps to protect the bone from excessive loss (Scipioni et al., 1994, Elnayef et al., 2015, Starch-Jensen and Becktor, 2019).In terms of two-stage SRT, Dohiem et al. (2015), explored the concept of using a full-thickness flap in the first stage to enable better control during the surgical steps, and a partial thickness in the second stage during implant placement to protect from further bone loss.

Osseous ridge splitting can be performed using a variety of instruments, both manually and motor-driven. The use of manual instruments, like blade No. 15, razor-sharp chisel, and beaver blade, is challenging when dealing with cortical bone and attempting to cut small amounts. However, they can provide a good control (Kumar et al., 2016, Li et al., 2017, Li et al., 2020). The use of either round burs or diamond disks can help to remove the bulk of the bone: however, these instruments generate heat, which might affect bone healing or lead to bone necrosis (Kumar et al., 2016, Li et al., 2017, Li et al., 2020). New modalities have been proposed and used in SRT-like laser (erbium: yttrium–aluminum- garnet, erbium, chromium-doped: yttrium-scandium-gallium-garnet), micro saw devices, and piezoelectric devices (Vercellotti, 2000, Zhang and Huang, 2020).

Piezoelectric devices are fast, safer and more accurate than other modalities. These devices work at a 25-30kHz frequency that makes it possible to control the splitting of bone close to vital structures as mental foramen and maxillary sinus (Kumar et al., 2016). Also, the oscillating frequency makes it possible for practitioners to perform a selective and less invasive cut, while the micromovement cuts bone but not soft tissue (Agrawal et al., 2014, Li et al., 2017). Unlike motor-driven and micro saw devices, the piezosurgical saw does not produce heat, which reduces the probability of postoperative bone necrosis (Crespi et al., 2014, Kumar et al., 2016, Li et al., 2020).

Practitioners have traditionally created the split in the bone before expanding it using a hand mallet. However, in more recent times, piezoelectric devices have been used. The piezo-surgical system helps to overcome the risk of displacement of bone fragments and vertigo (benign paroxysmal positional vertigo -BPPV-) associated with hand mallet percussions. As such, the piezo-surgical system can help to reduce the discomfort of patients (Crespi et al., 2014, Kheur et al., 2014, Li et al., 2020).

The ridge split technique is usually carried out in the maxilla, where the bone is more spongier and the cortical plates are relatively thin compared to the mandible (Kumar et al., 2016). The majority of the published cases that describe the use of SRT involved the replacement of a single tooth or multiple teeth in the arch in the maxillary and mandibular arches. The next section presents two case reports that describe full edentulous maxillary arches restoration using the ridge-splitting technique.

Case Presentation: Two edentulous patients attended the dental college of King Saud University. After an assessment of the patients' medical and dental conditions, the split ridge technique was chosen to place dental implants in the upper jaw. Consent forms were signed to proceed with the planned treatment.

Case I: A 47-year-old Moroccan female visited the Dental University Hospital (DUH) at King Saud University, Riyadh, SA. The patient was unaware of any medical condition and was seeking a fixed replacement for edentulism. The patient had been referred from the prosthodontic. After a clinical examination of the hard and soft tissue quality, a radiographic stent was constructed, and CBCT scan was performed (Figure 1). The CBCT scans showed a relatively good height (11-14 mm) in the anterior upper, pneumatized sinuses with 5 mm of bone, and a lower arch of acceptable length and width (Figure 2).

Figure 1: Initial clinical and radiographic presentation

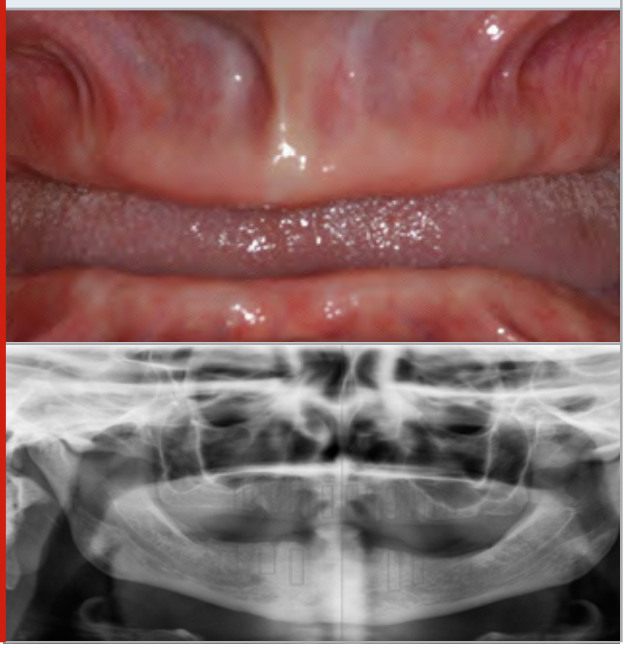
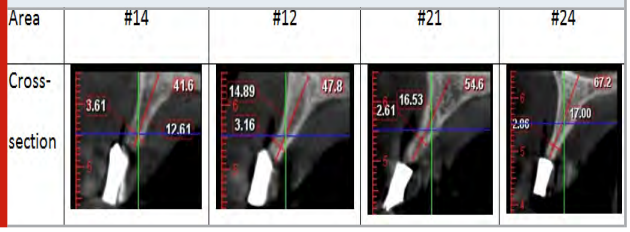
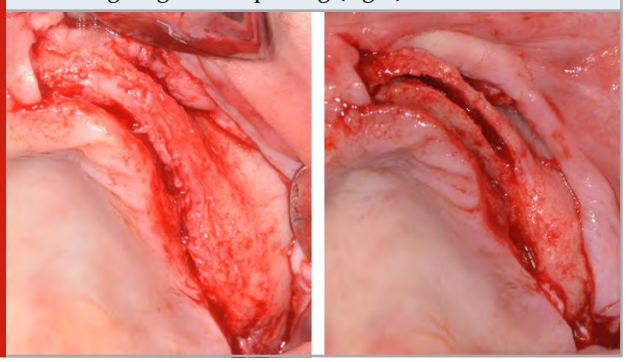


Figure 2: Cone-beam CT scans (CBCT) showing the narrow width of planned implants in the upper arch at upper first premolars and incisors



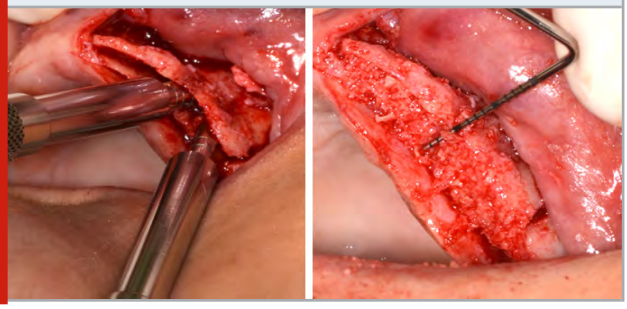
A treatment plan was discussed with the patient who signed the consent for upper-arch two-stage ridge splitting and a conventional implant placement in the lower jaw.One hour prior to surgery, the patient was given 1 g amoxicillin. Post-surgery, she was prescribed 1 g two times per day for a period of seven days. Surgery was performed under local anesthesia (Lidocaine 20 mg/mL with adrenaline 1:80,000).On the day of the surgery, a crestal incision was made, and a full-thickness flap was raised (Figure 3). A longitudinal mid-crestal osteotomy was performed using the piezosurgical saw in a side-to-side cutting motion. The depth of the first cut was 8-10 mm in relation to the anterior and premolar areas (Figure 3).

Figure 3: Full thickness flap reflection (left), Arrows indicating ridge after splitting (right).



Two vertical bone incisions were made: one at the mesial and other at the distal aspect. The osteotomy site was expanded using expansion osteotomes until it was 6-7 mm wide (Figure 4).

Figure 4: Expansion of the osteotomy (left), and filling the site with allograft (right)



The site was grafted with allogenic cortical particulates allograft and covered with a resorbable membrane (Biomend Extend, Zimmer).After achieving primary closure, the site was sutured with horizontal mattress and interrupted sutures using 3-0 silk suture material. Healing was uneventful and within normal limits. The same surgical technique was performed on the other side.Unfortunately, the patient did not attend follow-up appointments for a couple of months due to family issues, but later returned to the clinic seeking completion of the treatment. A new CBCT scan was taken of the upper arch (Figure 5). The gain of the bone after the first split-ridge procedure was (1-2 mm).

Figure 5: cross sections of the new CBCT for the planned areas to place dental implants

The new plan consisted of a one-stage ridge split with simultaneous implants placement. The same surgical protocol was followed as that performed during the first procedure. However, the implant was also placed (Figure 6).

Figure 6: Implant placement simultaneously with ridge splitting

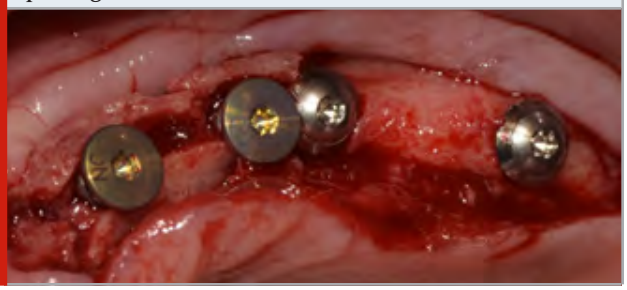
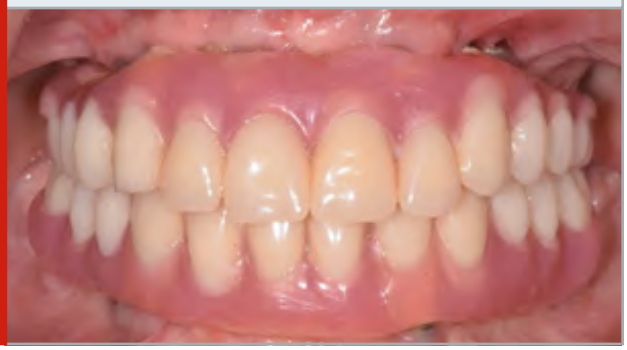


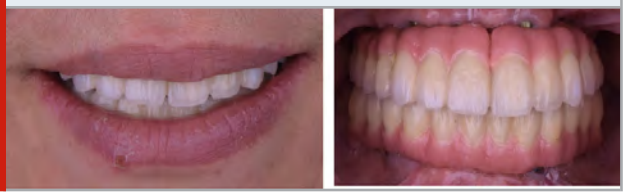
Figure 7: Upper and lower temporary dentures



The patient was given a temporary denture until complete healing and maturation were observed (Figure 7). A

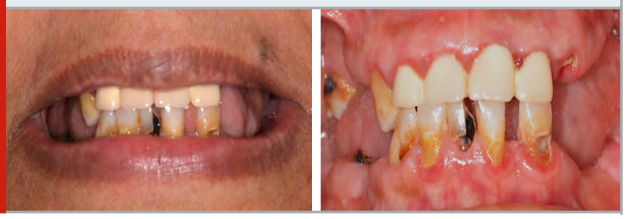
screw-retained final prosthesis was delivered to the patient six months later (Figure 8).

Figure 8: Final prosthetic treatment in intra and extra oral views



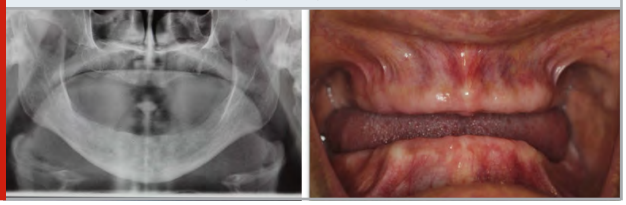
Case II: A 52-year-old Saudi female visited the dental clinic at the Dental University Hospital seeking treatment for her missing teeth and was referred for implant placement (Fig9). Consent forms were signed to proceed with the planned treatment.

Figure 9: Initial extra and intra oral status



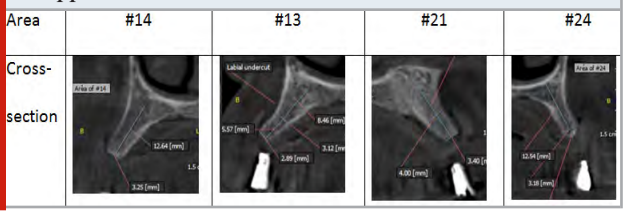
A full mouth extraction was done in addition to soft tissue grafting (free gingival graft) for lower right and left sides (Fig10).

Figure 10: Clinical and radiographic appearance after extraction and tissue grafting



The CBCT was taken after the construction of complete dentures, and some measures are illustrated in Figure 11. The treatment plan consisted of the placement of eight implants in the upper arch with simultaneous ridge splitting from Area of #14 to Area of #26.

Figure 11: Cross sections for some implants planned areas as appeared in CBCT



The same pre-operative medications were given to the patient as those administered to the patient in the first case. Local anesthesia was administered (Lidocaine 20 mg/mL with adrenaline 1:80,000), and a full-thickness flap was reflected before ridge splitting was performed using piezosurgery (Figure 12).

Figure 12: Full-thickness flap reflection (left) and splitting of the ridge using piezosurgery tip (right) .Arrow pointing to the splitting of the ridge

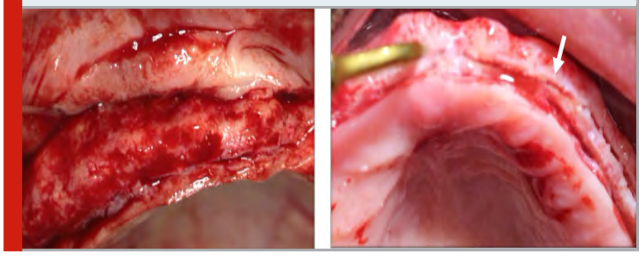
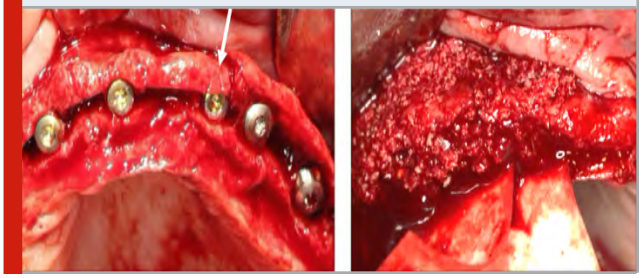


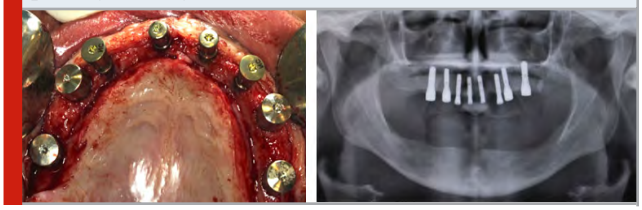
Figure 13: (left) implants placed after ridge expansion. Note the vertical fracture between #23 and24 implant fixtures(arrow). (Right) particulate bone graft and membrane were applied over the implants and secured.



After ridge expansion, 3.3*10 mm Straumann implants were placed in the areas planned for the surgical stent. However, a vertical fracture occurred between Implant 23 and Implant 24 implants (Fig13). Cancellous particulate bone graft was packed and covered with a collagen resorbable membrane (Figure 13).

After 6 months, implant exposure was carried on placing healing abutments (Fig14) and final prosthesis was delivered after appropriate healing time.

Figure 14: Clinical and radiographic presentation after placing healing abutments



DISCUSSION

Different techniques have been developed to provide patients with high-quality dentition replacement. Dental implants are considered the ideal treatment modality in most clinical scenarios. Ridge splitting was developed to utilize existing bone to expand the ridge width dimensions and, thereby, aid implant placement. Since

this procedure was first introduced in the 1970s by Tatum, many instruments have been developed for use with the ridge-splitting technique; for example, peizosaws and ridge-splitting osteotomes (Mechery et al., 2015, Li et al., 2020). In the 1990s, Summers and Scipiono et al. published data in which the five-year survival rate reached 98% (Scipioni et al., 1994, Summers, 1994). At that time, the criteria for case selection were more definite for the bone type to be more trabecular (D3 or D4) with proper vertical bone quantity.

In this paper, two patients' clinical and radiographic findings mandate increase in bone thickness prior to dental implant placement. The decision of bone grafting technique was determined according to bone height and width present. The two patients' dental condition as shown from CBCT sections were high enough to place a ten-millimeter ling implant but the width was compromised. Yet, the bone density and the bone form ,narrow crest and wide base, were key factors in selection of ridge splitting technique(Tolstunov et al., 2019, Zhang and Huang, 2020).

Moreover, a recent systematic review concluded the efficacy of bone splitting with high implant survival(Starch-Jensen and Becktor, 2019). In the first clinical case, ridge splitting was done in two-stage technique. The decision was based on the bone width presented initially as 2-3 mm, which in turns, mandate another stage with wider bone volume (Li et al., 2020). In the second presented case, there was an evidence of bone fracture while placing the implants. This fracture as shown by Yao et al. (2018), aid in decreasing the tension on the buccal bone in anterior maxilla. Improving the surgical operations with digitalized techniques will decrease the possible complications of exposing the bone and jeopardizing the blood supply, yet the accuracy of these approaches is sometimes questioned.

CONCLUSION

The fast and non-invasive nature of ridge splitting, and the superior bone healing observed after the application of this technique entails that it represents a preferred solution when the bone height and quality are adequate to allow the safe separation of the plates. Careful planning and utilization of instruments when splitting and expanding the ridge can provide a high standard treatment for function and esthetics with low morbidity and a short treatment time.

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Not applicable

Competing interests: Authors declare that they have no competing interests.

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Evaluation of Basic Medical Curriculum Integration Based on the Training of Chinese Excellent Category Doctors

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ABSTRACT

The traditional discipline-centered teaching mode is no longer adapt to the changes of current medical modes and the social demand for medical services owing to disjointed from clinical practice, knowledge separation and lack of connection between basic medical courses and clinical medical courses. Therefore, China has implemented the National Excellent Doctor Training plan and vigorously promoted the integrated reform of clinical medicine curriculum. In this study we compared the change of teaching effect between reform class and control class. We randomly selected 1 class from the 5-year clinical medicine major to carry out a series of pilot teaching reforms with curriculum integration, and at the same time, 1 class was selected for parallel control. Then the effect of the reform was evaluated from the aspects of test scores and 6-STATION OSCE. Student achievement and clinical skills are effectively improved through the integration of basic medical courses. The results showed that student achievement and clinical skills are effectively improved through the integration of basic medical courses It is concluded that we further need to integrate the various foundational and clinical disciplines into an organ-system based curriculum for the National Excellent Doctor Training plan.

KEY WORDS: CURRICULUM INTEGRATION, BASIC MEDICAL CURRICULUM, MEDICAL EDUCATION, DISCIPLINE-CENTERED TEACHING MODE.

INTRODUCTION

Under Flexner's influence, medical curricula around the world came to be structured into: Preclinical medicine learned in lecture theatres, laboratories, dissecting rooms, libraries and Clinical medicine learned in wards and operating theatres of teaching hospitals. Since the 1950s, medical colleges in Europe and The United States have proposed and implemented the teaching reform featuring the integration of medical curriculum. Curriculum integration involves the organization of teaching to interrelate or unify subjects frequently taught in separate academic courses or departments (Harden, et al. 1984, Scheffer, et al. 2012 Seethe and Khan 2019).

Most of the medical colleges in China are following the traditional system that is teacher centered, discipline based and opportunistic. With the development of global medical education and interdisciplinary integration, the model of Chinese medical education has also changed in the past decade. There were some defects in the traditional medical education pattern such as overlapping content of teaching, more time span, students learning burden, and comprehensive ability between various disciplines. Integration is an important means of dealing with overload of information, fragmented teaching of basic and clinical sciences, and the need for relevant and meaningful learning (Yamani and Rahimi 2016).

In this study, we have analyzed the problems in the process of integrated medical foundation course, and then really

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broke the barriers between disciplines, and integrated the systematic anatomy, histology and embryology, physiology, pathology, pathophysiology, pharmacology to "Two Introduction and Multiple Systems". We put forward the training of clinical ability, which is helpful to realize the training of students' clinical in the whole process of medical education. It is of great significance to realize the educational goal of "early clinical, multiple clinical and repeated clinical" and improve the training quality of medical talents.

MATERIAL AND METHODS

This study was granted an exemption by our Institutional Review Board since it evaluated outcomes of an existing mandatory component of the curriculum. We made an analysis the current research about the integration of basic medical courses based on published literature, and then carry out empirical research on medical colleges and universities that have integrated their courses, so as to provide scientific theoretical guidance and reference for the subject research.

Description of course integration: Six basic medical courses including *systematic anatomy, histology* and *embryology, physiology, pathology, pathophysiology* and *pharmacology* were selected as the subjects of course integration. The knowledge content of subject was decomposed into "knowledge points" to form "granulated resources". Guided by the cultivation of clinical thinking ability, the "granulated resources" will be centered on "organ-system", and the systematic teaching content will be reintegrated and reconstructed to form a modular curriculum system of "Two Introduction and Multiple Systems". Two Introduction is an introduction to human body form and function and the Multiple Systems are the respiratory system, circulatory system, digestive system, urinary system, blood system, nerve system, endocrine system, sensory system and reproductive system. According to the relevant requirements and procedures of the curriculum standards, the curriculum standards of the integrated curriculum were formulated. Then we organized the research team to compile "Introduction to human body morphology and mechanics" and 9 "organ-system" modules as case textbooks.

Teaching implementation of basic medical curriculum integration: In the five-year clinical medicine class of 2015, a 36-person pilot class for teaching reform was established based on the principle of mutual selection between the two sides, and other classes of the clinical medicine undergraduate course were taken as the control group. The pilot class of educational reform was organized for teaching according to the integrated teaching contents of basic medical courses, while the control group was taught according to the current talent training program. The teaching reform pilot class was taught based on integration of "teaching of early clinical probation" and the integrated course of basic medicine to form the idea of early clinical probation.

The process of "setting questions, seeking answer and basic clinical combination" was designed and directly applied to teaching. The teaching mode combining case-based teaching and PBL teaching was adopted to carry out teaching based on suitable early internship cases and combined with PBL or CBL teaching. The goal of the implementation of special teaching was to cultivate students' clinical thinking ability and improve students' ability to solve practical problems. The teaching methods of the control group were carried out according to the discipline-centered methods.

Evaluation of teaching effect: The teaching reform pilot class adopts the method of formative evaluation, summative evaluation and comprehensive evaluation. In the teaching process, the formative evaluation was highlighted, and the existing problems in the learning process are fed back to the students in time. The formative evaluation runs through the whole teaching activity. After the teaching activity of each course is finished, the summative evaluation based on comprehensive and case questions was adopted. After the learning of all the integrated courses of basic medicine, the comprehensive evaluation of learning effect was carried out by means of the basic stage assessment of simulated clinical practitioners. In addition to formative evaluation, summative evaluation and comprehensive evaluation were carried out in both the teaching reform class and the control class. The summative evaluation and assessment contents are generally consistent, but the teaching reform class was assessed according to the integrated curriculum, and the control class was assessed according to the unintegrated curriculum. Comprehensive evaluations were conducted in the same manner.

OSCE setting: A comprehensive 6-station OSCE was administered to the teaching reform and control class of five-year clinical medicine class of 2015. The examination was conducted after the clinical practice. The assessment of clinical skills includes the following aspects: Patient care skills, Interpersonal and communication skills, Professionalism skills, Practice-based learning and improvement skills, Systems-based practice skills and Medical knowledge skills; The OSCE consisted of 6 clinical problems; each clinical problem consists of six core competencies defined by the Accreditation council for Graduate Medical Education (ACGME) (Yang, et al. 2011).

Standardized patients should be used as a reference in the specific assessment. At each station, the summary scores were the sum of all the checklist items, and the six core competency sub scores were the sum of specific items for each competency. When presented, all scores were translated into 100 percentages. Please refer to the article of Yang et al. for more details (Yang, et al 2011).

Statistical analysis: All data were processed by SPSS 18.0 (SPSS Inc., Chicago, IL, USA). All data were presented as mean ± standard deviation. Comparison between groups was conducted using single-factor ANOVA followed by

Tukey's test. P<0.05 indicated significance, and P<0.01 indicated extreme significance.

RESULTS AND DISCUSSION

We have a teaching reform pilot class size of 36 students per year and control class size of 47 students. The students of teaching reform pilot class studied "Two Introduction and Multiple Systems" and the students of control class studied Six basic medical courses. Biochemistry and molecular biology as a comparative analysis course are taught in every class (see Table 1).

Average score (75.25) not including Biochemistry and molecular biology in teaching reform pilot class was higher than that (71.63) in control class. And however, *Biochemistry* and molecular biology was lower than that in control class. This results showed our teaching reform increased students' score. Next, the score of teaching reform pilot class and control class was further analyzed based on *Biochemistry* and *molecular biology* as a comparative analysis course are taught by same teacher. The calculating method is Relative performance= (Teaching reform subject $_{\text{Average score}}$/ *Biochemistry* and *molecular biology* score) / (Traditional subject $_{\text{Average score}}$/ *Biochemistry* and *molecular biology* score). The results from Figure 1 showed that relative performance in teaching reform pilot class was higher than that in control class.

Analysis of OSCE: In Figure 2, a significant difference in the performance between different aspects of core competency ($p < 0.05$) was noted. Teaching reform pilot class had the higher pass rate in the aspect of Practice-based learning and improvement skills (83%), Systems-based practice skills (75%) and Medical knowledge skills (91%), whereas the lower pass rate was noted in the aspect of professionalism (52%). Interestingly, teaching reform pilot class had the higher pass rate in the OSCE than that of control class.

Table 1. Average score in teaching reform pilot class and control class

Class	Students number	Course	Semester	Average score
Teaching reform pilot class	36	Introduction to Human Morphology	1,2	71.3
		Introduction to human mechanics	2	67.47
		Respiratory system	3	77
		Digestive system	3, 4	81.72
		Circulatory system	3	68.25
		Blood system	4	76.72
		Urinary system	4	79.94
		Sensory system	4	79.83
		Nerve system	5	73.19
		Endocrine system	5	75.25
		Reproductive system	5	77.11
		Biochemistry and molecular biology	2	71.8
Control Class	47	Systematic anatomy	2	51.98
		Histology and embryology	3	76.36
		Physiology	3	73.09
		Pathology	5	78.47
		Pathophysiology	5	72.3
		Pharmacology	5	77.55
		Biochemistry and molecular biology	4	77.91

In 1989, Shoemaker proposed a concept about integrated curriculum that is "Education that is organized in such a way that it cuts across subject matter lines, bringing together various aspects of the curriculum into meaningful association to focus upon broad areas of study (Betty 1989)." To this day, there is an ongoing discussion about whether medical curriculum should be discipline based or integrated. Abraham Flexner thought that students should first learn basic and biomedical sciences and then move to clinical sciences; however, a common criticism of this approach was that students would not see the relevance of basic and biomedical sciences applied to clinical practice, and it was preferable to encourage students to think as doctors from the day they enter medical school (Harden 1986).

Integration of medical curriculum was importance for medical education because basic science learning was placed in the context of clinical and professional practice and was considered by students to be more meaningful and relevant (Quintero, et al. 2016). After a discussion of the health-illness concept, we constructed a theoretical basis of this process that changed our traditional discipline-based learning perspective. The meaning of the health-illness process changed was defined as a social, cultural, biological, and psychological process

embedded and determined socially and culturally by group of human beings (Fanwei, et al. 2019).

Figure 1: Relative performance in teaching reform pilot class and control class

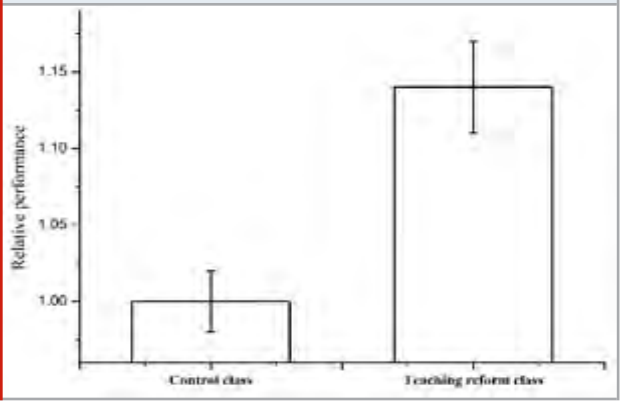
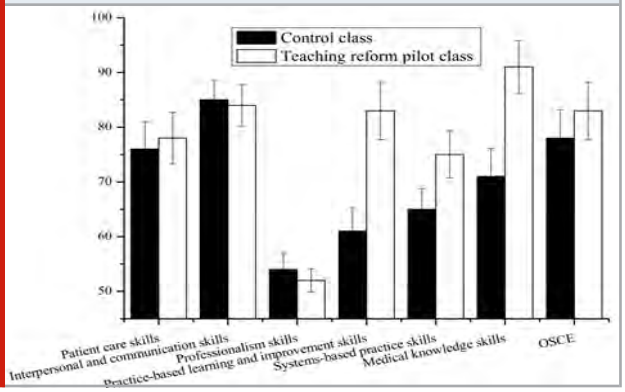


Figure 2: Overall pass rate (%) of each domain of ACGME competency and overall OSCE



As this approach implies that society and culture are no longer simply risk or etiological factors, our medical curriculum had to evolve into a new structure based on a "Two Introduction and Multiple Systems" concept of health and illness.In this study we randomly selected 1 class from the 5-year clinical medicine major to carry out a series of pilot teaching reforms with curriculum integration, and at the same time, 1 class was selected for parallel control. Then the effect of the reform was evaluated from the aspects of test scores and 6-STATION OSCE. The results showed that student achievement and clinical skills are effectively improved through the integration of basic medical courses.

CONCLUSION

The results demonstrated that student achievement and clinical skills are effectively improved through the integration of basic medical courses. We need further to integrate the various foundational and clinical disciplines into an organ-system based curriculum for a better National Excellent Doctor Training plan (China).

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Prevalence of Multidrug-Resistant Gram-Negative Bacteria in Saudi Arabia: Meta Review

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ABSTRACT

Antibiotic resistance bacteria developed abilities to resist antibiotics designed to kill them and mainly spread in hospitals compared to community. One of the biggest risks is getting an antibiotic-resistant infection from healthcare facility such as a hospital where patients are exposed to antibiotics. Moreover, resistant bacteria are more difficult to treat speciallyin immunocompromised patients Prevention of the spread of resistant bacteria can be done by recommended practices for identifying these bacteria, cleaning hands, wearing gowns and gloves, and cleaning medical equipment in addition to patient care areas. This article reviews the relevant knowledge of the epidemiology and molecular characteristics of resistant bacteria in Saudi Arabia. Multidrug-resistant Gram-negative (MDR-GN) bacteria are serious threats to public health especially extended-spectrum β-lactamase *Klebsiella pneumoniae, Acinetobacter baumannii,* and *Pseudomonas aeruginosa* which increased morbidity and mortality in hospitals. These pathogens raise serious concern in both hospitals and community settings and have become endemic in many tertiary hospitals and health care units worldwide. Moreover, the emergence and rapid spread of MDR-GN bacteria in hospitals have a significant impact on treatment outcomes and pose challenges to health care systems and medical care cost and effectiveness.

KEY WORDS: ANTIBIOTICS, RESISTANCE, K. PNEUMONIAE, A. BAUMANNII, P. AERUGINOSA.

INTRODUCTION

Multidrug-resistant Gram-negative bacteria (MDR-GN) are among the most serious threat to public health, due to their resistance to nearly all available antibiotics (Ventola, 2015; Exner et al., 2017; Alagna et al., 2020; Nijsingh et al., 2020). The Infectious Diseases Society of America (IDSA) has identified four Gram-negative pathogens of particular importance, extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae (E. coli)*, *Klebsiella pneumoniae, Acinetobacter baumannii*, and *Pseudomonas aeruginosa* (Bassetti et al., 2016; Fodor et al., 2020; Morris and Cerceo, 2020). Also, treatment options for these Gram-negative pathogens are rapidly declining, which leads to significant increases in morbidity and mortality (Karaiskos et al., 2019). These pathogens raise serious concern in both hospitals and community settings and have become endemic in many tertiary hospitals and health care units worldwide (Peleg and Hooper, 2010; Gray and Mahida, 2016). Moreover, the emergence and rapid spread of (MDR-GN) in hospitals pose challenges to health care systems, medical care cost and effectiveness (Santajit and Indrawattana, 2016; Serra-Burriel et al., 2020).

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Multidrug-resistant Gram-negative bacteria have been detected in Saudi Arabia since the 1990s. Many published studies from Saudi Arabia have focused on the molecular epidemiology of these pathogens (Zowawi et al., 2014; Zowawi, 2016). Several studies from different regions in Saudi Arabia have reported increasing carbapenem resistance among MDR-GN bacteria (Yezli et al., 2014, Faidah et al., 2017). Carbapenem-resistant *Acinetobacter baumannii* is the most common pathogens associated with nosocomial infection followed by *Pseudomonas aeruginosa*. Recently, the rate of carbapenem-resistant Enterobacteriaceae has been increasing (Alotaibi et al., 2017). The four Gram-negative pathogens identified by IDSA are the most frequent in KSA hospitals (Zowawi et al., 2014, Zowawi, 2016, Khan et al., 2018). This article reviews the relevant knowledge of the epidemiology and molecular characteristics of the four MDR-GN pathogens, extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae, Klebsiella pneumoniae, Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, in Saudi Arabia.

Multidrug-resistant gram-negative bacteria: A systematic search was conducted in specific online databases, including PubMed, Google Scholar, and Science Direct. The search strategy was focused on publications from the 2015 to 2020. Therefore, we used English key terms related to Multidrug-resistant gram-negative bacteria, molecular epidemiology, and antibiotic resistance. Different forms of the main terms were included in our search for example, MGN- extended Spectrum β Lactamase (ESBL), carbapenem resistant Enterobacteriaceae (CRE). The names of the four MDR-GN pathogens: *E. coli, K. pneumoniae, P. aeruginosa, A. baumannii* were also included. Since we are targeting studies about the Multidrug-resistant gram-negative bacteria in Saudi Arabia, the official name of Saudi Arabia, "Kingdom of Saudi Arabia" or "KSA", was included in the list of the key searching terms.

A total of 80 studies were selected for this review within the time window of the five-year. Most of these studies (23%) were published in 2019. The retrieved results for this review were classified based on the four MDR-GN pathogens. Each subsection starts with a brief background of MDR-GN pathogens under the study. The reported findings of the antimicrobial resistance rates and the resistant genes presented in each MDR-GN bacteria from all the studies collected in this review were summered. The retrieved results for this review were classified based on the four MDR-GN pathogens. Each subsection starts with a brief background of MDR-GN pathogens under the study. The reported findings of the antimicrobial resistance rates and the resistant genes presented in each MDR-GN bacteria from all the studies collected in this review were summered.

Multidrug-resistant Klebsiella pneumoniae: Over the years, *K. pneumoniae* has become an important opportunistic pathogen, that belong to the *Enterobacteriaceae* family, and a member of ESKAPE pathogens. Three to eight percent of hospital-acquired bacterial infections are related to *K. pneumonia* (Ashurst and Dawson, 2019). It is responsible for several diseases such as urinary tract infections, cystitis, pneumoniae, surgical wound infections and septicemia. *K. pneumoniae* demonstrated a significant resistance to antimicrobial groups such as β-lactam antibiotics, Cephalosporin's, aminoglycosides, fluoroquinolones, and Carbapenems (Dsouza et al., 2017). The emergence of *K. pneumoniae* strains resistant to broad-spectrum antimicrobial agents are a serious threats to the public health due to the limited treatment options (Navon-Venezia et al., 2017).

Numerous studies reported the prevalence of MDR-*K. pneumoniae* in Saudi hospital settings. In Riyadh Medical City, out of 227 of *Enterobacteriaceae* isolates 60% were MDR pathogens. *K. pneumoniae* accounted for 33% of infections. 51.4% of the total isolates were ESBL producers and10.1% were Carbapenemase-producing *Enterobacteriaceae* (Alkofide et al., 2020). At King Fahad Medical City at Riyadh, the most identified isolates were *K. pneumoniae* (47.4%) and E. coli (31.6%) (Alzomor et al., 2019). Another study by Bandy and Almaeen, (2020) was conducted in two specialist hospitals in Aljouf region, 222 non-duplicates Blood stream infections (BSI) samples from hospitalized patients, 62.2% were caused by gram-negative bacteria. *K. pneumoniae* was the most frequent (28.4%) pathogen. Moreover, 46% of *K. pneumoniae* isolates were carbapenemase producers and 52.2% of *E. coli* isolates were ESBL producers.

The prevalence of Carbapenem-resistant *K. pneumoniae* was 92.8%, followed by E. coli in 6.7%, and Enterobacter in 0.6%. In KAUH the percentage of CRE increased from 8% in 2017 to 13% in 2018. While in KAMC, the percentage was much higher throughout this study 43.2% in 2018 and 39% in 2019 (Taha et al.,2020). Another study performed by Ghanem et al.(2017) at King Fahd Hospital in Madinah, showed that *K. pneumoniae* species 100% resistance to Ampicillin. Among 15708 *K. pneumonia* isolates collected from 1149 patients at King Fahad Hospital in Medina, resistance rate was 38.4% for imipenem and 46.1% for meropenem, as well as high resistance rates for 40.7% and 53.3% for colistin and tigecycline, respectively (Al-Zalabani et al.,2020).

In Abha, a study conducted that *K. pneumoniae* isolates were highly resistant against ciprofloxacin, piperacillin-tazobactam, ceftazidime, cefepime, amikacin, and gentamicin (Al-Zahrani and Alasiri, 2018). At Aseer Central Hospital, *K. pneumoniae* had high rates of resistance to ampicillin, extended-spectrum β-lactamases-sulbactam (ESBL-SCM), piperacillin (100%), and to a lesser extent ceftazidime (92.5%), minocycline (80.2%), ceftriaxone (80.1%), and tetracycline (80%) (Al Bshabshe et al., 2020). *K. pneumoniae* ESBL-producing isolates (n-23) were collected from various body sites of patients at King Khalid University Hospital, Riyadh (Azim et al., 2019). Also, *K. pneumoniae* was one the most common UTI-causative and showed the highest resistance to ampicillin (97%) sulfamethoxazole/ trimethoprim (35%) and cefuroxime (30%) (Balkhi et al., 2018).

Several studies from Saudi Arabia have reported the prevalence of antimicrobial resistance genes and detected multiple resistance genes among *K. pneumoniae* isolates, such as CTX-M, TEM, BES and SHV genes that are associated with extended spectrum β-lactamases. NDM-1, OXA-48, SME, IMI, NMC, GES, and KPC are the predominant mechanisms of carbapenem resistance (Azim et al., 2019). Table 1 describes the molecular characteristics of MDR *K. pneumoniae* isolates, the regional distribution and number of cases from several studies from Saudi Arabia hospitals.

Table 1. Types of β-lactamase and carbapenems resistant genes carried by *K. pneumoniae* collected from various clinical specimens of patients at Saudi Arabia hospitals.

Region	City	Year of sampling	Setting	No. isolates	Types resistant genes	Refs.
Central	Riyadh	2016	KKUH	24	blaSHV blaCTX-M blaTEM blaKPC blaIMP	Azim et al., 2019
	Riyadh	2015	2 hospitals	4	OXA-1 TEM-1-BSBL AAC(6′)-Ib	Al-Agamy et al.,2019
	Riyadh	2011-2012	KAMC	54	NDM-1 OXA-48	Zaman et al., 2018
	Riyadh	2014	3 hospitals	21	blaOXA-48 blaNDM	Al-Agamyet al., 2018
	Riyadh	2011-2013	KKUH	5	blaNDM OXA-48	Alotaibi et al.,2017
Southern	Abha	2015	2 hospitals	49	VIM blaIMP blaOXA-48 blaVIM	Al-Zahrani and Alasiri, 2018
Western	Jeddah	2017-2019	KAUH KAMC	-	NDM OXA-48	Taha et al.,2020
	Jeddah	-	Private hospital	1	OXA-48-mediated CAZ-AVI	Al Dabbagh et al.,2019
	Jeddah	2018-2019	KAMC	1	blaKPC-2	Halaet al., 1019

KAMC: King Abdulaziz Medical City,KAU H: King Abdulaziz University Hospital, KKUH: King Khalid University Hospital, KFUH: King Fahad University Hospital, KFH: King Fahad Specialist Hospital

Multidrug-resistant Pseudomonas aeruginosa: *P. aeruginosa* is important opportunistic pathogen and a frequent cause of hospital-acquired infections mainly in patients with immunocompromised condition, which result in high mortality and morbidity rates in critically ill patients (Kaye and Pogue, 2015). *P. aeruginosa* is common agents of respiratory system infections, urinary tract infections, dermatitis, pneumonia, cystic fibrosis, bacteremia, surgical infections, soft tissue infections, and a variety of systemic infections (Rabani, and Mardaneh, 2015). The bacterium, *P. aeruginosa* is considered a multidrug-resistant if the isolate is resistant to three or more of the following antimicrobial agents: piperacillin, cephalosporins, fluoroquinolones, carbapenems, and aminoglycoside (Defez et al., 2004). These agents are representatives of the primary antibiotic classes used to treat *P. aeruginosa* infections.

In recent years, a considerable increase in the prevalence of MDR *P. aeruginosa* has been reported in Saudi Arabia. Furthermore, several studies have identified this prevalence of *P. aeruginosa* to be the most frequent pathogen in KSA hospitals (Khan et al., 2018). A study conducted at the ICU of King Khalid University Hospital in Riyadh reported a significant increase in resistance of *P. aeruginosa*. This resistance was reported as 84% to imipenem, 48%to meropenem, 40% to ceftazidime, and 32% to levofloxacin. Ciprofloxacin and piperacillin/tazobactam showed the same percentage of resistance (28%), followed by 4% to amikacin (Azim et al., 2019).

Cephalosporins proved to be ineffective with significant increase in resistance rate to cefuroxime and ceftazidime during the study period. Consistently, another study conducted at the Hammadi hospital and Habib hospital in Qassim, found that *P. aeruginosa* isolates were resistant

to multiple antimicrobial classes, including cefepime, ceftazidime, amikacin gentamycin, tobramycin, piperacillin/tazobactam, and carbapenem groups (Vijayakumar et al., 2016). Another study from Madinah, confirmed that *P. aeruginosa* tends to be resistant to several antibiotics (Saeed et al., 2018). Recent study performed over a 5-month period to determine quinolones susceptibility patterns. The *Pseudomonas* isolates were collected from different medical departments at a tertiary care hospital in Taif. The 42.4% (39/92) *P. aeruginosa* isolates were resistant to 1-7 of the tested quinolones. Gemifloxacin resistance rate was the lowest (28.3%) while the resistances to the other six quinolones were ≥ 35% (El-Badawy et al., 2019).

P. aeruginosa showed a gradual increase in carbapenems resistance due to its ability to develop resistance mechanisms to carbapenems and other antibiotics. Many studies informed the increasing rates of resistance to carbapenems among *P. aeruginosa* in KSA (Abdalhamid et al., 2016; Bosaeed et al., 2020). A study from Makah, 4803 Gram negative isolates collected from patients in Al-Noor Specialist Hospital. The rate of resistance to carbapenem was among *P. aeruginosa* (62.4%), K. pneumoniae (38%) and E. coli (5.59%) as reported by Faidah et al. (2017). Another study from the Western region was conducted by (Alkeshan et al., 2015). Clinical isolates of *P. aeruginosa* (n=121) were obtained from eight different hospitals in Makkah and Jeddah, *P. aeruginosa* isolates were highly resistant to meropenem (30.6%), ticarcillin (22.3%), imipenem (19%), piperacillin (17.3%), and (22.3%) to ticarcillin.

Another study carried out in tertiary care hospitals of Makkah and Jeddah over a 3-month period to determine the pattern of antimicrobial resistance of *P. aeruginosa* confirmed these findings(Khan and Faiz, 2016).The resistance rates in P aeruginosa isolates were 100% for carbapenem and most of them (89%) were non-susceptible to both ciprofloxacin and piperacillin-tazobactam (Bosaeed et al., 2020). During 2011, thirty-four isolates of *P. aeruginosa* collected from patients hospitalized in a tertiary hospital in Riyadh, were found to be highly resistant to carbapenems (Al-Agamy et al., 2016). Other study by Abdalhamid et al. (2016) evaluated the prevalence of carbapenem-resistant *P. aeruginosa* (CRPAE) colonization in the ICU patients at admission in two hospitals, found in Dammam and Khobar cities. hey reported the prevalence of CRPAE was 6.5% with resistance rate 45.1%.

Table 2. Types of resistant genes carried by *P. aeruginosa* collected from various clinical specimens of patients at Saudi Arabia hospitals.

Region	City	Year of sampling	Setting	No. isolates	Resistant genes	Refs.
Central	Qassim	2015	2 hospitals	11	cepA qacE	Vijayakumar et al.,2018
	Riyadh	2011	1 hospital	34	VEB-1a VEB-1b OXA-10 OXA-2 IMP	Al-Agamy et al.,2016
Western	Taif	2016-2017	1 hospital	92	qnrD qnrS, aac(6′)-Ib-cr	El-Badawy et al., 2019

KFUH: King Fahad University Hospital, KFH: King Fahad Specialist Hospital

Additionally, the major types of acquired b-lactamases that have been identified in *P. aeruginosa* strains including class A, B, and D β-lactamases, such as VEB-, PER-, GES-, TEM-, SHV- and OXA-types. Carbapenem resistance in P. aeruginosa was attributed to MBLs including IMP, VIM, SPM, GIM, AIM, and DIM enzymes and other enzymes, including KPC, GES, and OXA (Yezli et al., 2015; Sawa et al., 2020). Several studies from Saudi Arabia have been characterized by the molecular basis of β-lactamase and carbapenemase production in *P. aeruginosa*. Table 1 demonstrates the available data regarding the genetic determinants for ESBL and carbapenemase production by *P. aeruginosa*.

Multidrug-resistant Acinetobacter baumannii: *A. baumannii* is responsible for outbreaks and nosocomial infections such as ventilator-associated pneumonia, burn wound infections, bacteremia and urinary tract infections which occur in patients in intensive care units (Bassetti et al., 2016; Almasaudi, 2018; Ayoub Moubareck and Hammoudihalat, 2020). *A. baumannii* is one of the most troublesome bacteria due to its remarkable natural and acquired resistance to nearly all major antibiotics classes including broad-spectrum penicillins, cephalosporins, carbapenems, most aminoglycosides, fluoroquinolones, chloramphenicol, and tetracyclines, which compromises the ability to treat patients who are infected by this pathogen (Karaiskos et al., 2019).

Several reports on the epidemiological studies of nosocomial infections from different regions in Saudi Arabia have focused on the emergence of *A. baumannii* in healthcare settings and the ICU environment (Kharaba, 2017). At King Abdulaziz Medical City in Riyadh, the most prevalent Gram-negative bacteria in intensive care units was *A. baumannii* (17.97%). Ibrahim (2018) reported that the most secluded pathogens in ICU King Abdullah Hospital was *A. baumannii* (27.2%) followed by *P. aeruginosa* (23.8%) and *K. pneumoniae* (18.6%). In Ministry of National Guard Health Affairs (MNGHA) hospitals in Riyadh, Jeddah, Alhassa and Dammam, the highest MDR- Gram-negative isolates were *A. baumannii* (58.3%), *Klebsiella* spp. (20.4%) and *E. coli* (16.3%) (El-Saed et al., 2020).

Table 3. Types of β-lactamase and carbapenems resistant genes carried by *A. baumannii* collected from various clinical specimens of patients at Saudi Arabia hospitals

Region	City	Year of sampling	Setting	No. isolates	Resistant genes	Refs.
Central	Riyadh	2010	1 hospital	27	GES-11 GES-5 OXA-23	Al-Agamy et al., 2017
	Riyadh	2006-2014	1 hospital	503	bla -PER-1 bla -TEM	Aly et al., 2016
	Riyadh	2011	1 hospital	62	OXA-23 OXA-40	Alsultan, 2015
Southern	Abha	2013-2014	1 hospital	108	OXA-51 OXA-23 OXA-40 OXA-58	Elabd et al., 2015
Western	Jeddah	-	1 hospital	135	blaOXA-23 ISAba1 blaOXA-51	Shah et al.,2019
	Taif	2017	1 hospital	32	blaOXA-51	El-Badawy et al.,2019
Eastern	Dammam	-	1 hospital	103	OXA-51 OXA-23 NDM, VIM,	AlAmri et.al.,2020
	Al-Hassa	-	1 hospital	5	OXA-23	Alhaddad et al., 2018
	Eastern Region	2014	1 hospital	10	blaOXA-23 ISAba1 blaADC blaNDM-1	El-Mahdy et al., 2017

A. baumannii antimicrobial resistance rates in KSA have increased dramatically over the years to many antibiotics including carbapenems. The susceptibilities of *A. baumannii* to meropenem and imipenem in 2006 ranged between 64-81.2% while the susceptibility in 2012 ranged between 8.3-11% (Al-Obeid et al., 2015). Almaghrabi et al. (2018) recorded 94 clinical *A. baumannii* isolates collected from Aseer Central Hospital, 69% of these isolates were resistant to all antibiotics except colistin. A hospital-based, matched case–control study from Makkah, showed the highest resistance rate of *A. baumannii* was for imipenem (83.3%) followed by gentamicin (72.7%) (Al-Gethamy et al., 2017). *A. baumannii* isolates were highly resistant to carbapenem (99.13%), followed by P. aeruginosa (62.4%), K. pneumoniae (38%), and E. coli (5.59%) (Faidah et al., 2017).

Among 290 Gram-negative isolates collected from ICU at King Abdullah Hospital, Bisha, found that that *A. baumannii* was the most frequent pathogen with resistance rates from 93.4% to 97.5% for all tested antimicrobial agents except for colistin (Alsultan, 2015, Ibrahim, 2019). In the Aljouf region, all *A. baumannii* isolates revealed extended drug-resistance, with 70.6% resistance rate to trimethoprim/ sulfamethoxazole and showed resistance to gentamycin, and carbapenems (Bandy and Almaeen, 2020). A study conducted at a large tertiary care hospital in Taif, confirmed that *A. baumannii* tends to be resistant to different antibiotics (El-Mahdy et al., 2017, El-Badawy et al., 2019). Also, 66% of A. bumannii isolates were resistant to almost all tested antibiotics and no resistance to colistin was reported (Doi et al., 2015, Halwani et al., 2015).

Resistance to carbapenems is mainly due to carbapenemases and metallo-β- lactamases (MBLs) production (Leite et al., 2016; Vrancianu et al.,2020). In Saudi Arabia, many studies have shown prevalence of the different β-lactamases, with an emphasis on

carbapenemases among *A. baumannii* isolates and these studies reported that bla OXA-23 gene and a VIM-type metallo-β-lactamase are the most common genes responsible for resistance in *A. baumannii* (Shah et al.,2019; AlAmri et al., 2020). Table 2 summarized the distribution of β-lactamase and carbapenems resistant genes carried by *A. baumannii* collected from different regions across KSA.

CONCLUSION

The high prevalence of multidrug-resistant Gram-negative bacteria in hospitals and community settings has become a serious health concern and a growing threat in Saudi Arabia. The high morbidity and mortality associated with MDR-GN infections resulted in a significant impact on care cost and treatment effectiveness. Therefore, several measures need to be taken to control the spread of these pathogens, including improving infection control programs, early and accurate laboratory detection, judicious use of antimicrobial agents, and enhanced national disease surveillance. Finally, for better detection and control in Saudi Arabia, these procedures need to be combined with molecular typing methods of MDR-GN bacteria.

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Exploring the Role of Leadership Styles in Innovation Teams: A Case Study of King Abdullah Medical City Makkah, Saudi Arabia

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ABSTRACT

Innovation teams have received increasing interest from academia and practice. However, little is known about how the performance of innovation teams is fostered. We investigate the role of leadership styles (i.e., autocratic, participative, and laissez-faire) in promoting the team performance during each stages of team growth (i.e., forming, storming, norming, performing and adjourning). A qualitative approach with different data collection techniques has been used in this study. The data were collected from four (4) focused groups, eight face-to-face interviews and unstructured researcher's non-participatory observation and it was generated from King Abdullah Medical City (KAMC), a healthcare organization located in Makkah, Saudi Arabia, in March 2019. The findings demonstrate that the participative style is the most influential. In contrast, autocratic and laissez-faire styles have fallen short to keep the members move forward to the final stages of the project.have been

KEY WORDS: DESIGN THINKING; INNOVATION CHAMPION; INNOVATION TEAMS; LEADERSHIP STYLES; TUCKMAN'S MODEL.

INTRODUCTION

Prior studies have found that leadership plays a significant role in improving followers' satisfaction, commitment and performance (Limsila, and Ogunlana, 2008; Ribeiro et al., 2018, Mwesigwa et al., 2020). The extant literature has also informed a critical impact of leadership styles on employees' job performance (Mohiuddin, 2017; (Mwesigwa et al., 2020), creativity (Herrmann, & Felfe, 2013), motivation (Fiaz et al., 2017 and organizational innovation (Alblooshi et al., 2021).

Boosting innovative ideas has been increasingly a critical goal that every organization aspires to achieve. Organizations pursue creative ideas and encourage creativity by fostering innovation teams to compete successfully in a dynamic, fiercely, and highly competitive markets . However, health organizations strive for ways to promote innovation teams to produce innovative solutions to health problems (Varkey et al., 2008; Ferguson et al., 2019; Mitchell and Boyle, 2020).

Improving the effectiveness of teams in the organization has been increasingly an essential goal for organizations to survive in a rapidly increased global competition (Kozlowski, 2018). Innovation team effectiveness is critical to physical and virtual organizations in general and health organizations in particular (West et al., 2003),

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which strive to bolster innovative projects to improve human beings' health and life (Varkey et al., 2008; Ferguson et al., 2019). Improving teams' performance is not viable without understanding the development stages and factors that could enhance the teams' development. Previous research has found that leadership styles influence teams' creativity (Pei, 2017) and performance (Morgeson et al., 2010); (Gyanchandani, 2017).

However, limited attempts exist in exploring the impact of leadership styles on innovation team development and functions (Eisenbeiss et al., 2008; Morgeson et al., 2010). Besides, most of the research conducted on leadership behavior theories were in western countries, studies exploring leadership styles in eastern countries are scarce (Memon, 2014) Moreover, although a substantial amount of academic papers on leadership behaviors have been conducted over the past half-century, a lack of clear-cut practical leadership actions exists (Yukl, 2012). Further, Day (2012) emphasizes that "Context matters, especially with leadership." Hence, this study aims to investigate behaviors that are valid and useful to improve the development of innovation teams.

The current study seeks to achieve two primary purposes: first, to delve into the influence of leadership styles on Tuckman's development stages of innovation teams (i.e., forming, storming, norming, performing, and adjourning). Second, the study explores the team members' insights into the role of the apparent leadership style in impacting the innovation project's progression. To achieve the above purposes, we need to underscore the process of team leadership and to question how the involved sources of interaction are functioning side by side with the challenges the team members are facing (Morgeson et al., 2010, Mitchell and Boyle, 2020, Alblooshi et al., 2021).

Leadership style, also referred to as behavior, is a phenomenon that attracted scholars and practitioners' attention. This is attributed to leadership influence on employee motivation (Fiaz et al., 2017), team performance and well-being (Alblooshi et al., 2020) and project success (Raziq et al., 2018). Several decades ago, a substantial body of literature had differentiated the two approaches of leadership styles: task-oriented styles and interpersonally oriented style (e.g., Lewin and Lippitt, 1938; Bales, 1950; Hemphill and Coons, 1957; Likert, 1961). While the former is concerned with fulfilling tasks organized around task-relevant activities, the latter is mainly concerned with interpersonal relationships by taking into consideration workers' conditions (Eagly et al., 2003).

Prior studies have explored a wide variety of taxonomies which elucidate leaders behaviors, frame these styles into groups (Yukl, 2012; Behrendt et al., 2017) and explicate its impact on followers (Dierendonck et al., 2004; Jong and Hartog, 2007). In 1945, a pioneering attempt to explore leaders' styles was initiated by the Ohio State University leadership studies group (Carter 1958). The study's significant contribution is identifying two dimensions of leadership behavior: Consideration and initiating structure. Following the steps of the Ohio state university, the University of Michigan group has found two styles of leadership: Employee- Centered and Production-Centered (Katz and Kahn, 1950). Most of the earlier leadership theories are consistent with leadership dimensions by which four basic leadership styles surfaced: an Autocratic (or authoritarian) leader (High emphasis on performance and low emphasis on people), Laissez-Faire Leader (low emphasis on performance and people), Human Relations Leader (low emphasis on performance and high emphasis on people), and Democratic (or participative) Leader (high emphasis on performance and people) (Lewin and Lippitt, 1938; Warrick, 1981).

Autocratic leaders are characterized by their style that enforcing control over their followers and ignore personal relationships. Autocratic leaders focus predominantly on performance with low or no consideration on people (Warrick, 1981). Decision-making is often centralized with the leader without any thought of the opinion of the followers (Hassan et al., 2016). On the contrary, democratic leaders put a high emphasis on people and performance (Warrick, 1981). Democratic leadership is described as the performance of three functions: the distribution of responsibilities among members, the empowerment of group members and the support of the decision-making process of the group"(Gastil, 1994, p. 953).

Unlike autocratic leaders, democratic leaders encourage followers to participate in decision making. Prior studies have shown that autocratic leadership, relative to democratic, negatively influences the stability of the groups. It shows that many members exit their groups when supervised by autocratic leaders (Van Vugt et al., 2004). However, prior studies have found that democratic leaders positively impact group member satisfaction (Foels et al., 2000). Moreover, a study by Somech (2006) has also found that the participative leader in heterogeneous teams assists team members to exploit better heterogeneity of the groups in terms of the variety of professional backgrounds, knowledge, skills, and abilities.

Laissez-faire is a leadership style that mainly circumvents decision making, avoids problem-solving, and elope engagement. It is also described as "a general failure to take responsibility for managing" (Eagly et al., 2003, p. 571). The impact of Laissez-faire leadership style on followers' performance is yielding mixed results. Opponents of Laissez-faire leadership style has found that laissez-faire leaders are negatively impacting followers (Skogstad et al., 2007; Nielsen, et al., 2019) while proponents of this style argue that it has a positive impact on followers (Yang, 2015).

A modern view of leadership styles was following the older approach by studying new types of styles during the 80s and 90s. This updated perspective of styles focuses on how effective leaders can inspire and

foster followers' abilities and skills (Eagly et al., 2003). A pioneering attempt conducted by Burns (1978) and elaborated by Bass (1985; 1998) has yielded a new style called transformational leadership. Transformational leaders are characterized by considering themselves as role models to their followers by fostering their trust and confidence, empowering them to utilize and unleash their full potential (Eagly et al., 2003) Transformational leadership has consistently been related to employee satisfaction and empowering work environment (Boamah et al., 2018). In contrast, transactional leadership is concerned with managing, clarifying, rewarding, and correcting (Bass,1998; Eagly et al., 2003).

Team leadership: The impact of leadership in organizations has been a phenomenon that attracted the attention of management and organization scholars over a hundred years (Eisenbeiss et al., 2008) and historian and philosopher a millennia ago (Kozlowski et al., 2016). An exploding number of empirical studies has shown a substantial impact of leadership on employees' performance, motivation (Ullah et al., 2019) and innovative behavior (Jong and Hartog, 2007). Leadership is a process of substantial importance as it could influence organization's performance (AL Khajeh, 2018) and employee outcome behaviors such as commitment (Mwesigwa et al., 2020; Yahaya and Ebrahim, 2016) and satisfaction (Huey Yiing and Zaman Bin Ahmad, 2009).

Prior studies have emphasized leadership's role in team performance and innovation (Dackert et al., 2004; Somech, 2006). In particular, functional leadership theory is employed to explore the essence of team leadership; Moreover, team leadership is previously described as ". . . leader as completer . . . the best a leader can do is to observe which functions are not being performed by a segment of the group and enable this part to accomplish them" (Schutz, 1961, p. 61). McGrath also asserts that "the primary purpose of leadership is to ensure that the group fulfils all critical functions necessary to its own maintenance and the accomplishment of its task" (1962, p. 5).

Accordingly, team leadership is defined as the process of the team need satisfaction in the service of enhancing team effectiveness (Morgeson et al., 2010). Besides, team leadership refers to "teams with a clearly identified leader who sets the team's tone or culture. The leader engages and motivates the team, ensures that communication is free-flowing, and ensures that all members can participate in the team and feel supported. Through this, they elicit a commitment to the team and its objectives. The leader provides a safe climate for constructive disagreement and ensures conflicts are resolved. They provide feedback on team performance and encourage reflection, openness, and learning culture." (Sims et al, 2015b, p. 212). Salas et al. (2018) have also emphasized that coordination, communication, and adaptability are critical competencies to increase teamwork efficiency.

Morgeson and colleagues (2010) have contributed to team leadership literature by modeling sources of leadership in teams based on the interaction of structural dimensions of locus of leadership and formality of leadership. The locus of leadership dimensions describes the leader's role as an engaged member of the team (internal) or an (external) member of the team who does not participate in any related teams' tasks. However, the formality of leadership dimension represents whether the leader is responsible for team performance as formalized in the organization (formal) or whether the leader is informally accountable for a team's leadership and performance (informal) (Morgeson et al., 2010). According to Morgeson et al. (2010), team leaders in the current study are considered internal and informal. In particular, internal and informal leadership are characterized by shared leadership responsibilities among team members (Day et al., 2004) or when a member occurs informally as a leader (Foti and Hauenstein, 2007).

Morgeson and colleagues have also described team leadership functions based on two phases of team development: transition and action phases. The leadership functions that were manifest in the transition phase are: composing team, defining mission, establishing expectations and goals, structuring and planning, training and developing, sense-making, and providing feedback. However, the action phase is characterized by the leader who mainly monitors, manages, and challenges teams. Add to that; the leader should be occupied with performing team tasks, solving problems, providing resources, encouraging team self-management, and supporting social climate (Morgeson et al., 2010).

Moreover, leadership style plays a critical role in cross-functional team processes and performance (Somech, 2006). Innovation in organizations is considered the process to create and develop new methods for getting things done. Innovation enables idea generation, and ideas' implementation, leading to the best methods, practices, or products. Creativity and innovation appear at levels such as individuals, work teams, organizations, or in a combination of all these levels. However, the results can be recognized in one or more of these levels (Anderson et al., 2014).

Cross-functional teams are essential for innovation projects, where organizations need diverse team with variety of perspectives and experiences to solve complicated problems (Thayer et al., 2018; Usher and Barak, 2020). However, functional diversity can also lead to conflicts, which may hinder an optimal performance of team's (Driessen et al., 2015). According to Edmondson and Nembhard (2009), using teams in organization for developing new products can promote both internal and external success (Hayes et al., 1988; Wheelwright et al., 1992). Internally, successful teams have accelerated the product development cycle, reduced development costs, and increased new products quality (Cooper and Kleinschmidt , 1994; Gupta and Wilemon , 1990; McDonough, 2000; Sarin and Mahajan, 2001; Valle and Avella, 2003).

A recent study that explored the challenges facing innovation teams during the staging of the innovation teams has identified the following: leader selection criteria, leader personal characteristics, communication, cross-functionality, and task distributions as the major challenges (Kutob and Alhothali, 2020). Team development is described as a vernacular practice by which team members attempt to establish their way of conducting work with compelling social structures, norms, and practices (Kozlowski and Ilgen, 2006). It is considered holistic as all members go through this process together and it has been long related to team performance (Zhu et al., 2020). A pioneering attempt to structuring team development is demonstrated in Tuckman's stage model (1965).

The Tuckman's model, first published in 1965, demonstrates the progress of the team in four consecutive stages: forming, storming, norming, and performing which were based on clinical therapy and T-groups. Later on, the model has been updated by Tuckman and Jensen (1977) to involve a fifth stage called adjourning. The first stage forming represents the phase where the members are selected and where the design thinking occurs. Second, the storming stage in which the team faces conflicts and issues in dealing with each other. Then, the norming stage through which the team becomes more stable and familiar with each other. While the team becomes more effective and efficient at the stage of performing, in which the team improves substantially and provides valuable outcomes. The final stage of the Tuckman model is the adjourning stage in which the team approaches the closure, and the opportunity of starting a new project with the same team arises.

Tuckman's model is one of the influential models as "it responds to the growing importance of groups in the workplace and to the lack of applicable research.... useful for practice by describing the new ways that people were working together, helping group members understand what was happening in the development process, and providing consultants a way to predict the stages of growth in groups" (Bonebright, 2010, p. 112). Hence, in this study, Tuckman's model will be utilized to frame the stages of innovation team development.

Working in teams has increasingly become a typical structure of work in organizations (Hiller et al., 2006). This unprecedented use of team-oriented work is attributed to its impact on the organizational success and its agile response to uncertainty (Kozlowski, 2018). Health organizations are among the pioneering organizations in utilizing teams to achieve tasks and daily work activities. Failures of team leadership, coordination, and communication are the leading causes of major crisessuch as air crashes, medical errors, and technical failure. Therefore, innovation teams are considerably significant to the health industry.

A large body of research has investigated the efficiency of working in teams to produce innovative solutions to medical and health problems(Hewitt et al., 2014; Sims et al., 2015a; Sims et al., 2015b). Sims and colleagues (2015b) have utilized realist synthesis theory to explore the underlying processes used by inter professional teamwork to improve team efficiency. The findings reveal that 13 mechanisms, such as (e.g., leadership, shared purpose, innovation, and critical reflection), when used together, would enhance teams' direction and focus.

MATERIAL AND METHODS

To collect data for the current study, a qualitative approach with different techniques of data collection has been administered. In particular, four (4) focused groups' members, eight (8) face-to-face interviews and unstructured researcher's non-participatory observations have been used for this study. Semi-structured interviews allow participants to freely talk about their experience as innovation team members and the ramification of leadership style on the projects' progress and performance. The use of interviews is justifiable as it enables the members to express their views without feeling embarrassed by their leaders. Conducting interviews also helps the researcher to meet the participants at their convenience.

The reason behind using focused group to gather data is that the respondents worked within small teams, they feel more comfortable to respond and for researchers it is faster to collect their answers at once. Besides, data collected from researcher's non-participatory observation is also considered during the program life cycle. Data were collected from King Abdullah Medical City (KAMC), a healthcare organization located in Makkah, a Holy City on the Eastern side of Saudi Arabia in March 2019.

The innovation champion's program owner has approved permission to start data collection. Further, the researcher received permission from the Research and Innovation Center in KAMC for data collection. The data collected from six innovation teams with the total number of 36 of KAMC staff from different departments, positions and backgrounds (medical and non-medical) who participated in the Innovation Champion in 2019. Table 1 below shows the participants' demographic information. A convenient sampling is used to get participants to participate in the study. An invitation email was sent to participants and direct managers to confirm the approval of their participation in the research. Participants' description in terms of their role in the team (i.e., member or leader) and the department where they work are displayed in (Table 1).

Participants were contacted via phone to schedule an appointment for the interview or the focus group. The interviews were recorded after taking the participants' permission. The interview questions are focused on the role of leadership from three main perspectives: the first is the leader selection mechanism and criteria. Second, the leader's characteristics and its impact on team performance in the project. Lastly, the main concerns and disagreements on the leader's characteristics.

The five-stages of Design Thinking model proposed by the Hasso-Plattner Institute of Design at Stanford (Plattner et al., 2009) was used as a reference guide for innovation teams in managing and conducting their projects. See Table 2 below.

Table 1. Description of participants

Team Code	Type of team member	Participant Code	Department
1	Member	1	Executive Administration of Operation
	Member	2	Associate Executive Administration of Patient Affairs
	Member	3	Associate Executive Administration of Patient Affairs
	Member	4	Innovation Center - Taif
	Member	5	Innovation Center - Taif
2	Member	6	Executive Administration of Operation
	Member	7	Executive Administration of Research and Innovation
	Leader	8	Executive Administration of Medical and Clinical Affairs
	Member	9	Executive Administration of Medical and Clinical Affairs
3	Member	10	Executive Administration of Medical and Clinical Affairs
	Leader	11	Executive Administration of Medical and Clinical Affairs
	Member	12	Executive Administration of Operation
4	Leader	13	Executive Administration of Operation
	Member	14	Health Economics Department
	Member	15	Patient Experience Center
	Member	16	Executive Administration of Medical and Clinical Affairs
5	Leader	17	Executive Administration of Operation
	member	18	Executive Administration of Administrative and Financial Affairs
	member	19	Executive Administration of Operation
	member	20	Marketing and Corporate Communication Department
6	member	21	Legal Affairs Department
	member	22	Executive Administration of Medical and Clinical Affairs

Table 2. The Innovation Champion project phases as adapted from d.school model (Kutob and Alhothali, 2020)

D.school Model	Empathize	Define	Ideate	Prototype	Test
Definition	Gain an empathic understanding of the problem trying to solve.	Analyze the observations and synthesize them in order to define the core problems the team have identified.	Identify new solutions to the problem statement and evaluate the options then select the suitable option for the problem.	Implement the solution and investigate either accepted, improved and re-examined, or rejected on the basis of the users'experiences.	Alternate and refine the solution in order to rule out problem solutions and derive as deep an understanding of the product and its users as possible.
KAMC Model		Design		Develop	Deliver

Data Analysis: The steps of Miles and colleagues (Miles et al., 2018) were utilized to analyze data for this study.

A holistic approach emphasized by the overall research questions followed by what the participants have discussed when answering the main questions is conducted. Then, drawing conclusions and checking the raw data to verify assumptions was established. The respondents were asked to explain their experience in light of the five stages of Tuckman's model. Specifically, they are encouraged to discuss the problems that face them during the five stages of the team development model.

The first stage forming represents the phase where the members are selected and where the design thinking occurs. Second, the storming stage in which the team faces conflicts and issues in dealing with each other. Then, the norming stage through which the group becomes more stable and familiar with each other. While the team becomes more effective and efficient at the stage of performing, in which the team improves

substantially and provides valuable outcomes. The final stage of the Tuckman model is the adjourning stage in which the team is closing its project, and the opportunity of starting a new project with the same team members in the next project aris.

Table 3. Demographic characteristics of the participants

Variable	Criteria	Number	Percentage (%)
Age	18-24	0	0%
	25 to 34	9	41%
	35 to 44	10	45%
	45 to 54	3	14%
	55 and above	0	0%
Gender	Male	11	50%
	Female	11	50%
Education	Diploma	2	9%
	Bachelor	14	64%
	Master	6	27%
	PhD	0	0%

RESULTS AND DISCUSSION

Table 3 describes the demographic data of the participants. The findings reveal that the sample was balanced in terms of gender (50% female and 50% male). Participants were relatively young as (41 %) of them aged between 25 to 34 and (45 %) were between 35 to 44 years old. The majority of the sample (91%) is university educated. The themes were generated through using the thematic analysis and were classified into three major classes: the leader selection mechanism and criteria, the leader personal characteristics and its impact on team performance in the project. Table 4 below summarizes the themes discussed with the participants during their experience in the Tuckman's model of team building in comparison with three different leadership styles.

A group of respondents has demonstrated a set of leader characteristics that align with the autocratic leadership style. As displayed in table 2., during the forming stage, respondents emphasize that a member of the team assigns himself to be the group leader.

Being autocratic by nature, the leader assigns tasks and roles to other team members without prior discussion. "We were surprised that one of the team members had selected himself to be the leader without considering all the things that we have learned in the workshop to help us select the right leader. I would not be so sure about selecting him as a leader, but was obliged to accept that." (Team member (20) – male).

This finding corroborates with the existing literature on the characteristics of autocratic leaders. They take the sole duty to make choices and relevant decisions without seeking input from followers in the organization (Gandolfi and Stone, 2017; Harms et al., 2018). Besides, the leader assigns the tasks to team members without participation in any of the tasks. This result confirms prior studies where the leader of this group is taking an external role where he/she does not participate in any related teams' tasks (Morgeson et al., 2010). Moving to the storming stage, respondents emphasized that this stage is critical as it involves conflicts and arguments. Respondents stress that the leader imposes (his/her) opinions and ideas over the other members' views and ideas. Although he/she has not participated in any tasks, he still has the power to enforce his opinion. "Our leader always refuses any other opinion: He is refusing our attempts to reach an agreement and finding any reason to complicate the situation and refuse our opinions." (Team member (3) – female).

The norming and performing stages are parallel with the developing phase in the innovation project, where the participants asked to brainstorm their ideas relative to the main problem and the need identified in the discovery phase. Respondents have emphasized that Develop phase in the project is synchronizing with the norming stage in the development model. During the norming stage, the leader becomes more understandable and acceptable to team members' contributions.

"we have reached now a point of agreements by all members" (Team member (3) – female).However, during the performing stage where the members are functioning as capable teams, respondents emphasized that the leader becomes more focused on task accomplishment (Warrick, 1980), and the team members become more comfortable with each other. "Sometimes, they are positive and sometimes negative, but the group members are like one family. We all talk together to reach something that satisfies all members of the team" (Team member (3) – female). Respondents stressed that through the adjourning stage, the members start feeling of achievement and being proud; however, the team members are unwilling to continue with the same team members in future projects. This finding also corroborates with prior studies, which emphasize that the viability of the team is an indicator of team effectiveness (Hackman, 1987). "I do not think we are going to participate in other projects together" (Team member (10) – female).

Researcher observation has been taken into consideration. The main finding is that autocratic leaders in innovation projects could decrease team performance. It could be inferred from delayed reports that should be submitted by their leader. Besides, the leader refuses the team's opinions and suggestions in situations that require mutual decision-making. Finally, the findings of these focus groups confirm the characteristic of the autocratic leadership style is not good for growth. Autocratic leadership style, where the leader is responsible for taking all the decisions, has full authority over the work and team, assigning tasks, and control the communication within the group (Lewin and Lippitt, 1938; Gandolfi and Stone, 2017).

Respondents of focus group two ascribe one of the democratic leaders' main features, where the leader

encourages shared opinion. In particular, (see table 4 above), the leader of this group was selected by voting during the team members agree to choose a leader to the group based on his previous experience and specialties. This result confirms the characteristics of democratic leaders, where the leader seeks the followers' opinions when making decisions (Gandolfi and Stone, 2017; Rifaldi et al., 2019). Respondents also emphasized that the leader discusses the tasks and roles with the team members before assigning them. Hence, the characteristics of the democratic leader come to the surface.

Table 4. Leadership style in comparison to the team building stages

Tuckman Model of Team Building	Leadership Styles		
	Autocratic Scenario 1	Democratic Scenario 2	Laissez-Faire Scenario 3
Forming (team formation, setting ground rules and finding similarities)	The leader was selecting himself as a leader. The leader assigns tasks to members without prior discussion.	The leader was selected by voting or based on his previous experience and specialties. The leader discusses the tasks and roles with the team members.	Has unclear leader selection criteria. The tasks assignments have unclear goals and deadlines of the tasks.
Storming (Dealing with issues of power and control and suffering differences)	The leader assigns all the tasks to team members, and he did not participate in task accomplishment. The leader imposes his opinions and ideas over the other members' opinions and ideas.	The leader was sharing the decision-making opportunity regarding excluding one of his team members. The leader is trying to satisfy each member of the team on the expenses of the project due dates.	The team members are not a response to leader instructions, and the leader does not have enough skills to manage the conflicts. The leader tends to take all the work and do by him/herself.
Norming (Managing the team conflicts, finding the team norms, and refusing the similarities)	The leader becomes more understandable and acceptable to team contributions.	The leader and the team become more productive and achievable.	The leader becomes more aware of the things that are motivating her team members and try to increase their engagement.
Performing (functioning as a capable team)	The leader becomes more focused on tasks accomplishment, and the teams become more comfortable with each other.	The leader was sharing tasks based on the strong points of the team members.	The leader and the team are helping each other to resolve the conflicts and focusing on the results.
Adjourning (finding closure)	Feeling of achievement and proud, but the team are not willing to continue with the same team in future projects.	The leader and the team members are feeling as one family, and they are willing to continue to accelerate their project to the implementation phase.	The leader and the team members are wanted to stop by the deadline of the Innovation Champion program.

"The leader nominated by voting, and we are all agreed that this person was the best among us." (Team member (19) – female). Respondents of this focus groups stress that the leader was open to their opinions and seeking their contribution in decision making. A female team (7) member (team 2) indicates that "the leader asked us to help him to decide excluding one of the team members." Respondents have also emphasized that the leader was very flexible and cooperative. They also indicate that he was trying to satisfy each member; however, this has negatively impacted the project progress as it delays task submissions. This result contradicts previous results in the literature (Hackman, 1987).

Even though members' satisfaction is an indication of team effectiveness, the findings of this study show that satisfying each team member impedes the progress of the project.During the norming stage, respondents give attention to the congruity level that becomes evident among the members. Hence, team members become more productive and capable of achieving their tasks. "The team performance after excluding the trouble maker

member, everyone becomes helpful and powerful; the task assigned by the leader and all team members were accepting and working on it. The leader asked the team to provide him with any notes on the way of managing the project." (Team member (7) – female). During the performing stage, respondents reveal that the leader was open and democratic in that he divides tasks and responsibilities, considering the strong points and characteristics of the team members.

"He was able to resolve issues once appeared. Moreover, find a suitable solution. He was perfect, energetic, and enthusiastic" (Team member (9) – female).Through this stage, respondents have expressed the feeling of harmony, agreement, and assurance. They emphasized that the leader and the team members are feeling as one family, and they are willing to continue to move their project to the implementation phase. This finding indicates the team's effectiveness as the members are satisfied and willing to move to the next stage. Their willingness to move forward corroborates with prior studies in terms of the viability of the team as a facet of team effectiveness (Hackman, 1987). The findings underscore the features of the democratic style of a leader. The leader of this group demonstrates the feature of democratic leaders where the leader sought team members' collaboration throughout the progress of the project. These features confirmed the characteristic of the democratic style as described by (Lewin & Lippitt, 1938; Rifaldi et al., 2019).

According to the researcher's observations, the democratic leader had positive influences on his/ her team, especially in innovation projects, which need more flexibility and dynamism. One of the most critical findings is that the team member selection at the beginning of the project has a significant impact on the members who have a democratic leader, which facilitates the team to pass the storming stage faster and easier than the other teams. Respondents expressed that they were struggling in the forming stage as the goals were not clear. Tasks are also blurred; no one was sure what to do or when to start. This finding confirms the characteristics of Laissez-fair leaders, where they avoid leadership (Eagly et al., 2003; Skogstad et al., 2014). This finding confirms the destructive role of the Laissez-Faire leadership style as it positively related to role ambiguity (Skogstad e al., 2014), role stress, interpersonal conflicts, and health problems (Skogstad et al., 2017).

Through the forming stage, the leader was not clear about goals, roles, and tasks, so respondents were not cooperating with him nor responding to his requests and instructions. Respondents also emphasize that the leader lacks the required skills to manage the conflicts. So, to proceed, the leader tends to take all the tasks and do them by him/herself. The results also approve previous studies' findings into the failure of Laissez-fair leaders to solve problems (Eagly et al., 2003)."...When it comes to the tasks and reports, he did not allow us to see it before sending it to the program manager. When he shows us, it seemed not familiar to us, and he assigned himself the rest of the tasks without any prior discussions, how would I do the content of the report? We are not familiar with anything." (Team member (20) – male).

During the forming stage, the leader and the team are helping each other resolve the conflicts and focus on the results. "The team leader did not arrange the meetings. During the two hours at the meeting, I did not know what to do. In the first two weeks of the project, the picture was very blurry, meaning I would leave the team and join another team. We were already lost, and I do not want to say that the leader is the reason because he is trying hard not to fail, but he told us that he was like we lost until we started the project with what we know." (Team member (10) – female). Despite the leader's and members' efforts to move to the next stage, they fail, and their project has stopped without completion. The finding confirms prior studies that of Laissez-fair leadership style can deteriorate the effectiveness of the teamwork (Skogstad et al., 2007; Skogstad et al., 2017) as it leads to the failure of completing the project.

The findings of these focus groups indicate the salient feature of the Laissez-Faire leadership style, where the leader is more receptive to other opinions. This style of leaders does not have any constraints regarding who is taking the roles and decisions. This leadership style enables the team member to work freely, and the leader does not tend to engage in the process of leading (Lewin & Lippitt, 1938; Omolayo, 2007). The most apparent finding from the observation is that the members led by the Laissez-fair leader felt lost, reflecting on their ability to make the right decisions. As a concrete example, the team leader expressed his feelings of losing control and confusion "I am lost just like you (his team)." The team members were in a critical condition, and they needed to complete the project to participate in the contest. Another observational example is that one of the team leaders has closed his mobile while his team needs the project files that they are working with him to be submitted to the program manager.

CONCLUSION

Innovation teams have received increasing interest from academia and practice. However, little is known about how the performance of innovation teams is fostered. Hence, the current study explores the role of leadership styles (i.e., autocratic, participative, and laissez-faire) in promoting the team performance during the stages of team growth (i.e., forming, storming, norming, performing and adjourning). A qualitative approach consisted of four (4) focused groups, eight (8) face-to-face interviews and non-participatory observation have been used for this study. Data were collected from King Abdullah Medical City (KAMC), a healthcare organization located in Makkah, Saudi Arabia, in March 2019.

The findings demonstrate that the participative style is the most influential type of leadership. In contrast, autocratic and laissez-faire styles have fallen short as to keep the members moves forward to the final stages of the project. Moreover, the results indicate that all

the innovation teams passed through the team-building stages; however, the impact of leadership style differs from one group to another. The findings also demonstrate that the autocratic leadership style is the most effective as it is the only team members who are satisfied and have completed the project and are willing to move to the implementation stage. The study also has confirmed the destructive role of the laissez-faire leadership style as it leads to the failure of the innovation team project (Skogstad et al., 2007). For leaders to be adaptive, they must be aware of the critical contingencies that necessitate shifts in leadership functions. They must possess the required skills needed to help the team maintain its fit with their task environment and resolve challenges.

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Ethical Clearance Statement: Data were collected from King Abdullah Medical City (KAMC), a healthcare organization located in Makkah, a Holy City on the Eastern side of Saudi Arabia in March 2019.

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Insight into the Keratinase Enzymes from Microbial Origins and their Applications

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ABSTRACT

One of the environmental pollutants in the word is Keratin waste which is produced mostly from the poultry farms, slaughterhouses, and leather industries. Beneficial organisms are found in nature and considered as a well-known microflora (fungi and bacteria), which have the capability to degrade the keratin waste. This review deals with the different application of microbial keratinase. The keratinolytic microflora has been qualified to produce keratinase enzyme for biodegradation (enzymatic degradation). Keratinases are proteolytic enzymes accomplished of degrading insoluble keratin protein present in skin, hair, nail, or feather. Keratinases are active over wide range of conditions and are useful in bio recycling of keratin wastes into feed and fertilizers. They also have potential applications in leather, cosmetic, textile, biomedical and detergent industries. The applications of keratinases extend to energy generation and green synthesis of nanoparticles. Due to their ubiquitous biotechnological applications, techniques such as immobilization, optimization strategies, protein engineering and DNA recombinant technology have been used to improve their activities and stabilities thereby widening the scope for commercialization. This review records recent multi-functional applications of keratinases.

KEY WORDS: KERATIN, WASTES, KERATINASE, MICROORGANISM, APPLICATION, ENVIRONMENT, POLLUTANTS.

INTRODUCTION

All over the world, production of livestock is increasing quickly because of population growth, increasing incomes, changes in lifestyles and nutritional habits. The leftover from animal meat production consists of keratinous materials such as chicken feathers, pig bristles, wool and horns and millions of tons of these co-products are produced each year (Jingwen et al., 2020). Chicken feathers from poultry processing plants is classified as low-risk materials for animals, the public, and the environment because of high producing of keratin (Verma et al., 2017). Consequently, they can be considered as an abundant protein or amino acid source for new cycling processes targeting potential use in feeding, fertilizer in cosmetics and other applications (Callegaro et al., 2019). Searching for better and "green" ways to support the world health by motivation and utilization of natural byproducts are available. Unique keratinous materials come from the rich of certain amino acids, including in

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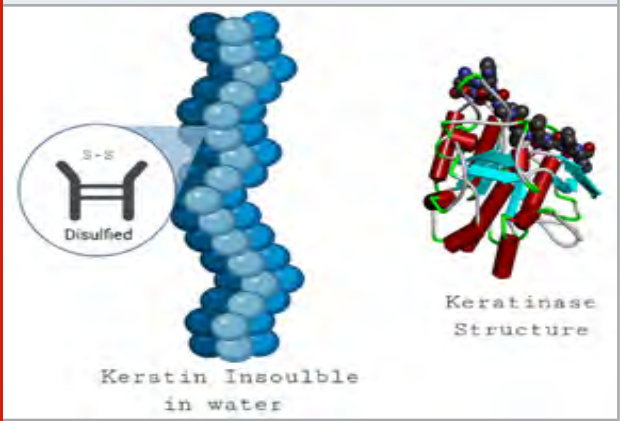
particular the sulfur-containing amino acid, cysteine, other amino acids like glycine, proline, arginine, and the essential amino acids valine, leucine, and threonine. The highly stability of keratin is due to disulfide bridges formed among cysteine residues within and between keratin polypeptides (Callegaro et al., 2019). Keratins considered as insoluble materials and partial or complete degradation without denaturizing the amino acids are needed (Gindaba et al, 2019). The degradation processes provide useful bio- refinery methods for both protein and amino acids (Koentjoro and Prasetyo, 2021).

Keratin and its derivatives: Keratins are a major class of structural proteins that are highly resistant to biological degradation. Common enzymes, which break down protein, such as trypsin do not affect Keratins. They are water insoluble proteins. Like other proteins, they are made of a long string of various amino acids, which fold into a final three-dimensional form. There are two types of keratins; α-keratins and β-keratins, consisting of tightly packed protein chains in α-helices and β-sheets, respectively (Parry and North, 1998; Esawy, 2007). Additionally, keratins filament structures are stabilized by their high degree of cross-linking of disulfide bonds, hydrophobic interactions and hydrogen bonds. Due to their extremely rigid structures, Keratins are insoluble and hard to degrade (Esawy, 2007). Chicken feathers are composed of over 90% of Keratin protein, small amounts of lipids and water. Feathers Keratin consists of high quantities of small and essential amino acid residues (Pencho, 1990).

Figure 1: Keratin structure shows the disulfide bond which makes the keratin insoluble in water and keratinase structure.



It is also the main protein components of hair, wool, nails, horn and hoofs. Animal hair, hoofs, horns and wool contain α-keratin and bird's feather contains β-KRT. The polypeptides in α-KRT are closely associated pairs of α-helices, whereas β-KRT has high proportion of β pleated sheets (Figure 1) (Asquith, 1977; Morris et al., 1992; Savitha et al., 2007). Keratins are macromolecule comprises a tight packing of supercoiled long polypeptide chains with a molecular weight of approximately 10 kDa. High degree of cross linked cystine disulfide bonds between contiguous chains in keratinous material imparts high stability and resistance to degradation (Schmidt and Barone, 2004; Coward-Kelly et al., 2006; Tamilmaniet al., 2008; Weidele, 2009). In summary, a keratinous material is a tough, fibrous matrix being mechanically firm, chemically unreactive, water insoluble and protease-resistant (Savitha et al., 2007;Callegaro et al., 2019; Gindaba et al, 2019; Koentjoro and Prasetyo, 2021).

Production of keratinases:

Keratinase production in liquid medium (submerged fermentation): Onifade et al., (1998) reported that when bacteria or fungi were grown in liquid medium containing keratinous substrates or feathers as carbon sources, extracellular intracellular keratinases were predominantly obtained (submerged fermentation). It is clear that keratin or feather act as an inducer for keratinases production however soy meal (non-keratin material) may also induce the enzyme release. Keratin was degraded in two steps, sulfitolysis, meaning the decrease the number of the disulfide bonds and protein hydrolysis (Gupta and Ramnani, 2006). Under submerged conditions using either shaking or static methods, production of Keratinase is mainly obtained (Lateef et al., 2010; Cai et al., 2011, Aly and Tork, 2018, Aly et al., 2019). Keratinolys is activity of each bacterium differed according to production conditions, the used microorganisms and cultivation techniques. Additionally, addition of simple carbohydrates like glucose suppresses the keratinase production (Daroit et al., 2011, Aly et al., 2019). On contrast, polysaccharide as starch improves the production of keratinases (Syed et al., 2009). Figure 2, showed the steps used for production keratinase in submerged fermentation.

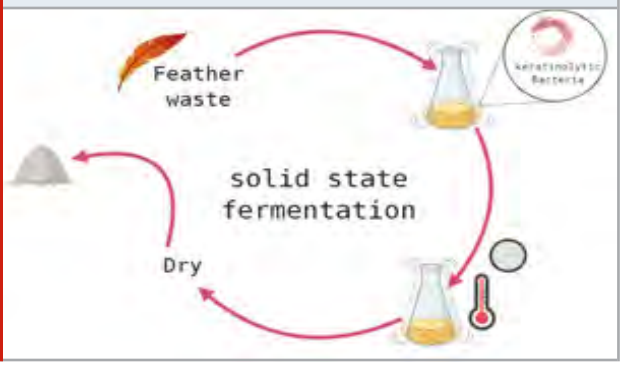
Figure 2: Production keratinase in submerged fermentation.

Keratinase production in solid state fermentation: Paul et al., (2013) reported that feathers, hair, horn and sugarcane bagasse can be used as rich carbon source for induction of keratinases from bacteria (solid state fermentation).Likewise , it was reported that under solid state fermentation, presence of 0.1% soybean powder in the growth medium in addition to feather enlarged keratinase production by *Bacillus* sp. PPKS-2 (Prakash et al., 2010, Tork et al., 2016) while El-Gendy (2010) found that under solid state fermentation and using different agricultural and poultry wastes, the endophytic keratinolytic fungal,Penicilliumsp., extensively produced keratinase. Figure 3 showed the steps for Keratinase

production during solid state fermentation. Fermentation method is used to biodegrade whole chicken feather sby keratinolytic microorganisms.

For example, *Fervidobacterium islandicum* AW-1 is keratinolytic microorganism has been considered as an important microbe for biodegradation of feathers and keratinous wastes. Potential fermentation process enables this bacterium to degrade feather with excellent activities (Yeo et al., 2018). The used technique is advantageous by reason of its ability to apply substrates quite rapidly and is best suited for the bacteria that need high moisture contents (Koentjoro and Prasetyo, 2021). In this method, the screening and isolation of bacteria are the most power step to start the process. The ability of the isolates to degrade the feather polymers directly and highly fermented was selected. The ability of the microbe to degrade feather is affected by optimization of feather concentration, incubation time, pH and temperature (Osman et al., 2017).

Figure 3: Keratinase production in solid state fermentation.

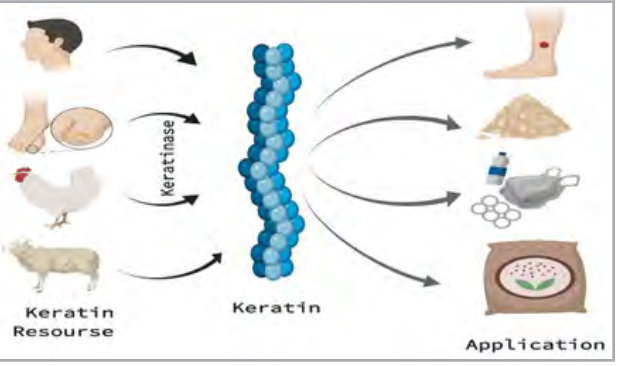


Keratinase application:

Wound dressings: Keratins is a rich source of proteins and have a clear functions in wound healing because Keratin material found mainly as fundamental components of the skin (Kelly, 2016). Proteins of Keratin may be obtained from wool using processes that do not hydrolyze peptide bonds, which allows the keratin proteins to retain a form and function similar to native keratins. Topical creams can then be prepared from purified keratinase which can be incorporated into dressings and Keraplast. These treatments are often used for the treatment of persistent wounds and have been found to be therapeutic (Kelly, 2016) as shown in Figure 4.

Keratinase for Keratin waste management: The huge increase in the population is linked to increases in wastes production. Biodegradation of different wastes to useful materials or products is important for meeting the demand of the crowded population. Accordingly, there is an enlarged amounts of agro- industrial wastes, including keratin and feather wastes from slaughterhouses, poultry farms and leather industry (Tesfaye et al., 2017; Srivastava et al., 2020). It is clear that, feather wastes increased every minute and feather treatments are urgent to decrease bad environmental impacts and maintain healthy environment. Thus, keratinases from bacteria or fungi can degrade different waste components in addition to prions by using cocktail enzymes from active microbes. Microbial keratinase had very remarkable feather degrading ability and thus could be usefully used in management possess (Lateef et al., 2015).

Figure 4: Keratin sources used for keratinase production and its applications.



Keratin wastes may be keratins, feathers, collagens, elastins, wool and prion proteins all these wastes are generated during meat industries and considered dangerous wastes must be efficiently degraded by the keratinolytic enzymes (Zhao et al., 2012). Also, wool waste can be degraded by keratinase obtained from *Stenotrophomona smaltophilia* as reported before (Fang et al., 2013). Therefore, using a mixture of bacterial keratinases can be used as potential application for bio-treatment of slaughterhouse or abattoirwaste stream/effluents to simple important materials. Thus, professional management of keratin and feather wastes through recycling into value-added and proper products is important. The costs of chemical management process may be high, thus efficient means of biological treatments can be applied to prevent the harmful effects of these wastes on the environment (Nnolim et al., 2020).

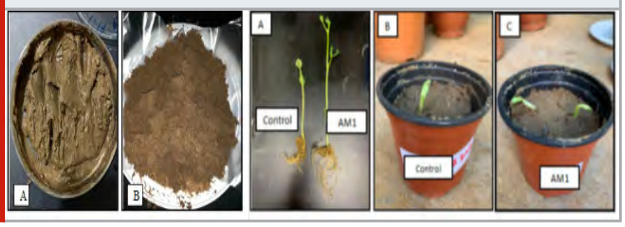
Animal feed production: For decades, it is well know that feather meal can be used as supplements or a feed but the nutritive value of the meal is different due to the used protein material in the feed. Proteins structure in feathers or other keratinous materials are difficult to be broken or digested by ruminants or livestock due to structural orientation and presence of different chemical groups (Nnolim et al., 2020). Chemical degradation of feather wastes can be used to prepare feather meal but this method is costly high and need high energy input in addition it denatures important heat sensitive proteins (Dong et al., 2017). Thus, keratinases from keratinolytic microorganisms can be used for the recycling techniques used for waste managements (Gegeckas et al., 2018).

keratinolytic wastes are rich in many amino acids and the hydro-thermal treatments of keratinolytic wastes reduces nutritional values of the products as they destroyed definite essential amino acids like methionine, lysine, histidine and tryptophan and inability to release some

amino acids from the keratins. Consequently, keratinases from microorganism origins are a good alternative for bio-treatment and may be used for keratinolytic wastes degradation. as reported before, the keratinolytic isolate *B. subtilis* may be used for degradation of keratins and production of proteinous hydrolysate with as potential values from wool and feather wastes (Fakhfakh et al., 2013; Volik et al, 2020). Keratinase degradation of feather is more beneficial than microbial degradation, as it avoids the possibility of exposure of the environment to the microbe which may be a pathogen. Nutritional uses of keratinase daily in growing and nursery pigs as supplements improve immune response, weight gain, nutrient digestibility, intestinal morphology and ecology (Wang et al., 2011).

Bacillus licheniformis hydrolyzed feather to free amino acids which increased the growth chickens (Williams et al., 1991) while feather hydrolyzate obtained by B. licheniformis LMUB05 showed no significant effect on birds performance (Adetunji and Adejumo, 2018). For these reasons, biodegradation of keratins which serves as an actual source of nutrient-rich feeds, and feed supplements with lots of potential applications in animal husbandry needs more detail studied to enhance to biodegradation process using the best bacterium or keratinases (Nnolim et al., 2020).

Figure 5: Feather degradation production biofertilizer to enhance plant growth (Khalel et al., 2020).



Production of bio-fertilizer: Keratin composed of different amino acids and many microorganisms are able to degrade it by production of keratinase. An example for a keratinase-producing strain is *B. subtilis* which is demonstrated plant growth-promoting and broad-spectrum antimicrobial activities, as it produced indole acetic acid (IAA) and antifungal activities in the course of keratinase production (Jeong et al., 2010). Feather meal produced from the recycling of keratinous wastes is still applicable as a slow-release biofertilizer. Khalel et al. (2020) obtained feather hydrolysate through hydrolysis from *Streptomyces enissocaesilis* AM1which enhanced plant growth. It also enhanced inorganic elements and microbial activities in the soil, while the N, P, K and the C/N ratio was increased, thereby improving soil fertility (Figure 5).

CONCLUSION

Microbial keratinases have many industrial and biotechnological applications including management of wastewater, treatments and recycling leathers, feathers and agricultural wastes. They can be used in textile technology, food and feed preparations, medical and pharmaceutical applications, agriculture industries (bio-fertilizers and composting, plant-growth promotion), and bio catalysis preparations. Search for new sources of keratinases from bacteria using new techniques to increase enzyme production, decreased the production costs, enhanced the enzyme characters in addition to enzyme stability at high temperature and different pH are urgently needed. The multi-functionality of keratinases may encourage scientists for more studies which guide us for production of keratinase with excellent characters for bioconversion of feather or different keratinous wastes to free amino acids or bio-fertilizers.

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An Updated Review on Harmful Effects of Bisphenol a on Human Nervous System: Molecular, Cellular and Behavioral Aspects

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ABSTRACT

Bisphenol A (BPA) is an endocrine disrupting chemical that is widely used in the synthesis of polycarbonate and epoxy resins and plays A role in the manufacture of small plastic bottles, food packaging, water bottles, medical devices and dental sealants. In recent years, studies have shown that BPA has different harms on various human systems, including nervous system, urinary system, reproductive and immune system, digestive system, behavioral development, etc., and the harm of BPA to human body may last for several generations. In recent years, studies have shown that BPA has different harms on various human systems, including nervous system, urinary system, reproductive system, digestive system, and behavioral development, etc. the harm of BPA to human body may last for several generations. So we critically reviewed the recent literature on the role of BPA in the nervous system. At the same time, the role of BPA in nervous system is reviewed. This paper reviews the effects of bisphenol A on the nervous system from three aspects: molecular, cellular and behavioral development. It provides a material basis for the subsequent study of bisphenol A and nervous system.

KEY WORDS: BISPHENOL A, NERVOUS SYSTEM, NEURON, BRAIN ESTROGEN, NEURAL STEM CELLS.

INTRODUCTION

The nervous system is composed of the central part and the peripheral part, which plays a leading role in regulating the physiological functions of the human body. The functions of various organs and systems are directly or indirectly controlled and coordinated by the nervous system, making the human body into a unified whole (Jalilian, et al. 2019). The nervous system can accept all kinds of information about changes in the internal and external environment, analyze and integrate them, and make the body respond to stimuli accordingly, so as to maintain the unity between the body and the internal and external environment (Heuckeroth, Schafer 2016). In addition, the high development of cerebral cortex makes the brain become the highest center and the higher organ of thought and consciousness. Accordingly, nervous system has crucial effect to human body, substances that do damage to nervous system can produce adverse effect to many aspects of the human body. Bisphenol A inhibits the proliferation of neurons and neural stem cells (Liang, et al. 2020).

BPA has estrogen-like effects, and its exposure affects the synthesis of endogenous estrogen, leading to endocrine disorders and interfering with the human metabolic

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process (Spackova, et al. 2019). In this review, we mainly summarized the effects of BPA on the nervous system and the possible mechanism, which can provide a reference for future studies on the effects of BPA on human body.

Effect of BPA on estrogen in the brain: Aromatase is the rate-limiting enzyme in the process of estrogen synthesis, which can catalyze the conversion of androgen to estrogen. Bisphenol A increases the level of aromatase by enhancing the expression of brain-specific aromatase, thus increasing the endogenous estrogen level in the developing brain (Chung, et al. 2011). So BPA, a form of estrogen, interferes with estrogen dependent processes by binding to estrogen receptors (ERS) (Adewale, et al. 2011). As A powerful estrogen analogue, bisphenol A not only interferes with normal hormonal regulation in synaptic plasticity and memory in female mice but also shows estrogen-damaging effects at different concentrations of circulating estrogen (Xu, et al. 2015).

BPA exposure interferes with the development of signaling pathways in the brain such as estrogen, oxytocin and vasopressin that are critical for synaptic organization and delivery (Arambula, et al. 2018). Corticosterone and its role in the brain are easily influenced by the programming effects of current acceptable doses of BPA. Corticosterone levels rose in body exposed to low doses of BPA (Poimenova, et al. 2010). In addition, bisphenol A enhanced mRNA expression levels of extracellular signal regulated kinase (ERK), fatty acid amide hydrolase (FAAH) and sodium gated channel (NAV 1.8), leading to changes in the expression of estrogen and pain-related genes and increased migraine in mouse models (Vermeer, et al. 2014).

Environmental BPA exposure enhanced the biosynthesis of local estrogen in the brain and further inhibited the ER-β signaling pathway in the nervous system. Perinatal exposure to BPA not only significantly inhibited the expression of Nr1, Nr2A, and 2B of the NMDAR subunits in the developing hippocampus, but also decreased the expression of ER-Beta (Xu, et al. 2010, Xu, et al. 2010). Prenatal exposure to low doses of bisphenol A alters estrogen receptor expression levels in newborns of both sexes (Cao, et al. 2013). Fahrenkopf and others in the maternal exposure to bisphenol a mouse fetal estrogen receptor (ERalpha) dependence were used in this study the effects of progesterone receptor (PR) expression bioassay method, the results show that the gestation maternal exposure to bisphenol A (BPA) side inside the offspring pronucleus (MPN) of PR has enhanced the role of immune responses (PRR) level, and this effect is done through activation of estrogen to ERalpha, instead of ERbeta (Fahrenkopf, et al.2020). The results of Tonini, C et al showed that prenatal BPA exposure did not affect ERα phosphorylation in female fetuses but did affect ERα phosphorylation in male fetuses. In conclusion, the effect of BPA on the induction of estrogen receptor alpha is sex-dependent and more significant in males than females (Tonini, C, et al. 2020).

Effect of BPA on neurotransmitters: Perinatal exposure to low doses of BPA disrupts overall metabolism and brain function in CD-1 mice (Cabaton, et al. 2013). Low doses of BPA disrupt the serotonin system in prenatal and lactating mice and significantly increase dopamine, serotonin, and metabolites in the putamen, thalamus, and plasma of mice at 3 weeks (3 weeks) and/or 14 -- 15 weeks (14 weeks) postpartum (Sarrouilhe, Dejean 2017, Nakamura, et al. 2010). When the perinatal female mice exposed to bpa, like the 3-4-2 hydroxy benzene acetic acid (DA metabolites), temperature, acid (HVA DA metabolites), 5 - hydroxy indole acetic acid (5 - hydroxy indole acetic acid) and 5 - hydroxy ethyl amine acid ester (5 - glycolic acid) and many other chemicals in its descendants in the brain or blood increased, however, the proportion of HVA/DA only in certain areas of the brain (Honma, et al. 2006). So, not only are the effects of BPA different in different areas of the brain but the effects of BPA exposure are different in different genders.

Exposure to low doses of bpa causes of male mice, the amygdala and hippocampus of GABA levels drop, but some areas of Glu levels rising, however for perinatal and lactation female mice low dose of bisphenol A is not only the brain almost all areas of GABA (gamma-aminobutyric acid) and Glu (glutamic acid) levels, and lead to the cerebral cortex and the hypothalamus NE levels (Ogi, et al. 2015). Therefore, the effect of bisphenol A on females and males is different. Perinatal exposure to low doses of BPA resulted in reduced levels of neurotransmitters such as gamma-aminobutyric acid and glutamate in serum and brain samples of neonates with PND21 (21 days postpartum) (Cabaton,et al 2013). BPA may affect monoamine (which increases serotonin and dopamine) levels in newborns, although the dose of BPA is lower than the prescribed environmental level, 28 days after injection and 2 days after birth (Matsuda, et al. 2010).

Therefore, in the Tang and others with blood clam (Tegillarca granosa) as the research object, using exposure to accord with the actual concentrations of BPA and environment MPs to observe BPA and MPs alone or with the effects of exposure on the immune and nervous biomarkers of this study found that BPA not only has obvious immune toxicity, but also lead to treatment of BPA and MP NFkappaB signaling pathways in four immune related gene expression level serious inhibition (Tang, et al. 2020).

Exposure to BPA and/or DEHP induces apoptosis and histopathological changes in hippocampal cells. In addition, the mechanism of neurotoxicity induced by BPA and DEHP may be changes in oxidative/antioxidant status as well as neurotransmitters and related enzymes (Yirun, et al.2021). Maternal exposure to BPA reduced the levels of hippocampal neurotransmitters such as Glu/GABA in F1 offspring. The adverse neurodevelopmental effects of maternal exposure to environmental doses of BPA persisted into the offspring (Zhang, et al.2020).

BPA affects the dopamine system: BPA has an effect on the dopamine system. Studies have shown that when mice are exposed to BPA, the dopamine system of mice changes, and the changes are more evident during organ development and lactation (Narita, et al. 2007, Suzuki, et al. 2005). Long-term chronic exposure to BPA during organogenesis or lactation enhances the function of dopamine D1 receptors and activates G proteins in the peripheral brain (Suzuki, Mizuo, Miyagawa, Narita 2005, Suzuki, et al. 2003). Prenatal and neonatal exposure to BPA in rats results in enhanced central dopaminergic system, hypersensitivity to the abuse of reward-acting drugs, and hyperactivity (Suzuki, Mizuo, Miyagawa, Narita 2005).

BPA reduces the expression of the dopamine transporter gene in adult mice (Ishido, et al. 2007). Castro et al. found that BPA, BPF, and BPS had different effects on the expression of genes related to the 5alpha-R and DA/5-HT systems in female PFC (Castro, et al. 2015). The results show that BPA causes behavioral changes in zebrafish and the mechanism of these changes is the high accumulation and dysregulation of the neurotransmitter systems of serotonin, globulinergic, dopaminergic, cholinergic and GABAergic (Kim, et al. 2020).

Toxic effect of bisphenol A on DNA methylation: Environmental chemicals can affect human health and disease in ways that affect DNA modification. The epigenetic effect of bisphenol A was sufficiently demonstrated by the reduction of CpG methylation upstream of the acanthopteris gene, and in female acanthopteris the epigenetic effect is multigenerational (Singh, Li 2012). Prenatal exposure to low doses of BPA can cause long-term epigenetic damage to the brain (Kundakovic, et al. 2013). Early exposure to BPA causes epigenetic dysfunction and neurodevelopmental disorders by altering the brain's epigenetic mechanisms and gene expression levels (Kubota 2016). Exposure to BPA during the early stages of development leads to the continued accumulation of fat by reducing the methylation of fat genes (Shimpi, et al. 2017).

Exposure to bisphenol A in the fetal period can damage the naturally occurring bifeng DNA that is associated with obesity-related DNA methylation (Taylor, et al. 2018). 2.6- dibipa, tripipa and tibipa had the effect of increasing lipid accumulation and the expression of specific protein 2 in adipocytes, which increased dose dependence of PPAR gamma transcriptional activity. In addition, TeBBPA, debromide and bromide accumulating in breast milk play an important role in promoting adipocyte differentiation (Akiyama, et al. 2015). In addition, exposure to BPA resulted in reduced DNA methylation of germ cell imprinted genes IGF2R, PEG3, and H19 in fetal mice (Zhang, et al. 2012). Exposure to BPA altered the expression level of microRNA in human placental cell lines and the treatment of bisphenol A had a strong induction effect on miR-146a in particular (Singh, Li 2012).

Reactive oxygen species (ROS) are closely related to oxidative damage and carcinogenesis of cells, and bisphenol A can cause the production of ROS (Lei, et al. 2018). Low-dose BPA significantly promoted DNA damage (Pfeifer, et al. 2015). When human breast epithelial cells are exposed to BPA, it results in increased methylation of genes associated with the development of most or all tumor types, such as BRCA1, CCNA1, THBS1, TNFRSF10C, and TNFRSF10D (Qin, et al. 2012). After mothers were exposed to BPA, their offspring showed brain cell DNA damage, and this damage was only seen in the F1 generation (Zhang, et al. 2020). In addition to influencing the methylation patterns of genes such as those that encode proteins associated with reproductive physiology, BPA can have a direct effect on genes responsible for DNA methylation (Cariati, et al. 2020).

Effect of bisphenol A on neurons: BPA had no effect on nerve survival and nerve cell size in postperinatal mice, but the apoptosis of dopaminergic neurons in midbrain of weaned mice and even reduced motor neuron pool volume in adult mice (Lin, et al. 2006, Jones, et al. 2016). Fetal exposure to low doses of BPA inhibits the release of excitatory neurons in offspring and disrupts cortical neurogenesis and neuromorphic development during neuronal migration in the hippocampus, thus disrupting the localization of mouse offspring neurons and forming between the thalamus and cerebral cortical networks. The damage may even continue into adulthood (Mathisen, et al. 2013, Ling, et al. 2016, Kimura, et al. 2016). BPA is not an anti-androgen mechanism but acts through A non-androgen receptor-dependent mechanism (Jones et al 2016).

Bisphenol A phosphorylates NR2B of the NMDAR subunit through the estrogen receptor mediated pathway, and thus rapidly increases the activity and density of cultured hippocampal neurons (Xu, et al. 2011, Xu, et al. 2010). Bisphenol A reduces the differentiation of dopaminergic neurons by inhibiting the expression of IGF-1 (Huang, et al. 2017). After local injection of bisphenol A into primary visual cortex A17, the targeting selectivity of neurons was significantly increased, while the activity of neurons was rapidly inhibited and the activity of other neurons decreased (Xu, Ye, Li, Chen, Tian, Luo, Lu 2010). Exposure of Hess-derived embryoids to bisphenol A resulted in A decrease in the number of neural precursor cells (NPC) and Hess-C-derived large neurons (Huang, Ning, Zhang, Chen, Jiang, Cui, Hu, Li, Fan, Qin, Liu 2017).

The effect of bisphenol A on the number of different neurons is different. Studies have shown that 50 mg/kg or 50 μg /kg (BW) BPA can increase the number of oxytocin immune response neurons in PVN, but hardly change the serotonin fiber density or the number of eralphair neurons (Adewale, et al 2011). BPA affected an area of the MPFC (medial prefrontal cortex) associated with neurological disorders, but only in men and not in women (Sadowski, et al. 2014). Acute exposure to BPA, not through cortical interactions but through altered projections of the lateral geniculate nucleus, leads to limited visual perception in cats (Xu, et al. 2018).

BPA may interfere with the normal development of the cerebellum by affecting the developing cerebellum granule neurons (Mathisen, et al 2013). Maternal exposure to BPA resulted in a decrease in the number of hippocampal neurons and spinal density in the offspring, and this effect was observed in both F1 and F2 (Zhang, et al. 2020). Tang, C et al. showed that long-term exposure to low doses of BPA could inhibit the activation of AVPV-kisspeptin neurons, which were induced by Eralpha and prolonged the oestrus period and reduced ovulation in adult female mice (Tang, et al. 2020).

Effect of BPA on the proliferation of neural stem cells (NSC): NSC proliferation and differentiation are changed by BPA in vivo and in vitro studies (Tiwari, et al. 2015). Chronic exposure to BPA impairs autophagy-mediated mitochondrial transformation and leads to apoptosis of hippocampal neural stem cells (NSC) (Agarwal, et al. 2016). BPA had adverse effects on NSC proliferation and neuronal differentiation in hippocampus and SVZ. BPA inhibits the proliferation and differentiation of rat NSC through Wnt/ β-catenin signaling pathway, and enhances neurodegeneration. (Tiwari, et al 2015) (Tiwari, et al. 2016). Exposure to different concentrations of BPA has different effects on neural stem cells. High concentrations (> 400 microns) of BPA have cytotoxic effects on neural stem cells, while low concentrations of BPA have estrogenic activity and stimulate the proliferation or differentiation of NSC (Kim, et al. 2009, Kim, et al. 2007).

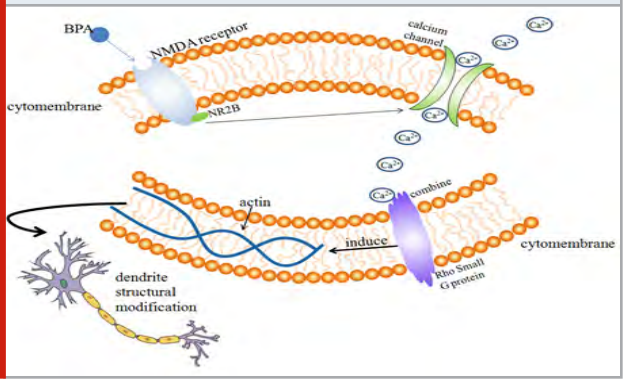
Estrogen mediated the proliferation of NS/PCs through nuclear cell mediated induction and had a positive effect on the proliferation or differentiation of neural stem cells (NS/PC) (Okada, et al. 2010). Bisphenol A enhanced the proliferation or differentiation of NS/PCs when the cells are poorly supplied with mitogens or differentiation factors such as FGF-2 in the early stages of neurogenesis (Okada, et al. 2008). Exposure to bisphenol A had positive effects on the cell cycle outlet of irradiated glial cells and iPS cells, and significantly reduced the proliferation caused by prolonged iPS cell cycle length in SVZ (Komada, et al. 2012). BPAF showed the strongest cytotoxicity on hesc and hesc derived neural stem cells (NSCs), while BPS showed the least cytotoxicity. Exposure to BPA and its derivatives causes lengthening of neurite length in neuron-like cells (Liang, et al. 2020).

Effect of BPA exposure on oligodendrocytes: The proportion of oligodendrocytes generated by neural stem cells/progenitor cells in total cells increased after treatment with E2 or BPA (Okada, Murase, Makino, Nakajima, Kaku, Furukawa, Furukawa 2008). Both in vivo and in vitro BPA exposure at postnatal days 21 and 90 altered the proliferation and differentiation potential of OPCs, and reduced the gene expression and protein levels associated with myelination (Tiwari, et al. 2015). Bisphenol inhibits OPCS differentiation, which is caused by thyroid hormone exposure (Seiwa, et al. 2004).

The proliferation and differentiation of central nervous system astrocyte progenitor cells and non-serum rat embryonic cells were not affected by low levels of BPA in serum-free environment. However, at a dose of 1-100 pg/ mL, low levels of BPA resulted in overactivation of signal transduction, transcriptional activator, and anti-decapida-paralysis homologue 1 (Smad1) mother cells, and significantly increased GFAP expression in SME cells (Yamaguchi, et al. 2006).

Effects of BPA on dendritic filament and dendritic spine: Dendritic spines are spinous processes on dendrites of neurons. The postsynaptic area of excitatory synapses receives external stimuli and regulates synaptic transmission by changing its shape and size (Koshida, et al. 2018). Actin cytoskeleton plays an important role in the morphological development of dendritic spines, actin is one of the main components of dendritic spine cytoskeleton (Hlushchenko, et al. 2016). Rho small G protein is an important cytoskeletal regulator of actin, which is involved in the regulation of neuronal morphological changes. The ability of dendritic filaments and dendritic spines to move rapidly in a short period of time is largely dependent on the regulation of actin cytoskeleton by environmental factors (Bryan, et al. 2004). After the activation of NMDA receptor, with the participation of Ca^{2+}, actin localization in dendritic filament and dendritic spine is regulated, resulting in of the morphological changes in dendritic spines (Furuyashiki, et al. 2002).

Figure 1: Possible mechanism of BPA on dendritic morphology and developmen



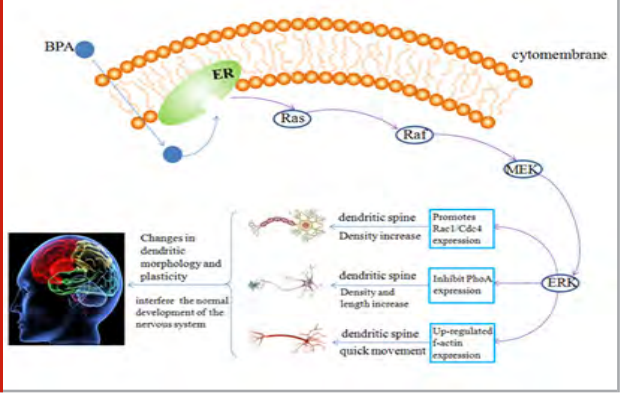
According to the previous experimental studies (Figure 1), it can be speculated that the possible mechanism of BPA's influence on dendritic morphology is that acute exposure of BPA for 30 min increases the phosphorylation level of NMDA receptor subunit NR2B by means of extracellular estrogen receptor mediation, after the activation of NMDA receptor, Ca^{2+} concentration increases and binds to Rho small G protein (actin cytoskeleton regulator) on the surface of the cytoskeleton, Rho GTPase regulates the cytoskeleton structure, so that the positioning and dynamics of actin in the dendritic filament can be changed, resulting in rapid movement of dendritic filament dendritic spine and ultimately affecting

dendritic morphology, (Bryan, et al 2004, Furuyashiki et al 2002).

Waters et al. found that chronic or acute BPA exposure is mediated by ERs inside or outside the nucleus of neurons, leading to changes in density of dendritic spines in hippocampal neurons, synaptic number and hippocampal dependent cognitive function (Waters, et al. 2009). The morphology of dendrites in neurons is closely related to Rho A Rac1 and Cdc42. Inhibition of Rac1 or Cdc42 expression can lead to decreased density of dendritic spines and dendritic filaments, while inhibition of Rho A expression leads to increased density and length of dendritic spines and dendritic filaments (Tashiro, Yuste 2004). BPA can promote the Rac1/Cdc42 hippocampal neuron to inhibit the expression of Rho A, and also greatly increase the expression of f-actin in dendritic filaments, so as to increase the length of dendritic branches and the density of dendritic filaments and promote the mobility of dendritic filaments. These changes are related to ERs mediated erk1/2 signaling pathway (Guangxia, et al. 2013).

According to the previous experimental studies (Xu, et al 2011, Waters, et al 2009, Guangxia, et al 2013), it can be speculated that the possible mechanism of BPA on dendritic morphology and development of hippocampal neurons is that chronic or acute BPA exposure promotes Rac1/Cdc42 inhibition of Rho A expression in hippocampal neurons through erk1/2 signaling pathway mediated by ERs inside and outside the nucleus of neurons, and increases the density and length of dendritic branch lengths and dendritic filament dendritic spines. At the same time, the expression of f-actin in the dendritic filament of hippocampal neurons was also up-regulated, which increased the number of synaptic spines density and hippocampal dependent cognitive function changes caused by the kinematics of dendritic filament, thus affecting the morphological development of hippocampal neuron dendrites and interfering with the normal development of the nervous system (Figure 2).

Figure 2: Possible mechanism of BPA on the morphological development of hippocampal neurons



Effect of bisphenol A on behavioral development: Even below the current reference safe daily limit of 50 µg/kg day set by the USEPA, some behaviors and neuronal morphology are also changed by the exposure of BPA in puberty and these changes can continue into adulthood (Bowman, et al. 2015). Perinatal exposure to BPA during brain development is obvious damage to the gender recognition memory space, resulting in female rats' spatial memory impairment and passivity male mice from memory, and this kind of behavior change is permanent (Poimenova, et al 2010, Jardim, et al. 2017).

The disruption of sexually dimorphic behaviors is related to persistent, sex-specific social and anxiety-like behavior of the BPA exposure (Kundakovic, Gudsnuk, Franks, Madrid, Miller, Perera, Champagne 2013). During exposure of rodents during perinatal period, long-term anxiety behavior occurred in adulthood (Zhou, et al. 2013, Xu, et al. 2012). Exposure to bisphenol A in pregnancy and lactation enhanced anxiety and depression in both genders. However, the difference was that exposure to bisphenol A in pregnancy had a stronger effect on women's anxiety, (Xu et al 2012).

Paternal exposure to bisphenol A strengthened the anxiety behaviors of F1 female as well as depression behaviors in both sexes of F1 rats (Fan, et al. 2018). Myelination in the hippocampus of the rat brain can be altered by exposure to bisphenol A during fetal and postnatal periods, which leads to cognitive deficits (Tiwari,et al 2015). When the mice were chronically exposed to low doses of bisphenol A, the brain cells numbers were damaged, and adolescent mice had lower learning and memory skills (Zhou, et al. 2017). One of the factors leading to the development of neurobehavioral disorders such as autism spectrum disorder, is thought to be exposure to bisphenol a during gestation (Harris, et al. 2017). Among children in the United States, there was a link between higher urinary BPA concentrations and ADHD, and these associations were particularly evident among boys (Tewar, et al. 2016).

Rats exposed to bisphenol A showed a range of behavioral changes, for example, the decrease in locomotion, the increase in the dislike of light and sound, the change of grooming habits and the enhancement of startle reflex (Vermeer, Gregory, Winter, McCarson, Berman 2014). During the development of zebrafish, the exposure of BPA changed the spontaneous movement, and significantly reduced touch response and swimming speed in response to light stimulation (Wang, et al. 2013). Rat fetuses exposed to BPA led to adult-onset obesity, this adult-onset obesity phenotype may be caused by the destruction of the physiological bimodal nature of epigenetic regulation of fggy in mouse WATs by prenatal exposure to bisphenol A (BPA), (Taylor, et al 2018).

When animals are exposed to low doses of bisphenol A, the development of their reproductive organs are disrupted and the effects on the brain's physiology are long-lasting (Panagiotidou, et al. 2014, Kawai, et al. 2003). During courtship, the treatment of bisphenol reduced the male locomotion, and was related to the decrease of female courtship behavior but more

aggressive toward mating with rivals (Wang, et al. 2017). When female offspring are exposed to 25 weight/kg/day bisphenol A daily, the brain revealed masculinization (Hass, et al. 2016).

Because of bisphenol A's estrogenic activity and endocrine disruptor capabilities, prenatal exposure to bisphenol A (BPA), even at very low doses, can have an impact on vertebrates in terms of neurological and behavioral sex differences (Ponzi, et al. 2020). Studies have shown that even low dose maternal BPA exposure can produce sex dependent learning and memory ability of F1 male mice, but has no significant effect on learning and memory ability of F2 generation male mice (Zhang, et al. 2020).

CONCLUSION AND OUTLOOK

From the existing research results, it can be found that it has different effects on the nervous system due to the differences in the exposure period, dose, time, location, sex, age and population of BPA. The effect of BPA on the nervous system is very complicated. BPA is an exogenous estrogen, which has the role of estrogen and antiestrogen. BPA acts as analogue to the estrogen receptors and interferes with normal levels of hormones in the body, leading to endocrine disorders.

Bisphenol A has a high lipid solubility, which is easy to pass through the blood-brain barrier and placental barrier. BPA accumulates in the brain and damages the development of the brain, leading to some abnormal behaviors. BPA can also enter the fetus and affect the growth and development of the fetus. In addition, BPA also reduces the proliferation and differentiation of neural stem cells and oligodendrocytes. Numerous studies have shown that BPA may cause diseases such as obesity, birth defects, breast cancer and so on.

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Author contributions: R Wang and N Li conceived and wrote the manuscript while Y Dong, Y Cai and G Ye revised the manuscript critically with substantial intellectual input. Q Wang supervised the development of the work, critically evaluated the manuscript with intellectual input. All authors approved the final version. J Cheng?

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Biosimilars Pharmaceutical Market in India: Current Status, Challenges and Future Perspective

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ABSTRACT

Biosimilars are the subsequent adaptations of the original biologic medicines and, these are manufactured with the purpose to provide remedial effects which are similar to the original drug. In the upcoming decade, there would be an increase in the number of existing biologics going off patents which would provide an opportunity for several innovator firms to offer services, specially designed for biosimilars. The share of the biopharmaceutical sector is forecasted to undergo expansion both in the Indian and global pharmaceutical market. But the biosimilar firms face many problems in the development, clinical improvement, manufacturing, registration and product marketing. Also, fierce ongoing competition in the market obstruct the entry of new players and restrain the growth of this market. There are several challenges such as variability in structure, immunogenicity, and regulatory barriers which further impede the growth of the biosimilars market. Thus, they require separate marketing approval since they are not generic versions of biologics. Hence, they need full documentation on quality, safety and efficacy. The regulatory environment of pharmacy across the world is getting more stringent and impacting exports. Several factors, such as a discrepancy in regulations, the funds allocated for investment and the ambiguity regarding the level of market maturity attained, have served as deterrents for Indian biosimilar players to participate actively in global markets. This paper aims to highlight the biosimilars market scenario in India and worldwide. In addition to that, it also discusses the significant challenges involved with biosimilars. It also contemplates some trends that reflect the bright future for biosimilars pharmaceutical market.

KEY WORDS: BIOPHARMA MARKET; BIOPHARMACEUTICAL PRODUCTS, BIOSIMILARS; PHARMA EXPORT.

INTRODUCTION

The pharmaceuticals market in India is very exclusive and has demonstrated very high potential in the last couple of decades. The sector has ranked tenth globally in terms of value and ranked third in terms of volumes. The Indian pharmaceutical market has the potential to reach USD 70 billion in future growth scenario. India's ranking is among the 12 top biotech destinations in the world with the third position in the Asia Pacific region. India's biotechnology segment one of the fastest-growing sectors with a turnover of $ 7 bn during the year 2015, and since then it has been growing at a rate of 16.3% annually (India Pharma, McKinsey & Company Report, 2020). Due to the increase in patent expiries for biologic drugs, there exists a valuable opportunity for the development of more productive biopharmaceutical industry in India.

Also, the remarkable success of a few recent launches has demonstrated the true potential of patented products (India Pharma Report, 2020). As compared with the previous years, the Indian biotech industry is budding

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at a faster speed, in FY16, it witnessed the growth of 57.14 per cent. The total industry size stood at US$ 11 billion in FY16, and it has reached to US$ 11.6 billion in FY17. This growth depends on several factors such as rising demand, rigorous research and development activities and healthy government initiatives which fast-paced the growth of this sector (Biotechnology Industry in India, 2017).

Figure 1: Global Pharma Market Size. Source-https://www.ibef.org › Industry.

According to the FDA definition, biosimilars are licensed by the FDA as they are identical to the approved reference product, and shown to have no clinical difference from the biologic reference product. The first biosimilar got approval in the year 2006 in the European Union. And since then the number of approved biosimilar drugs have reached more than 700 in numbers. The biosimilar guidelines of India are in regulations with the EMA and WHO. India pharmaceutical companies are enhancing their manufacturing skills, and for clinical trials, they are working together with pharmaceutical companies worldwide. Also, due to the cost advantage of lower manufacturing cost, India has more benefit than its contesting nations which will further create a favorable scenario for the biopharmaceutical market.

The Indian biosimilar market includes product segments such as insulin, G-CSF, vaccines, erythropoietin, interferon-alpha, hormones, fibrinolytic and plasma proteins. (Table-1). Among these, insulin occupies the largest market share, followed by erythropoietin and GCSF. With the suitability of biosimilars, their demand is higher in the domestic market. These are consumed mainly as a part of remedial action and treatment of incessant illnesses such as rheumatoid joint pain, kidney problems, diabetes, tumours, CVDs, immune system illnesses, development hormone inadequacy, haematological maladies, and irresistible infections (Biosimilar and Interchangeable Products – FDA, 2017).

By the year 2025, it is predicted that the international biosimilar market will reach around USD 46 billion. Now, globally there are many biosimilars in the process of manufacturing. There is immense potential for long term growth of biosimilars in established markets of Europe, Japan and the United States and consumers can pay for the same. However, there are few challenges for biosimilars growth, such as acceptance by both doctors and patients in terms of its safety, quality, effects on health, and precision in regulatory compliance.

Despite these challenges, there is an enormous capacity and high demand for biosimilar drugs in developed economies. While in the developing economies, consumers have fewer resources to pay for highly-priced biosimilar medicines. Therefore, in emerging economies, consumers have limited financial capacity and low affordability for biosimilars. Many studies have shown that the cost of biosimilar medicines is the main impediment in emerging markets. The developing world has different healthcare structure in place, and their respective authorities are now focusing on reducing the cost of biosimilars and enhancing access to medicines for their populations. However, the approval and regulatory procedures for biosimilars is also a matter of concern for both developed and developing economies (Indian pharmaceutical industry report, 2016).

RESEARCH METHODOLOGY

The main objective of this paper is to analyze the Indian biosimilar pharmaceutical market and its growth prospects. The present study discusses the biosimilar market's current status, growth pattern, various challenges and future perspective in terms of exports. The research also focuses on strict regulatory requirements in developed and emerging markets which obstruct the entry of new participants and averting the growth of this market. This study is an outcome of extensive literature review and analysis of data from various databases like Export and Import Bank of India, IBEF, McKinsey, Company reports etc. The study also included reports of the survey conducted by multiple pharmaceutical research firms.

Indian Biosimilar Market: Biosimilar products should have resemblance with the reference product in terms of quality, stability, characterization, specification, efficacy, safety, preclinical attributes, clinical attributes, pharmacokinetics and pharmacodynamics, toxicity and immunogenic studies (Study on the Indian pharmaceutical industry, 2015). India showed its acceptance towards the concept of 'similar biologics' in 2000, by approving it's first 'similar biologic' for a hepatitis B vaccine. In India, the development of biosimilars cost around 10-20 million USD due to regulatory procedures for their approval. And biosimilar manufacturer faces many problems in the development, clinical improvement, manufacturing, registration and product marketing in contrast with generics drugs (Rushvi P et al., 2016).

India has a huge share of the biosimilar market, and they will be expected to become a progressively vital part of the pharmaceutical ecosystem (Ray Tanmoy, 2017). In the domestic market, there are above 20 biopharmaceutical companies actively working on biosimilars development. Till date, more than 70 biosimilar products have been approved in India, and these figures are continuously increasing. Among these, more than 50 biopharmaceutical products have been permitted for marketing in India which includes monoclonal antibodies, etanercept, filgrastim, development hormones, proteins, insulins, interferons and streptokinase. And, with more than 60 biosimilars in the development pipeline, the industry is bound to establish itself in therapeutic areas such as cancer treatment, immunological disorders and diabetes. And biosimilar makers are specifically interested in leading biologics like Avastin, Humira and Levemir with recent patent expiry. Now, the Indian manufacturers are directing their concentration on more biosimilars production as many follow-on biologics are going off patent in the coming years. And it is anticipated that there will be a rise in the market share of follow-on biologics in the global biopharmaceutical market (Study on the Indian pharmaceutical industry, 2015).

Indian pharmaceutical companies have an enormous scope in the biosimilar market over other firms. Presently, India is booming as a significant contributor in the world biosimilar industry. One of the main strength India has is that it has the most significant number of USFDA approved manufacturing plants outside the US. Booming clinical trials and clinical research have added another feather in the cap of Indian pharmaceutical companies. Very low-cost infrastructures and highly educated citizens, and day by day, an increasing number of skilled forces provide the ideal combination for entering such a complex and non-established industry. However, there is a lack of specific regulations for the approval of biosimilars in India. Hence, to raise India from a complex, competitive environment and shine as a leading producer of biosimilars calls for an immediate need for the establishment of proper regulatory standards in India (Biosimilars Market Global Scenario, Market Size, Outlook and Trend, 2018).

Biosimilars Market: Global Scenario: Globally, from 2017 to 2025, the biosimilars market is predicted to reach $46.0 billion, which is rising at a CAGR of 33%. The global biosimilar market has been segmented based on geography and product class. Geographically, it is segmented into Europe, Asia Pacific, North America and Rest of the world. Around 35% of the global market share is accounted for by Europe, which is the largest, followed by the Asia Pacific and North America, with 30% and 27% global market share, respectively. The rest of the world has a share of 8%. (Biosimilars Market Global Scenario, Market Size, Outlook and Trend, 2018).

And the product section of biosimilars is segmented as Recombinant non-glycosylated proteins such as insulin, interferons, G-CSF, development growth hormones. Recombinant glycosylated proteins such as monoclonal antibodies, erythropoietin, follitropin. And, recombinant peptides like glucagon and calcitonin (Biosimilars Market Segmentation by Product Type, Global Demand Analysis & Opportunity, 2019).

Europe has the largest market share in terms of revenue due to the presence of well-defined regulatory framework for major biopharmaceutical companies such as Pfizer, Merck, Johnson & Johnson, Novartis, AstraZeneca, GlaxoSmithKline and Sanofi. Furthermore, well-developed healthcare infrastructure and a rising number of product launches have driven regional market growth. The biosimilars markets in Europe have evolved to a great extent with a large area of single payors. Europe has been a pioneer in the biosimilar regulatory landscape and the European Medicines Agency corroborated a set of stringent rules and regulations that biosimilars manufacturers must adhere to evaluate and approve their biologics centrally.

Figure 2: Global Biosimilars Market Size and Forecast. Source-https://www.variantmarketresearch.com

The centrally governing bodies decide on the approval and indications of new biologics. And then the individual countries can postulate their specific policies about price, purchase, utilization of biosimilars and the decisions for interchangeability. Thus, the developed markets in Europe are more established, and they demonstrate mixed levels of biosimilars penetration. There is an increase in demand for biosimilars due to various factors such as level of awareness about biosimilars among doctors and pharmacists, different incentives, purchasing policies and distribution channels for these medicines. The incentive and purchasing policies for biosimilars are diverse across all European countries, which leads to special procedures to access different markets. The penetration level of biosimilars, in conjunction with other factors, will regulate the level of competition and price erosion in established markets.

In Europe, the level of biosimilars acceptance varies from country to country. The countries named Finland, Poland,

Bulgaria and Denmark are witnessing more extensive penetration of biosimilars products than other countries such as the UK, France and Germany. Some trends observed in the UK has the highest share for GCSF, i.e. 98% and only 6% share for Epoetin alfa. Another trend observed is that Insulins and Follitropin alfa biosimilars with the highest being 35% have less market penetration in most countries in Europe.

Table 1. Examples of biosimilars approved in India by top 20 pharmaceutical companies dealing with biosimilar segment

Product name	Active substance	Therapeutic area	Approval date in India	Pharmaceutical Company
AbcixiRel	Abciximab	Autoimmune disease	23-Apr-13	Reliance Life Sciences
Basalog	Insulin glargine	Diabetes	2009	Biocon
Biovac-B	hepatitis B vaccine	Hepatitis B	2000	Wockhardt
CanMab	Trastuzumab	Breast cancer	23-Oct-13	Biocon
Choriorel	chorionic gonadotrophin hormone	Female infertility	22-Jun-11	Reliance Life Sciences
Cresp	darbepoetin alfa	Anaemia, Cancer, Chronic kidney failure	23-Mar-10	Dr. Reddy's Laboratories
Epofit/Erykine	epoetin alfa	Anaemia, Cancer, Chronic kidney failure	Aug-05	Intas Pharmaceuticals
Exemptia	Adalimumab	Rheumatoid arthritis	25-Sep-14	Zydus Cadila
Glaritus	insulin glargine	Diabetes mellitus	Mar-09	Wockhardt
Intacept	Etanercept	Ankylosing spondylitis, Psoriatic arthritis, Rheumatoid arthritis	Mar-15	Intas Pharmaceuticals
MabTas	Rituximab	Lymphoma, Non-Hodgkin's Lymphoma	26-Feb-13	Intas Pharmaceuticals
Neukine	Filgrastim	Neutropenia, Hematopoietic stem cell transplantation, Cancer	Jul-04	Intas Pharmaceuticals
Neupeg	Pegfilgrastim	Cancer, Neutropenia	Aug-07	Intas Pharmaceuticals
Peg-grafeel	Pegfilgrastim	Cancer, Neutropenia	10-May-11	Dr Reddy's Laboratories
Razumab	Ranibizumab	Degenerative myopia, Diabetes complications	19-Jun-15	Intas Pharmaceuticals
Reditux	Rituximab	Leukaemia, Lymphoma, Rheumatoid arthritis	30-Apr-07	Dr. Reddy's Laboratories
Religrast	Filgrastim	Neutropenia	2008	Reliance Life Sciences
Repoitin	Erythropoietin	Anaemia, Chronic kidney failure	29-Nov-11	Serum Institute of India
RituxiRel	Rituximab	Non-Hodgkin's Lymphoma, Rheumatoid arthritis	12-Feb-15	Reliance Life Sciences
Wepox	epoetin alfa	Anaemia, Cancer, Chronic kidney failure	Mar-01	Wockhardt
Wosulin	human insulin	Diabetes mellitus	13-Aug-03	Wockhardt

Source: CDSCO (Central Drugs Standard Control Organization)

ABBREVIATIONS
FDA: Food and Drug Administration
EMA: European Medicines Agency
WHO: World Health Organization
G-CSF: Granulocyte-colony stimulating factor
CVD: Cardiovascular Disease
USD: United States Dollar
US: United States
UK: United Kingdom
CAGR: Compound Annual Growth Rate
DPCO: Drugs Price Control Order
NLEM: National List of Essential Medicines
CBDT: Central Board of Direct Taxes
MCI: Medical Council of India
CDSCO: Central Drugs Standard Control Organization

In the present era, Europe is leading in the global biosimilars market followed by the Asia Pacific, which accounted for a chief market share in this segment. In Asia Pacific countries, the low manufacturing cost of biosimilars, skilled labour force, rising demand for less expensive therapeutic products and high prevalence of chronic diseases are factors contributing to the provincial biosimilars market growth. Another critical factor that drives this market is increasing focus on biosimilar product developments by countries such as China, India, and South Korea. North America is also expecting to experience substantial growth in the biosimilars market due to rising efforts from manufacturers to tap growth opportunities in the U.S. and Canada. In March 2009, the U.S. biosimilar regulatory pathway was established, and from then only this segment has gained significant drive in the market, which presents new challenges and opportunities. In March 2015, the U.S. FDA approved the first biosimilar product named Zarxio (Biosimilars Market Global Scenario, Market Size, Outlook and Trend, 2018).

Exports of Indian Biosimilar Products: By 2030, India will become the sixth-largest market for pharmaceuticals, and it has firmly established itself in the global biopharmaceutical market. Many of the Indian pharmaceutical companies are preparing to step into the global biosimilars market. As per the report of Associated Chambers of Commerce of India's 2017, biosimilars represent a 30% compound annual growth rate. They are worth $2.2bn out of the $32bn total Indian pharma market and are estimated to reach $40bn by the year 2030. The expiry of a range of biologic patents in the upcoming years will further aid this growth. At present, several pharmaceutical companies are starting to pursue regulated markets. In India, there is an active pipeline of biologics segment in the list of the top pharmaceutical firms named as Intas Biologicals, Biocon, Dr Reddy's Laboratories, Zydus Cadila, Reliance Life Sciences, Lupin Pharma, Wockhardt etc (Indian biosimilar market to be worth $40 billion by 2030, 2016).

As compared to small-molecule generics, wherein cutthroat competition and suppression of prices due to numerous extrinsic and intrinsic factors hinder the profit-making potential of the market, the growth opportunities are considerably more lucrative in the biosimilar sector. The Indian biosimilar industry is approaching substantial advancements owing to a peak number of approvals in the domestic market, active commitment in semi-regulated markets and a burgeoning position in the regulated markets. This development is being driven by growing market maturity in Europe, USA and other countries, and it requires a forthcoming environment for smooth regulatory approvals and high unmet clinical need across different markets. And there are many other factors like the significance of investment in this segment, discrepancy in regulations and insecurity about the market maturity levels have aided as constraints for Indian biosimilar players to engage in regulated markets actively (Nawrat Allie, 2018).

The renowned pharmaceutical firms worldwide are establishing partnerships with Indian pharma companies which reflects the growth of a promising market of biosimilars in India. The Roche Swiss-based pharmaceutical firm moves into an agreement with Emcure, which is an Indian firm to market the drug named Biceltis for cancer treatment. Another renowned pharmaceutical firm Mylan has established a partnership with Biocon, which is Bangalore based pharmaceutical firm. Both companies now working together and have made significant development by getting approval for biosimilars in both developed markets of Europe and the US. In the year 2018, Biocon revenue growth is $120m, and this firm recorded 36% growth from there biosimilars business. And in the same year partnership of these firms, Biocon and Mylan produced a biosimilar drug named fulphila (trastuzumab) which is approved by the US FDA. This drug is shown to decrease febrile neutropenia while cancer patients go through chemotherapy. It is the first biosimilar manufactured by an Indian pharmaceutical firm which got approval in the US. And currently, Fulphila is under review in Australia and the European Union, and many other biosimilars are going through these processes to enter the global market (Indian biosimilar market to be worth $40 billion by 2030, 2018).

In the upcoming decade, there would be an increase in demand for biosimilars worldwide which will drive the biopharmaceutical industry in India. As per the report by Crisil Research, from period 2015-2021, the pharma industry in India is expected to grow at 12-14% CAGR. There are various factors which improve the growth of the biopharmaceutical sector in India, which includes the introduction of new molecules by innovators, drugs going off-patent, upsurge in ageing population and increase in the number of chronic illnesses worldwide. With the emergence of private manufacturers, the industry landscape has undergone a drastic change, and the sector is being focused upon more. The augmentation in patent expiry for biologic drugs has birthed new opportunities for the Indian biosimilar industry. Furthermore, the domestic market highly favours the production of biosimilars due to lesser cost implications as compared to other players in the global market. This has led to improved manufacturing abilities of the Indian biopharmaceutical companies, quality standards, and well-nurtured collaborative relationships with MNCs for conducting clinical trials will further aid the robust growth of this market (Indian Biosimilars Industry-Roadmap to Actualize Global Leadership, 2018).

Challenges Faced by the Biosimilar Pharmaceutical Industry: The regulatory environment of pharmacy across the world is getting more stringent. And to compete in the global market, the Indian pharmaceutical industry needs a robust regulatory set-up in place. However, currently, the pharmaceutical sector is grappling with several issues like delays in clinical trial approvals, the new pharmaceutical pricing policy, a uniform code for sales and marketing practices, compulsory licensing, manufacturing quality, regularity uncertainty, reluctance in prescribing, complexities in the production and competition all of which need immediate attention.

Deferral of Clinical trials Approvals: These are the gold standard processes which determine the safety and effectiveness of these drugs, and they must establish before regulatory approval. India is becoming a knowledge hub for pharmacy, research and development, and clinical trials. These clinical trials are required for the growth of the pharmaceutical industry to foster cost-effective treatment for different ailments such as diarrhoea, tuberculosis, malaria, meningitis etc. to benefit from opportunities provided by biosimilar drugs. And regulatory delays in the clinical trials are severely hampering this possibility. It has disturbed the innovation curve as well as the growth of the clinical trial industry. Furthermore, issues such as ineffective regulatory oversight, need for safeguards for informed consent for vulnerable populations and compensation guidelines for patients for trial-related deaths have emerged as significant concerns. As a result, because of the mentioned limitations during clinical trials, our country is missing out on many opportunities.

National Pharmaceutical Pricing Policy: Pharmaceutical price controls are, in effect worldwide. The Indian government has developed the capacity of the Drugs Price Control Order (DPCO) by this policy to include all the drugs in the National List of Essential Medicines (NLEM). They have changed the formula from a cost-based method to a market-based approach to reach the maximum price limit. By this policy, the pharmaceutical firms are feeling the effects of the price controls on their top line drugs which will have a negative impact in short course. However, the adoption of refined strategic measures will negate this impact to a large extent in the long term. There is one issue which has severely impacted the pharmaceutical industry is the timeline for the implementation of DPCO. The pharmaceutical industry felt that the government did not provide sufficient time for implementing the new packaging and labelling with the revised prices. There is no clarity regarding location, when and where packaging and labelling exercises could be undertaken. Due to this, some pharma companies go to court to get an extension while others who couldn't go in time are still suffering. This problem can be avoided through the right consultation and by giving adequate time to the firms for the implementation of the revised prices.

Uniformity in sales and marketing practices protocol: The Department of Pharma has given guidelines on uniformity in sales and marketing practices protocol. These guidelines applied to all pharmaceutical firms to streamline marketing efforts and prevent corruption. But the Department of Pharma guidelines differs from the MCI guidelines on sales and marketing practices. And the tax authorities use the Central Board of Direct Taxes (CBDT) circular based on MCI guidelines to decide on permitted sales and marketing expenses. So, due to different benchmarks between the directions of Department of Pharma and MCI. There is an increased demand for clarity, both from the perspective of the tax authorities and the pharmaceutical industry.

Compulsory licensing: The pharmaceutical industry is already following strict rules and regulations on manufacturing and quality practices for drug development both in domestic and international markets. The blanket practice of compulsory licensing will destabilize both the Indian as well as foreign biopharmaceutical companies. There should be an equilibrium between the need for the affordability of drugs and intellectual property protection. The intention of the government to ensure the availability of patented medicines at a reasonable price is noble, but there are other ways of achieving the same goal. Manufacturing quality: The Indian pharmaceutical industry is efficient, which is making affordable medicines not only for the Indian market but also exporting these drugs to the world. The increasing confidence of foreign markets for the drugs manufactured in India is vital. For that, the authorities need to set quality standards as par with the global standards through appropriate legislation. Effective enforcement and compliance with these standards also need to be monitored.

During last year, the pharmaceutical export from India to the US increased to 32%, and India has become the biggest supplier of medicines to the US. Now Indian pharma firms are drawing more massive FDA scrutiny for manufacturing and quality compliance. For India to continue exporting to the foreign markets, the manufacturers will have to step up their quality and curate manufacturing compliance programs which are in line with the regulations. Addressing the above challenges, holistically will strengthen the sector, which constitutes a significant part of the Indian economy. Regulatory Uncertainty: Unpredictability and inconsistency exist in the regulatory scenario governing biosimilars. In the year 2010, the Biosimilars Act, also recognized as the Biologics Price Competition and Innovation Act, was passed to set a standard for the approval process for biosimilar medicines. The act defined the pathway for approval

and the timeline for biosimilars. The act also authorizes the FDA to undertake identical measures. This act has given six guidance documents to explain provisions of the Biosimilars Act, which strengthened the standards for some restrictions and added new restrictions.

The Reluctance in Prescribing: Biosimilar drugs are produced using a living system, or genetics which significantly affects the safety and efficacy of the therapeutic molecules. Even a minor change in the formulation or process can change the final product drastically as compared to generic drugs. Slight changes in the manufacture of biosimilar medicinal products can affect the efficiency and efficacy of therapeutic molecules to a very great extent. Physicians have been reluctant to adopt biosimilars unless these agents show useful clinical data. So, many healthcare providers are unwilling to prescribe biosimilar products, which pose a major challenge to the growth of the market.

Complexities in Production: The cost indulges in developing biosimilar drug is higher than generic drugs. Also, biosimilars production is a complex process which involves exact copying the structure of the original biologic. Thus, the biosimilar manufacturing incurs a high cost, time and risk in comparison to generic drugs. And this production cost is passed on to the consumers in terms of higher prices.

High Competition: The competition with the reference products threatens the position of biosimilars in the market. The discounts in this sector stem from rebates and service contracts for branded biologics, which is not the case in generic drugs that are often generously discounted, thus reducing the appeal of biosimilars. In case of complicated or chronic biologic treatments, the demonstration of the benefits of trading in biosimilars and convincing the stakeholders could consume more time. The biopharmaceutical companies must devise suitable strategies to mitigate the risk emanating from the above-discussed challenges for continual and compliant growth over the next decade (Indian pharmaceutical industry: Challenges and Prospects, 2016).

RESULTS AND DISCUSSION

Future Perspective: The growth of global biopharmaceutical market is influenced by various factors such as desired results of clinical trials, emerging pressure to diminish healthcare expenditure, increase in the incidence rate of chronic diseases, and rising demand of biosimilars for different ailments such as rheumatoid arthritis, blood disorders, cancer etc. The share of the biopharmaceutical sector is forecasted to undergo expansion both in the Indian and global pharmaceutical market. In the upcoming decade, there would be an upsurge in the number of existing biological drugs going off-patent, which leads to a rise in demand for biosimilar drugs. However, certain challenges such as manufacturing complexities, novel strategies by biologic drug manufacturers, costs, stringent regulatory requirements in developed and developing countries will hinder the entry of new players and restrict the development of this market.

By looking at the trends in the most optimistic scenario, it is predicted that in India by the year 2030, the biosimilar pharmaceutical market worldwide will be of $240 billion, and the domestic market reach will be around $40 billion. Therefore, the Indian biopharmaceutical firms can attain specialization in the biosimilar sector and further propagate it to established markets. Thus, making India a leading contributor towards this segment and this necessitates the existence of a specified and streamlined process so that the Indian manufactured products can be at par with the globally accepted standards, and more export opportunities can be harnessed. These measures will adequately supply India with ammunition to compete with other developed countries in terms of regulatory aspects and export of biosimilars. Thus, it is essential to foster the vibrant industry landscape and support the biosimilar pharmaceutical industry in real value realization. It is pertinent to impart necessary information and accurate inventory to all the participants. So, they can be well prepared to contest in commercial encounters both in domestic as well as international markets.

Conflict of Interest: The authors as named Gyan Prakash Ujalayan, Shibu John declares that there is no conflict of interest.

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The Deleterious Health Effects of Aluminium: An Updated Review

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ABSTRACT

Aluminium is a frequently used metal for food processing and packaging. It is a choice of metal for food processing as it is light-weight and possesses excellent heat conductivity. In addition, it can be easily molded and therefore used in food packaging. Aluminium foils and cans are very popular for storage of food for short and long duration of time, respectively. In spite of its voluminous use in food industries, there are growing concerns of aluminium-associated health risks in human. It is reported that aluminium leach-out from the storage vessel or foil and contaminate the food material. The aluminium leaching is more during heating and in the presence of acidic contents in food. Over the course of time, aluminium accumulates and is stored predominantly in lungs, bones, liver, kidneys and brain. Researchers are investigating the level of aluminium accumulation in body and its effect in developing diseases. Several reports highlighted that aluminium increases the risk of Alzheimer's disease and other neurological disorders. In addition, the high internal concentrations of aluminium may induce convulsions, esophagitis, gastroenteritis, kidney damage, liver dysfunction, loss of appetite, loss of balance, muscle pain, psychosis, shortness of breath, weakness, fatigue and birth defects in new born. However, a systematic investigation is required to stablish the relationship between aluminium and its deleterious effects in human. The present work highlights application of aluminium in food, its route of entry in body, affected organs and developing disease. The alternatives to the aluminium in food processing and packaging are also highlighted.

KEY WORDS: ALUMINIUM, ACCUMULATION, ESOPHAGITIS, GASTROENTERITIS.

INTRODUCTION

Aluminium is the third most abundant element in the earth's crust (Stahl, 2011). In 1825, it was isolated by the Danish physicist Hans Oersted. Most aluminium is stably bound as an ore in clay, minerals, rocks and gem stones This lightweight, non-magnetic, silvery white-coloured metal can be produced from the aluminium ore—bauxite—by a high energy consuming mining process; it is this process which provides the world its main source of the metal. As a consequence of this technological progress, aluminium has become increasingly bioavailable for approximately the past 125 years. Food additives, drinking water and leaching from aluminium cooking utensils are some of the sources of exposure to aluminium. Minimal exposure of aluminum to our bodies is not a problem. Human bodies can excrete small amounts very efficiently; an aluminum tolerable daily intake of 1 mg/kg body weight /day has been established by the World Health Organization (WHO) of the United Nation (UN) (Exley 2013; Gupta, 2019).

In the medicine field, aluminium compounds are now widely used being in the composition of numerous pharmaceutical conditionings (e.g., antacids, phosphate binders, buffered aspirins, vaccines, or antiperspirants), making them a potential threat (Spencer, 1979;

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Kramer, 2014; Gupta, 2019). To date, the main known toxicological effects of aluminium included anaemia, neurodegenerative disorders such as Alzheimer disease and dementia, amyotrophic lateral sclerosis, hepatotoxicity, or diverse reproductive disorders (Jabeen, 2016, Muselin, 2016). Other symptoms that have been observed in individuals with high internal concentrations of Aluminum are colic, convulsions, esophagitis, gastroenteritis, kidney damage, liver dysfunction, loss of appetite, loss of balance, muscle pain, psychosis, shortness of breath, weakness, fatigue and birth defects in new born (Khalil, 2014).The analysis of Al is challenging because of its low concentrations in some foods and the potential for contamination during sample preparation and analysis (Saiyed, 2005; Klotz, 2017).

Figure 1: A representation of human exposure to aluminium and its impact on the body. Source: (Kramer, 2014).

Exposure of Aluminium: Aluminium has long been established in medical applications as, e.g., an adjuvant in vaccines and an agent against pathological hyperhidrosis with a low side-effect profile. It is a natural component of drinking water and foodstuffs and is a component of many manufactured materials (Stahl, 2011). Aluminium compound is used in many diverse and important industrial applications such as alums (Aluminium sulphate) in water treatment and alumina in abrasives and furnace lining (Klotz, 2017).

The internal exposure levels of those exposed in workplaces where aluminum welding is carried out, during electrolysis in aluminum production, or in the processing industries (e.g., foundries, powder production) can be significantly higher compared with individuals not exposed to aluminum at work, meaning that the reference values derived for the general population may be exceeded in these workers. Longitudinal studies on aluminum welders revealed that the aluminum content in welding fumes correlated with aluminum concentrations in blood and urine. Aluminium exposure from drinking water has been extensively investigated in relation to the development of neurological disorders, including AD, due to the proposed enhanced bioavailability of aluminium in this form (Krewski, 2007; Klotz, 2017). Aluminium in foodstuffs: Aluminium has been shown to enter the human body predominantly through the oral route, as it is present in food, food additives, pharmaceuticals, utensils, and water (Landry, 2014).

Table 2. Aluminium content of various foodstuffs and spices (n = 3) (Semwal, 2006)

Food	Al concentration (mg kg−1)
Rice (*Oryza sativa*)	0.9 ± 0.001
Wheat (*Triticum aestivum*)	1.2 ± 0.10
Bengal gram dhal (*Cicer arietinum*)	4.7 ± 0.06
Kabuli channa (*Cicer arietinum*)	5.2 ± 0.02
Red gram dhal (*Cajanus cajan*)	3.2 ± 0.009
Black gram dhal (*Phaseolus mungo*)	4.1 ± 0.03
Onion (*Allium cepa*)	0.8 ± 0.004
Garlic (*Allium sativum*)	1.1 ± 0.01
Clove (*Eugenia caryophyllus*)	0.45 ± 0.002
Cinnamon (*Cinnamomum zelyanicum*)	1.6 ± 0.03
Red chilli (*Capsicum annuum*)	0.51 ± 0.003
Turmeric (*Curcuma longa*)	0.89 ± 0.007
Cumin (*Cumimum cymimum*)	0.72 ± 0.005
Pepper (*Piper nigrum*)	3.2 ± 0.06
Coriander (*Coriandrum sativum*)	0.95 ± 0.006
Cardamom (*Elettaria cardamomum*)	0.49 ± 0.002
Black cardamom (*Amomum subalatum*)	0.84 ± 0.003
Fenugreek (*Trigonalla foenum-graecum*)	0.78 ± 0.002
Mustard (*Brassica juncea*)	0.48 ± 0.08
Mean ± SD.	

Aluminium ingestion could result from: (1) contamination of food via leaching from cooking utensils; (2) storage of food in contact with aluminium; (3) aluminium salts added to water during purification; (4) aluminium compounds added to food, e.g., aluminium in baking powder; (5) aluminium in vegetables (plants assimilate aluminium to varying degrees depending on species, the availability of aluminium in the soil, soil pH etc.); (6) use of aluminium containing drugs (Tennakone, 1992). The most recent analysis shows that to meet the current annual global demand for aluminium 11 kg of the metal must be cast for every person on Earth (Exley, 2013).

It is reported that aluminium salts can be absorbed by the gut and concentrated in various human tissues including bone, parathyroid, and brain. Aluminium bioavailability from occupational inhalation exposure

is ~ 2% whereas oral aluminium bioavailability from water has been reported to be 0.1 to 0.4% (Krewski, 2007; Bassioni, 2012). Owing to acid rain, numerous metal ions, including aluminium are escaping from mineral deposits where they had been stored for billions of years as hydroxy-aluminosilicates (HAS), increasing the biological availability of aluminium to living organisms. According to this hypothesis, acid rain is acting as a key to the lock for aluminium release, causing its appearance in polluted waters (Crisponi 2013).

Table 2. Aluminium content of various foodstuffs and spices (n = 3) (Semwal, 2006)

Source	Amount
Natural sources	2 – 5 mg/day
Tea leaves	0.1 % – 1 %
Coffee from aluminium moka	0.8 – 1.2 mg/cup
Drinking water	0.07 mg/l
Beverages in aluminium cans	0.04 – 1.0 mg/l
Cooked spinach	25 mg/kg
Unprocessed food	0.1 – 7 mg/kg
Food additives	10 – 20 mg/day
Food cooked in aluminium pots	0.2 – 125 mg/kg
Soy-based infant milk formulas	6 – 11 mg/kg
Antacids	35 – 200 mg/dose
Buffered aspirin	9 – 50 mg/dose
Antidiarrhoeal drugs	36 – 1450 mg/ dose
Antiperspirants	50 – 75 mg (daily exposure)
Vaccines	0.15 – 0.85 mg/ dose
Parenteral nutrition solutions for Adults	40 – 135 µ g/l
Parenteral nutrition solutions for Infants	10 – 270 µ g/l

European Food Safety Authority (EFSA) issued an opinion on the safety of aluminium from dietary intake in which the typical aluminium content of unprocessed foodstuffs was reported at less than 5 mg per kg food, but it also referred to higher levels of 5 to 10 mg/kg. Based on animal studies, the EFSA derived a tolerable weekly intake of 1 mg aluminium per kg body According to the EFSA assessment, the dietary intake of aluminium in the general population is between 0.2 to 1.5 mg per kilogram of body weight per week, equivalent to a daily intake of 1.7 to 13 mg of aluminium for a 60 kg adult. Human exposure is divided into two categories, "external contact" and "dietary contact"; examples of these two categories are presented in Table 5 (Stahl 2017; Sander, 2018).

The beneficial effects of aluminium-containing antacids for the treatment of peptic ulcer are well recognized. However, these antacids can cause adverse reactions. It is the aluminium which interacts in the intestine with anions, such as phosphate and fluoride, and affects the absorption of these dietary and possibly also endogenously secreted elements. Aluminium forms insoluble complexes with the dietary phosphate which becomes unavailable for absorption (Spencer, 1979). It is one of the common practices to wrap meat items in Al foil for baking and grilling. Aluminium can be toxic to bone, bone marrow and the nervous system (Kaiser, 1985; Jabeen, 2016).

Routes through which aluminium enters into the human body: Ingested metals may be considered in two categories: those soluble throughout the potential pH range of the gastrointestinal lumen (approximately pH l-s), such as Na, Mg and Ca, and those susceptible to hydroxy-polymerization, such as Al, Cu, Fe, Mn and Zn (Kaiser, 1985). The inhalation of Al via mouth may result in absorption across the lung epithelia or the deposition of Al in the lung and its subsequent passage to the gut . the mucociliary pathway may be the principal mechanism by which Al in the lung become systemic (Exley, 1996; Flarend, 2001).

The skin: The outer epidermis or stratum corneum of the skin is an enucleated layer of keratin-rich cells held within a predominantly lipid intercellular matrix. Transport of topically applied aluminium, such as an antiperspirant or a sunscreen, across this layer would involve passive diffusion by both trans- and paracellular routes and is expected to be minimal. Aluminium chlorohydrate (ACH) is a water-soluble aluminium complex (Covington, 1990) which is the active ingredient in some antiperspirants. They suppress eccrine sweating by forming a hydroxide precipitate in the sweat duct or by denaturing keratin in the cornified layer that surrounds the opening of the sweat duct. Other antiperspirants are made from similar aluminium salts which may also contain zirconium (Laden, 1988; Robert, 2001; Flarend, 2001). It is believed that ACH acts as an antiperspirant by precipitating inside the eccrine sweat glands to produce insoluble aluminium hydroxide, which then plugs the gland and blocks the secretion of sweat (Flarend, 2001; Exley, 2013).

The Nose: It has been suggested that Aluminium may directly enter the brain from the nose through olfactory neurones, which run from the roof of the nasal cavity to the olfactory bulb. Inhalation exposure results from cosmetic, occupational and environmental Al sources (Robert 2001). The inhalation of Al via the mouth may result in absorption across the lung epithelia or the deposition of Al in the lung and its subsequent passage to the gut. This mucociliary pathway may be the principal mechanism by which Al in the lung becomes systemic (Emily 1994).The cilia of the olfactory epithelium are nonmotile and aluminium impacting upon this surface will be presented with a large surface area for association with this surface and for dissolution into the mucus layer covering the epithelium. The olfactory epithelium is essentially continuous with the olfactory nerve and olfactory bulb and presents an uptake route for aluminium, as complexes or particulates, into the brain (Exley, 2013).

The Lung: Absorption of Inhaled Aluminum Although inhalation exposure is not likely to be of concern to the general population, miners, smelters, and other metal workers can be exposed to toxic levels of aluminum through dusts and aerosols. It has been estimated that about 3% of aluminum is absorbed into the blood from the lung (Emily 1994).The lung epithelia are diverse in respect of their composition of different cell types and, in the alveolar epithelium in particular, myriad transport proteins and channels. The highly dynamic nature of the lung epithelium means that it must be a site for the accumulation of aluminium and a surface for the uptake of aluminium into lung tissues and access to the systemic circulation (Exley, 2013).

Table 4. Aluminium in foodstuffs (milligrams per kilogramme or milligrammes per litre). Source: (Stahl, 2011; Stahl 2017).

Product	Number	Minimum	Maximum	Mean value	Median value
Dates	18	1.23	6.72	3.39	2.57
Pine nuts	9	12.0	38.6	26.1	23.8
Wheat	65	1	19	4	3
Baking mixes	37	1	737	51	6
Bread	107	1	14	3	2
Spelt	28	BG	3.0	0.63	0.37
Loaf-shaped yeast fruit cakes	60	3	22	10	9
Fine pastries in aluminum trays	38	1	537	19	3
Salt pretzels and similar savory biscuits	185	2	218	13	4
Pasta	24	1	76	10	4
Herbal-teas	12	14	67	40	45
Cocoa powder	37	80	312	165	160
Chocolate	84	6	150	48	39
Confectioneries	115	1	184	17	8
Malt	50	1	12	7	7
Evaporated milk	49	0.08	0.66	0.290	0.205
Soft cheese	13	0.3	5.39	1.68	1.37
Harz cheese	22	0.15	0.78	0.400	0.438
Milk curd	53	0.03	1.73	0.224	0.109
Beer and mixed drinks containing 237	0.4	4.2	0.5	0.4	
beer, draught beer Fruit juice and fruit juice drinks	59	0.4	47	3	1
Wine and fruit wine	65	0.4	15	2	1
Mineral water, spring water and table water	171	0.1	0.07	0.01	0.006
Ready-cooked meals in aluminum tray	31	1	13	3	1
Soups	16	1	15	5	3
Pork (canned)	8	0.76	1.35	1.23	1.08
Beef (canned)	6	0.52	1.1	0.634	0.669
Game	149	<BG	1.1	0.110	0.025
Herring (canned)	32	0.16	5.99	1.99	1.60
Crustaceans	45	0.07	40.0	4.47	2.54
Flour	65	1	19	4	3

The Gut: Gastrointestinal absorption is not the only route of Al uptake. Other intake routes have been investigated including nasal, dermal, and respiratory. Some absorption of aluminum may occur in the stomach; the majority of aluminum absorption, however, is expected to occur in the intestine. In general, the two-step absorption process in the gut is 1) lumen to mucosa and 2) mucosa to bloodstream (Devoto, 1994; Peto, 2010). The reality of Al absorption in the GI tract may well be one of several mechanisms, both passive and active. The individual contributions of these processes to the net absorption of Al are dependent upon a number of factors including the chemistry of the gut lumen and the health of the individual. The rate of absorption of Al, for example, via the gut, will depend upon the route of uptake, with paracellular transport expected to proceed at a much faster rate than cellular internalization (Exley, 1996; Peto, 2010).

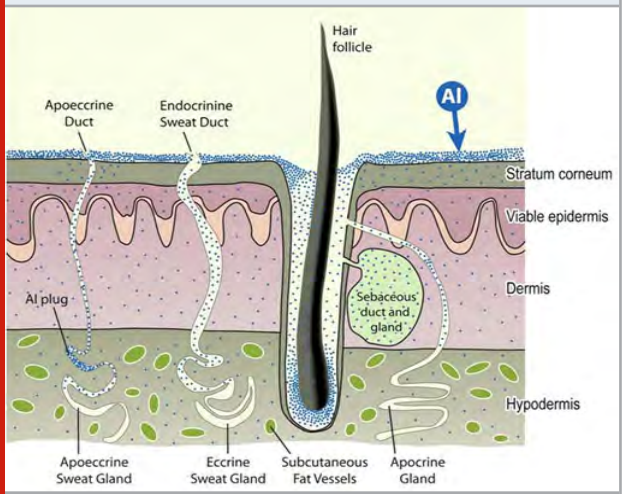
Absorption, Distribution, Metabolism, and Excretion: In humans, Al is absorbed and accumulated systemically via (1) the diet (including water and medications), with absorption occurring across the gastrointestinal tract; (2) the inhalation of partic- ulate Al through the nose

(Roberts, 1986), with absorption occurring across the olfactory epithelium; (3) the inhalation of particulate Al through the mouth, with absorption occurring via the gastrointestinal tract and, possibly, across the lung epithelia, and, controversially, the skin. The term "distribution" has been interpreted to encompass both the transport of Al and its accumulation in the various body compartments (Exley, 1996). About 90% of the Al circulating in the blood is transported bound to transferrin (iron-transporter protein), while the rest of Al binds to albumin and citrate in the blood (Igbokwe, 1919; Barr et al., 1993; Röllin et al., 1993; Ittel, 1993).

Table 5. Aluminium—external and dietary contact. Source: (Stahl 2017).

Examples of external contact	Examples of dietary contact
Construction materials, including alloys (e.g., vehicle construction, aerospace, suitcases, facades, tent construction)	Packaging and containers (beverage and food cans, coffee pots, outdoor cutlery and dishes, coffee capsules, household aluminium foil)
Electrotechnology, including alloys (e.g., electrical conductors)	Nanoparticles in sunscreens
Fuel for solid-fuel rockets (up to 30% Al) and pyrotechnics	Foodstuffs
Pigments for paints (e.g., "silver" bronze paints)	Toothpaste (e.g., AlF3: caries prophylaxis)
Metal polish (Al2O3: paste, suspension in MeOH or H_2O)	Pharmaceuticals (e.g., heartburn medicines-pH-regulation; vaccine adjuvants)
Organic syntheses (e.g., LiAlH4:reducing agent)	Vaccine adjuvant— (increases the immune reaction)
Jewellery and ornaments	Cosmetics(e.g., deodorants–antitranspirants) Food additives (e.g., as colorants or stabilizers)

Figure 3: The skin is a sink for topically applied aluminium and will act as a source of biologically reactive aluminium both to structures within the skin and to the systemic circulation (Christopher 2013).



The distribution of aluminum is better understood as accumulating mostly in bones and lungs (Krewski et al., 2007). Other affected areas are soft tissues (usually after intravenous fluid contamination), the spleen, liver, kidney, nervous tissues, muscles, and the heart (Greger, 1993). Within blood, Al is ~ equally distributed between plasma and cells. The higher concentration in lung of normal humans may reflect entrapment of airborne Al particles whereas the higher concentrations in bone, liver and spleen may reflect Al sequestration (Robert, 2001). The metabolism of Al might otherwise be defined as the systemic and cellular response to the body burden of Al. The metabolism of other nonessential, potentially toxic, metals is achieved through specific cellular responses such as the metal-induced metallothionein system (Exley, 1996).

The absorbed fraction of aluminum is bound rapidly to the tissues - the remaining free aluminum is excreted through the kidneys - however, aluminum clearance is about 5% of glomerular filtration rate secondary to protein binding (Sedman, 1992). Tissue accumulation of Al is reduced by citrates and fluorides through renal excretion when the transferrin-Al binding capacity of the blood is exceeded. Al is also excreted in the milk, bile, feces, sweat, hairs, nails, sebum and semen (Igbokwe, 2019). The kidneys eliminate the absorbed aluminium in amounts of 15-55 µg/day through urine and faeces. Al excretion is lower in people with reduced renal function and this can lead to toxic effects because of the nephrotoxicity of Al.

In dialysis settings, Al is eliminated from the dialysate by reverse osmosis and deionization since the early 1980s.5 The National Kidney Foundation–Kidney Disease Outcomes Quality Initiative (KDOQI) recommends measurement of serum aluminum level (SAL) at least once per year to assess Al levels and risk for Al toxicity.6 These guidelines also recommend measurement of SAL every 3 months for patients who take Al-containing medications (Hsu, 2016; Gupta, 2019).

Blood: The blood is probably the main distribution network for systemic aluminium though this statement is made with the proviso that there are no reliable data on the aluminium content of lymph. Because of the high concentration of potential ligands relative to the concentration of the metal, aluminum is expected to be entirely soluble in blood at concentrations up to

100 ug/1. Based on more than 50 literature sources, Ganrot reported that the most credible values for serum aluminum are in the range of 1-5 pg/1, or 0.037-0.185 uM; he judged that values much higher than these stems largely from contamination (DeVoto, 1994). During hemodialysis (HD), essential kidney functions such as the elimination of water and metabolic wastes as well as the correction of the electrolyte and acid/base state, are replaced by the artificial purification system (Kazi, 2008; Khalil, 2014).

Elimination of aluminum: Minimal exposure to aluminum isn't a problem; our bodies can excrete small amounts very efficiently, a tolerable daily intake (TDI) for aluminum of 1mg/kg body weight/day has been established by an international committee of experts under the auspices of the world Health Organization (WHO) and Food and Agricultural Organization (FAO) of the United Nations. Aluminium is excreted from the body, and hence removed from the body burden, by a number of routes including via the faeces, urine, sweat, skin, hair, nails, sebum and semen. The routes of excretion are mostly from the kidneys, which accounts for 95% of elimination, and bile. Urine accounts for .95% of excreted Al. Reduced renal function increases the risk of Al accumulation and toxicity in the very young, elderly and renally diseased human being (Greger and Sutherland 1997; Exley, 2013; Landry, 2014; Khalil, 2014).

However, absorption of Al in the gut can easily vary by a factor of 10 or more (Edwardson et al., 1993), and this is a reflection of both dietary and physiological differences among individuals. Gastrointestinal mucus was suggested to contribute toward the effective excretion of Al (Powell et al., 1994). The mucus acted as a sink for Al, with mucus sloughing ensuring the removal of the Al in the feces (Exley, 1996; Khalil, 2014).

Leaching of Aluminum: Aluminium cooking utensils are widely used in homes, restaurants and community kitchens and in the food industry, hence the intake of aluminium from utensils is of great concern (Semwal, 2006). Nowadays, it is a common practice to wrap meat and fish prior to oven cooking. Due to the possible relation between aluminum uptake and the specific diseases mentioned in many literatures, it is important to determine the aluminum concentration in the food wrapped with aluminum foil (Khalol, 2014). It was also reported that the breast piece of chicken comprises of more aluminum level than the leg. Aluminum is found to leach out from the foil due to different stimulants; particularly in distilled water as well as in acidic and alkaline media. Rise in temperature also enhance the rate of migration of aluminum in acidic media (Jabeen, 2016).

It is well established that Al dissolution is highly dependent on pH, temperature, and the presence of complexing agents. Al exhibits a passive behavior in aqueous solutions due to the protective compact Al2O3 film on its surface. However, the solubility of this protective film increases in acidic and alkaline medium. According to Bi (1996), Al leaching in aqueous solutions may be explained by the following chemical reaction occurring on the surface of the Al cookware (Verıssimo, 2006; Juhaiman, 2010).

$$Al2O_3 + 6H+ \rightarrow 2Al_3 + 3H_2O$$

where $Al2O_3$ is a protective film. The free aluminium in solutions reacts with organic acids found in food, like citric, oxalic and acetic acids, and other complexing ligands like fluoride ion and hydroxyl. These reactions may take place simultaneously and promote each other Regardless the type of food that is being cooked, the recipe and the way of preparing the food must play an important role on the aluminium leaching levels. The interaction between food and aluminium packaging can also be a potential source of aluminium release which can contribute to aluminium ingestion. Aluminium packaging in the food industry is very popular because it is impermeable, greaseproof, non-absorptive, inert, highly formable with excellent dead fold characteristics, and easily recyclable (Verıssimo, 2006).

Aluminium in pharmaceutical products: The route of intoxications with pharmaceuticals and agrochemical sources may be through inhalation of aerosols, ingestion of medications or by parenteral administration. Humans and animals are exposed to Al-containing medications such as phosphate binders, antacids, buffered analgesics, antidiarrheal and antiulcer drugs (Lione, 1983, 1985; Yokel and McNamara, 2001). Various intravenously administered pharmaceuticalproducts were reported to contain 684–5977 µg/g of Al (Sedman et al., 1985). Many antacids contain 104–208mg of Al per tablet, capsule or 5 ml of suspension (Zhou and Yokel, 2005; Krewski et al., 2007).

The use of aluminium in non-prescription drugs has increased substantially in recent time. Aluminium containing antacids are widely used in medicinal preparations. The most common form of aluminium in these preparations is aluminium hydroxide (Rajwanshi, 1997). The beneficial effects of aluminium-containing antacids for the treatment of peptic ulcer are well recognized. However, these antacids can cause adverse reactions (Spencer, 1979). A normal therapeutic regimen of antacids contains 5 g of aluminium hydroxide per day, a dosage several hundred times higher than the amount normally ingested from food. It is the aluminium which interacts in the intestine with anions, such as phosphate and fluoride, and affects the absorption of these dietary and possibly also endogenously secreted elements. Aluminum forms insoluble complexes with the dietary phosphate which becomes unavailable for absorption. In addition to the interaction of aluminum with the dietary phosphate and fluoride, the absorption of aluminum from these antacids and the deposition of aluminum in various tissues has been reported in recent years (Spence, 1979; Crisponi, 2013).

Children seem to absorb aluminium more readily than adults and there are several reports of children with renal

failure developing aluminium toxicity from aluminium-containing phosphate binders prior to commencing dialysis. Infants given aluminium-containing antacids showed significant aluminium absorption compared to controls, as shown by blood and urine aluminium levels. Aluminium toxicity should be suspected in individuals who have had pharmacological exposure to oral aluminium or contaminated parenteral fluid. Any individual with a serum level of aluminium by tameless atomic absorption of>100 l.tg/1, who has encephalopathy, should be assumed to be aluminium toxic. Children with failure to thrive and osteopenia, who have been exposed to aluminium, should have a bone biopsy followed by quantitative histology and aluminium staining (Sedman, 1992). Parenteral nutrition solutions are contaminated with aluminium. Aluminium can cause osteomalacia in patients who receive long-term parenteral nutrition. It can also lead to encephalopathy in newborns and osteopenia in premature infants (Popinska, 2010; Mudge, 2011).

Vaccination: Aluminum is added to vaccines to help the vaccine work more effectively, but unlike dietary aluminum which will usually clear rapidly from the body, aluminum used in vaccines and injected is designed to provide a long-lasting cellular exposure (Tomljenovic, 2013). Aluminum salts are used as adjuvants in preparations for vaccines and hyposensitization. An aluminum dose of 0.1–0.8 mg is absorbed upon oneoff application of a vaccine approved in Europe (Klotz, 2017).

Some concerns have been raised in recent years regarding the possible adverse effects of aluminium in childhood vaccines on the maturation of the immune system. In fact, aluminium is used as an adjuvant in multiple childhood vaccines, including DtaP, Pediatrix (DtaP, hepatitis B, polio combination), Pentacel (DtaP, HIB, polio combination), hepatitis A, hepatitis B, Haemophilus influentiza B (HIB), human papilloma virus (HPV) and pneumococcal vaccines (Crisponi, 2013).

Toxicological effects of aluminium on humans: The toxicological effects of Aluminum (Al) might depend, between others, of administration route, the time and level of exposure, and the speciation of the metal (Bernal, 2009). Aluminium can be toxic to bone, bone marrow and the nervous system (Yang, 2014).

Aluminium toxicity has been a topic of great interest since 1976 when the metal was first associated with neurological syndrome called dialysis encephalopathy (Rajwanshi, 1997). A causal role for aluminium in human pathology has been clearly established in at least three diseases: dialysis dementia, osteomalacia and microcytic anaemia without iron deficiency (Bernal, 2009). The principal symptoms of aluminium toxicity are:– diminished intellectual function, forgetfulness, inability to concentrate;– speech and language impairment;– personality changes, altered mood, depression;– dementia;– visual and/or auditory hallucinations;– osteomalacia with fracturing;– motor disturbances;– weakness, fatigue, mainly related to microcytic anaemia;– epileptic seizures (Crisponi, 2013; Rajwanshi, 1997).

The exact mechanism of aluminum toxicity is, however, not fully understood. It is considered certain that aluminum is potentially cell- and neurotoxic. Enzyme activity may be disrupted and mitochondrial function may be impaired. Toxic effects of Al arise mainly from its pro-oxidant activity which results in oxidative stress, free radical attack and oxidation of cellular proteins and lipids (Igbokwe, 2019). Current research indicates that oxidative stress may be a factor in various neurological diseases including AD (Campbell, 2002; Stahl, 2017).

Children seem to absorb aluminium more readily than adults and there are several reports of children with renal failure developing aluminium toxicity from aluminium-containing phosphate binders prior to commencing dialysis. In 2004, the U.S. Food and Drug Administration (FDA) set a limit for aluminum from parenteral sources for individuals with impaired kidney function and premature neonates at no greater than 4 to 5 µg/kg bw/day, stating that levels above those have been associated with CNS and bone toxicity (Mudge, 2011).

In addition, according to the FDA, tissue loading may occur at even lower levels of administration. What the upper limit for “safe” aluminum exposure might be for healthy neonates is not known. In spite of these above data, newborns, infants and children up to 6 months of age in the U.S. and other developed countries receive 14.7 to 49 times more than the FDA safety limits for aluminum from parenteral sources from vaccines through mandatory immunization programs (Tomljenovic, 2011).

Affected organs

Kidney damage: The effect of renal failure on aluminium (Al) accumulation in different organs and the subsequent systemic toxicity is well known (Mahieu, 2005). Aluminium causes oxidative injuries to the kidney and liver leading to tissue degeneration and necrosis, and associated serum biochemical derangements. Although the kidney appears to be able to excrete the aluminium in healthy persons it is not known the limit of this elimination capacity and it is certain that people suffering from chronic renal failure do not possess the ability to excrete it (Merta, 2006; Igobokwe, 2019).

Al accumulation in bone: Al has also been implicated in the development of osteomalacia (bone softening), especially in hemodialysis patients who experience high Al exposure from the Al-contaminated dialysate used in dialysis procedures (Peto, 2010). The skeletal system is a target for aluminum toxicity. Aluminum incorporates into the bone and causes physiochemical mineral dissolution as well as cell mediated bone resorption (Becaris, 2010). Bone Al concentration in normal human beings is a few times greater than brain Al, on a dry weight basis. Al increased more in bone than brain in haemodialysis patients (Alfrey et al. 1980; Paolo et al.

1997). Aluminum levels in bone tissue of healthy people range from 5 to 10 mg/kg (Jabeen, 2016).

Brain: Patients exhibited elevated aluminum concentrations in plasma and brain Those affected disorientation, memory impairments, and, at advanced stages, dementia. The cause of these effects lies, firstly, in the slow—compared with other organs—removal of aluminum from the brain and, secondly, in the multitude of biological processes affected by aluminum in the brain (Klotz, 2017). Aluminium may enter the brain through multiple routes: from blood, either through choroid plexus or across the blood brain barrier (BBB) and from the nasal cavity into olfactory nerves, followed by direct distribution into the brain (Crisponi, 2013).

Liver: For orally ingested aluminum, however, the tissues mostly affected are the bones, liver and the blood itself (Landry, 2014).

Disease due to aluminum: To date, aluminum has been linked to neurological and bone abnormalities, Alzheimer's and Parkinson's diseases, and cognitive impairments (Greger and Sutherland, 1997; Greger, 1993; Krewski et al., 2007)

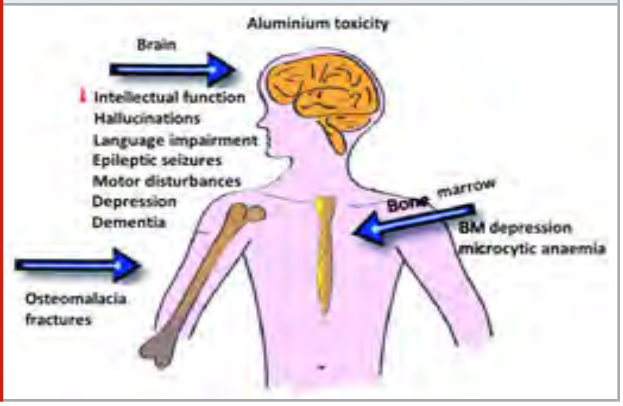
Neurodegenerative effects due to aluminium: Since aluminium is primarily excreted by the kidney, its accumulation is an important concern in patients with impaired renal functions. It can get accumulated in organs such as bones, brain and other tissues and is associated with toxic sequelae. Accumulation of aluminium in the brain appears to be a major cause in the development of a neurological syndrome called 'dialysis encephalopathy' or 'dialysis dementia' and a specific form of osteomalacia (aluminium bone disease) due to accumulation in the bone (Gupta, 2019).

Aluminum (Al^{3+}) exhibits a high affinity to proteins, which it is able to cross-link. In contrast to other ubiquitously occurring metals such as iron, manganese, and zinc, aluminum is not known to perform a physiological function in the human organism. In humans, Al accumulation in the brain and scalp hairs has been associated with neurodegenerative diseases such as dialysis-associated encephalopathy, Alzheimer's disease, Parkinson's disease (dementia), amyotropic lateral sclerosis, multiple sclerosis and autism (Exley, 2014; Klotz, 2017; Igbokwe, 2019).

As such, aluminum accumulation within the central nervous system (CNS) over the course of aging appears to reach a critical threshold in which sufficient amounts of this neurotoxin accumulates to induce proinflammatory signaling, dysregulation of gene expression (particularly in neurons), irreversible brain cell damage, and functional decline resulting in deficits in cognition, memory and behaviour. Aluminium is neurotoxic as the establishment of toxicity thresholds can result in neuronal dysfunction, neurodegeneration and ultimately neuronal cell death through a continuum of disruptive events from classical apoptosis through to sudden and violent necrosis (Exley, 2014). Cholinergic neurons are particularly susceptible to aluminum neurotoxicity, which affect synthesis of the neurotransmitter acetylcholine. In addition to these neurotoxic effects, a number of additional diseases, of which will be outlined, are being associated with aluminium as a causal relationship. However, the degree of evidence is somewhat weaker (Kramer, 2014; Klotz, 2017; Lukiw, 2019).

Oxidative stress: Oxidative stress is an event resulting from the formation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and the superoxide radical (O2¼) (Becaria, 2002). Oxidative stress is closely associated with the neuropathology of AD (Thathiah, 2009). Al-induced oxidative stress with the metabolic defects that accompanies it may incidentally be the crux of the toxicosis, to the extent that the use of antioxidant agents forms the fundamental basis for therapeutic interventions apart from chelating drugs. More generally, Al is also considered to be a mediator of oxidative stress, and efforts have been made to understand the underlying mechanisms of Al-catalyzed oxidative stress. For example, one study found that Al^{3+} ions augment iron-induced lipid peroxidation in rat liver microsomes at pH 7.4. This study also found Al^{3+} that accelerates the peroxidation of erythrocytes by hydrogen peroxide (H_2O_2). Another study found similar results (Peto, 2010; Igbokwe, 2019).

Figure 4: Principal targets of aluminium toxicity in humans (Crisponi, 2013)

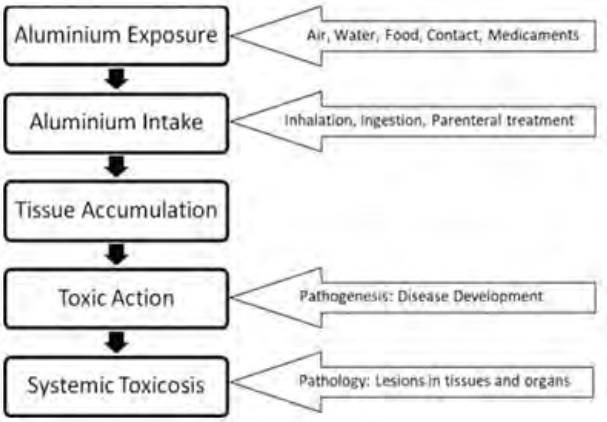


Alzheimer's disease: Alzheimer's disease (AD) is a progressive form of dementia of the elderly and the most prevalent neurodegenerative disease in the world. High concentrations of aluminum have been detected in brain tissues of patients with Alzheimer's disease (Tomljenovic, 2011). It is clinically characterized by the progressive loss of memory and other cognitive abilities and pathologically by severe neuronal loss, glial proliferation and amyloid plaques composed of β-amyloid protein (Aβ) surrounded by degenerated nervous terminations and neurofibrilar tangles. Neuropathologically, AD-afflicted brains are characterized by two proteinaceous aggregates: amyloid plaques, which are mainly composed of the β-amyloid protein (Aβ), and neurofibrillary tangles (NFTs), which are made up of hyperphosphorylated

aggregates of the tau protein (Ferrari, 2008; Thathiah, 2009; Bassioni, 2012).

Dialysis patients exhibited impaired speech, apraxia, and, in the further course, dementia syndrome as well as partly focal, partly generalized seizures. Ecological studies have suggested that concentration of aluminum in drinking water of 0.10-0.20 mg/l may increase the risk of alzheimer's disease (AD) with relative risk ranging from 1.35-2.6 (Rogers, 1991; Klotz, 2017).

Figure 5: Major themes for the literature search on aluminium toxicosis (Igobokwe, 2019).



Osteomalacia: One primary site of Al accumulation is in bone, where it contributes to the development of osteomalacia, especially in chronic hemodialysis patients (Peto, 2010). Osteomalacia, diagnosed histologically, affects about 20% of patients in terminal renal failure (Parkinson, 1981). Aluminium deposits are present at the mineralised bone front on both growing and resting bones. The association between increased aluminium bone stores in dialysed patients and the development of osteomalacia, previously known as 'renal osteodistrophy' has been well established (Crisponi, 2013).

Aluminium-related osteomalacia differs from classical vitamin-D-deficiency osteomalacia in that patients are resistant to treatment with even large doses of vitamin D, have an increased incidence of bone fractures, and are particularly likely to experience bone pain (Boyce, 1982). Hyperaluminernia and high tissue burdens of aluminum are frequently found in patients on chronic intermittent hemodialysis. It is suggested that aluminum produces chronic toxicity and that dialysis dementia and nonhypophosphatemic osteomalacic dialysis osteodystrophy are manifestations of this aluminum intoxication (Graf, 1981).

Aluminium can be detected at the interface between osteoid and calcified matrix (the mineralisation front) in bone from some patients with chronic renal failure'" after exposure to high levels of aluminium in the dialysis water or following treatment with aluminium containing phosphate-binding drugs (Boyce, 1992). The aluminium binds to the calcification front where it appears to inhibit mineralization of osteoid, and because skeletal uptake of calcium is blocked, there is a tendency to hypercalcemia and relative hypoparathyroidism. Because of low bone turnover and morbidity due to aluminium related anemia and neurotoxicity, it has been assumed that the prognosis is poor, although recently improvement in bone mineralization status has been reported after removal of aluminium from the dialysis water by reverse osmosis (Smith, 1987: Crisponi, 2013).

Figure 6: Disease caused by Aluminium

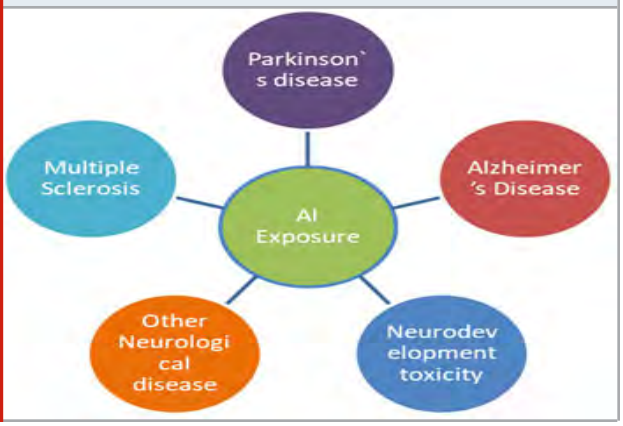


Table 6. Aluminum associated with neuronal injury (Jabeen, 2016)

Neurological findings	Neurotoxic effects
Amyotrophic lateral sclerosis Alzheimer's disease	Degenerative changes in motor neurons Loss of cognitive function
Dialysis encephalopathy	Myoclonic jerks
Hearing deficit	Cell loss in corti, spiral ganglion
Dementia	Intellectual debilitation

Anaemia induced from aluminum: Aluminum plays a role in the blood toxicity seen in patients with chronic renal failure. The usual anemia of chronic renal failure is normocytic and normochromic and is directly related to deficiency of erythropoietin Starkey, 1987) The causal relationship between anemia and aluminum intoxication was reported by Elliot et al. in 1978 (Sedman, 1992; Starkey, 1987). Anemia attributable to aluminum toxicity was first described in patients with marked aluminum overload characterized by basal serum aluminum levels over 250 pg/liter, severe bone fracturing osteomalacia and often, dialysis dementia (Bia, 1989). A microcytic anaemia is associated with dialysis encephalopathy and remits when exposure to aluminium is reduced (Parkinson, 1981; Starkey, 1987; Becaria, 2002).

CONCLUSION

Human beings are frequently exposed to Aluminium. It can be harmful if injected to living beings. At high

temperature aluminium leaching take place at higher rate and also dependent on food, salt, and pH values. In packaging of food and other related product, suppliers must be mentioned the level of aluminium in the product label. Despite its prevalence in the environment, no living organism is known to use aluminium salts metabolically, but aluminium is well tolerated by plants and animals. Because of the abundance of these salts, the potential for a biological role for them is of continuous interest and studies continue

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Emerging Applications of Nanotechnology in Neurological Disorders: Recent Review

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ABSTRACT

The neurological disorders include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, stroke, epilepsy, brain tumours, multiple sclerosis etc. which are the leading health concerns in today's world. The conventional therapies are not yet successful in treating these diseases because of the presence of intracellular and extracellular barriers across the central nervous system (CNS), which poses the major challenge of drug delivery to the CNS. The field of nanotechnology promises revolutionary advances of treating these devastating neuronal human disorders and has shown great potential to overcome the problems related to the conventional treatment approaches. Gold nanoparticles, micelles, quantum dots, polymeric nanoparticles, liposomes, microparticles, carbon nanotubes, fullerenes and several other types of nanoscale materials have been engineered and utilized for various purposes including improvement of diagnosis, delivery of neurotherapeutic agents, treatment-response assessment etc. The nanomaterials cross those barriers, target specific cell or signalling pathway, respond to endogenous stimuli, act as a vehicle for gene delivery and also support nerve regeneration. Such frameworks may serve as effective drug delivery systems and can pave the way for effective treatments in the neuronal disorders. It has been found that the drugs encapsulated with nanomaterials have better efficacy in eradicating the diseases than the bulk materials used in conventional therapies. But there are several basic concerns related to the therapeutic approach of nanotechnology, including health issues and other problems because of the very small size of nanomaterials. This review mainly aims to focus on the barriers which guard the CNS, the nanomaterials as effective drug delivery systems, their preparation, mechanism of action, nanoformulations of different neuroprotective agents, nano-neurotoxicity and future perspectives.

KEY WORDS: BLOOD-BRAIN BARRIER, NANOMATERIALS, NEURONAL DISORDERS, THERAPEUTIC DRUGS.

INTRODUCTION

The term 'Neurological Disorders' refers to central and peripheral nervous system diseases including the brain, spinal cord, cranial nerves, peripheral nerves, nerve roots, autonomic nervous system (ANS), neuromuscular junction and muscles. These disorders include cerebrovascular diseases, Parkinson's disease, Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, multiple sclerosis, brain tumour, stroke, neuroinfections, autism spectrum disorder and schizophrenia (Chhabra et al., 2015). Unfortunately, many potent neuropharmaceuticals aimed at providing a treatment for such disorders proved inefficient in large scale clinical trials. The reason, at least in part, is the unsuccessful delivery of substances to their targeted site of action inside the body. A wide spectrum of potential drugs has been investigated to treat several neurological disorders but their therapeutic success is still limited due to range of challenges (Sahoo et al., 2017).

The difficulty of crossing the peripheral barriers viz. the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB), particularly the BBB, is the key challenge

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in delivering therapeutic agents such as medicines, nucleic acids, proteins, imaging agents and other macromolecules to the CNS (Sahoo et al., 2017). Nanotechnology is an innovative and promising approach for delivering these neurotherapeutics across BBB. Although the assembly and use of nano-sized particles had taken place many years ago, nanomedicine was first established as an interdisciplinary science within the nineties of the last century. The nanotechnological approach was first framed within the 1950's and soon the constitutive force to determine nanomedicine gained importance as a paramount section in science and medical treatments (Krukemeyer et al., 2015 Sohail et al., 2020).

In the last few decades, due to its nano-size range, its unique physico-chemical properties and ability to exploit surface engineered biocompatible and biodegradable nanomaterials, drugs loaded inside nanoparticles (Table 1) have shown great potentials for efficient drug delivery to CNS (Aso et al., 2019). These nanoparticles can be made through different approaches which are illustrated in Table 2. Nanotechnology will gain importance in the coming decade in medical field, as it has the capability to improve the quality of life of the patients having neuronal disorders (Sohail et al., 2020). After the successful implementation of the strategies in nanotechnology, the growth of the field of neural circuitry has exponentially accelerated (Sohail et al., 2020).

Recent years have witnessed an explosion of research studies in the field of nanotechnology which opens up new probabilities in drug delivery, theranostics, tissue engineering, magnetofection and gene therapy (Krukemeyer et al., 2015). The effectiveness of nanotechnology is now well established and it has carved path for new and very efficient systems for drug delivery even to the most inaccessible regions such as CNS (Kumar and Singh, 2015). In this review, we strive to explain the applications of nanotechnology in neurological disorders by identifying the key principles, concepts and techniques, which will lead to further understanding in this topic and will call for much more research (Naqvi et al., 2020).

Blood Brain Barrier (BBB) and Blood Cerebrospinal Fluid Barrier (BCSFB): The brain has a very dense microvasculature with the average distance between the blood capillaries to be around 40 microns suggesting that each cell in the brain might have its own capillary (Duvernoy et al., 1983). The diffusion distances from nearest capillary to a neuron are approximately 10-20 nm (Schlageter et al., 1999). The epithelial cells of the choroid plexus (CP) contain tight junctions which limits the penetration of substances from blood to CSF.

But due to its low resistance (Saito, 1983), few substances penetrate from the blood into the CSF. For example, Azidothymidine (AZT) enters CSF through the choroid plexus epithelium but is tightly restricted at the BBB. The entry of a substance into the CSF may not allow its penetration into the brain parenchyma. The circumventricular organs are separated from the rest of the brain by the unique presence of ependymal and glial cells (Abbott et al., 2006) and penetration of substances from CSF to brain parenchyma is facilitated through diffusion. The large distances create a diffusion barrier that can be referred to as the CSF-brain barrier (CBB). The arachnoid barrier (AB) cells are present in subarachnoid space filled with CSF, which surrounds the brain and spinal cord which may act as a barrier and restrict the penetration of substances into the CSF (Yasuda et al., 2013; Sohail et al., 2020).

Strategies for Drug Delivery into CNS: Delivery of drugs through invasive techniques causes a number of problems like immunological inflammatory reactions, damage nervous system and many others. On the contrary, non-invasive technique such as nano drug delivery ensures drug delivery without damaging BBB (Jain, 2007). In this procedure, the targeted drug delivery in required quantity could be achieved by encapsulating the drug within a carrier system specially nanoparticles by the technique of nanotechnology. The nanoparticles should have a sufficient tensile strength to remain in the circulation for a long period without getting degraded. The delivery system may be either polymer based or lipid based (Naqvi et al., 2020).

Mechanism of Action of Drug Release with the help of Nanomaterials: Nanomaterials possessing positive surface charge electrostatically interact with the negative surface charge of endothelial cells present in brain, and further the lipophilic nature of nano-carriers facilitates adsorption process. The nanoparticles get absorbed by getting access to low density lipoprotein receptors on the brain capillary endothelial cells following normal endocytosis and transcytosis. Desorption occurs and then it re-enters into the blood stream, then on the surface of the blood brain barrier, the drug loaded nano carrier releases the encapsulated or adsorbed drug and further diffuses into brain parenchyma. Either passive, gradient-dependent (passive targeting) or active, energy-dependent (active targeting) pathways can lead to selective entry into the brain (Figure 1). The nano-carriers having a size less than 500 Da undergo transcellular transportation (Georgieva et al., 2014).

The nanoparticles can enter the cell through macropinocytosis, a vesicle mediated endocytosis or by phagocytosis which can be carried out through the following two pathways (Figure 2):

(i) Clathrin-Mediated Endocytosis: This mechanism occurs on all mammalian cells. The nano-carrier binds with a specific plasma membrane receptor, stimulating the polymerization of clathrin-1, a cytoplasmic protein just below the plasma membrane in order to form an inward budding leading to the engulf of cargo (Rappoport, 2008). The GTPase activity of dynamin pinches off the inward budding resulting into the formation of clathrin-coated vesicles. Actin helps in shedding of clathrin coat leading to the formation of early endosomes which deliver their content to late endosomes and finally to the lysosomes

where it is degraded off. During the transition from late endosomes to lysosomes, the pH gradually decreases, causing the release of the drug from the nano-vehicle and finally releasing the drug at the target site (Georgieva et al., 2014).

Table 1. Preparation of nanoparticles

Sr. No.	Techniques Used	Preparation Procedure	Types of Nanoparticles
1.	Solvent Evaporation	The polymer solutions are prepared in water-non-miscible, Organic volatile solvents ($CHCl_3$, $CHCl_2$, and C_4H8O_2). The Emulsion (o/w, w/o/w) undergoes evaporation of the solvent. The NPs are collected, washed, and lyophilized after ultracentrifugation	Poly (lactic-co-glycolic acid) (PLGA) nanoparticle isprepared by this method (Reis et al., 2006)
2.	Nanoprecipitation	A solution is prepared by dissolving polymer in water miscible organic solvent. For formation of colloidal suspension and its precipitation pipetting is done in stirring aqueous medium	Preparation of cyclosporine A loadedNPs (Allemann et al., 1998)
3.	Emulsification	The polymer is dissolved in partially water-soluble solvent in the presence of excess of water. This is then dissolved in aqueous solution having surfactant. Nano spheres or Nano capsules are produced depending on the concentration of oil and polymer	Doxorubicin (anti-cancer drug) loaded PLGA NPs is done by this method (Yoo et al., 1999)
4.	Salting Out	Drug and polymer are dissolved in a solvent(acetone). This is dissolved in an aqueous solution containing as calcium chloride or sucrose which acts as salting out agent and polyvinyl pyrrolidone acting as stabilizing agent. This forms o/w emulsion that is then diluted in excess water resulting in the production of Nano spheres	This technique is employed in formation of lipophilic drugs (Memisoglu et al., 2003)
5.	Supercritical Fluid Technology	In this process, rapid expansion of supercritical solution into liquid solvent (RESOLV) and rapid expansion of super critical fluids (RESS) was used	Submicron particles of cyclosporine, water insoluble drug (Young et al., 2000)
6.	Emulsion Polymerization	The monomer is dispersed in aqueous or organic non-soluble solvent followed by addition of surfactant. Polymerization is established either by adding an initiation molecule such as a free radical or by producing the radical by the monomer itself with the aid of radiation	Poly (styrene-methyl methacrylate) /SiO_2composite NPs (Mahdavian et al., 2007)

Table 1 Continue

7.	Ionic Gelation	A solution of a biodegradable polymer (chitosan or gelatin) and a di block polymer is produced and is then mixed with a solution of the drug to be incorporated. The molecules undergo electrostatic interactions resulting change of state form liquid to gel, the process is referred to as Gelation	Chitosan nanoparticles are produced by this process (Memisoglu et al., 2003)

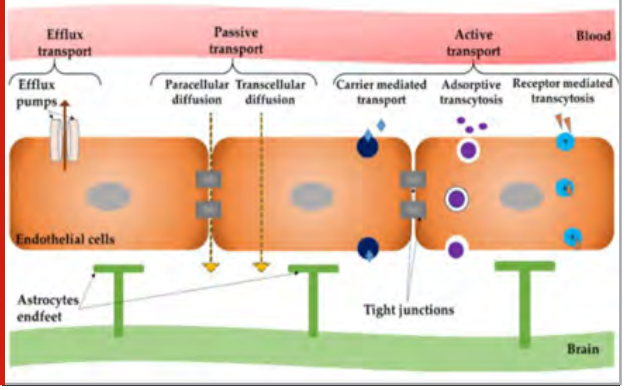
Table 2. Nano approaches towards CNS drug delivery

Sr No.	Nanoparticles	Description	Uses
1.	Micelles	Micelles are the vesicles ranging from10 to 100 nm with outer hydrophilic portion and inner hydrophobic core (generally polypropylene glycols, phospholipids, fatty acids). They may be made up of either amphiphilic surfactants (non-polymeric micelles) or amphiphilic copolymers (polymeric micelles)	They help in the loading of hydrophobic drugsfor CNS delivery (Torchilin, 2007)
2.	Polymeric Nanoparticles	Polymeric nanoparticles (10-100 nm) are solid colloidal dispersion of biocompatible, biodegradable polymers. These have a core of dense polymer and a hydrophilic outer covering to provide steric stability	*Encapsulates lipophilic* drugs which may be encapsulated, adsorbed or chemically attached to the surface (Sahoo et al., 2017)
3.	Solid Lipid Nanoparticles (SLN)	They are aqueous colloidal nano-carrier system composed of lipids (triglycerides, fatty acids, steroids, etc.), introduced aqueous surfactant solution or water and enerally solidify on cooling	Quercetin loaded to treat AD, Atazanavir loaded against HIV-encephalitis (Chattopadhyay et al., 2008)
4.	Nano Emulsions	Nano emulsions (100-500nm) are o/w or w/o colloidal particulate systems which are made up of edible oils, surfactants and water.	Modification of nano emulsions helps in overcoming the BBB, helping in rapid distribution of drugs to peripheral sites, mainly the brain (Shah et al., 2013)
5.	Dendrimers	Dendrimers have 3-dimensional symmetrical structure having an inner core from which there is a number of hyper branches, ('generations') with functional groups at the peripheral terminal surface to be easily functionalized with many ligands	Dendrimers are used for hydrophobic and hydrophilic drug delivery (Tripathy and Das, 2013; Sohail et al., 2020)
6.	Carbon Nanotubes and Fullerenes	These are carbon allotropes which are characterized by a hollow structure and striking thermal, electrical and mechanical properties. Fullerenes are of two types- Spherical Fullerenes and Cylindrical Fullerenes or Nanotubes	These are successfully used in neuronal disorders like AD, PD, and ischemic stroke and in vivo in many diseases like bone ants, rheumatoid arthritis, osteoporosis, and cancer (Boridy et al., 2009)

(ii) Caveolar Pathway for Delivery in the Brain: This pathway escapes lysosomal delivery thus making it different from different from the clathrin-mediated pathway.

Caveolae are flask-shaped invaginations in the plasma membrane and three isoforms of caveolin proteins are present in mammalian cells: caveolin-1, caveolin-2, and caveolin-3 helps in transportation through this pathway. The Nano carriers are internalized after binding to caveolar receptor forming a vesicular structure known as cavicle. The cavicle then is drived with the help of energy derived from actin and is ultimately fused with caveosomes which have neutral pH and then moves toward the endoplasmic reticulum penetrating into the cytosol and finally gaining access to the nucleus through the nuclear pore complex (Rappoport, 2008).

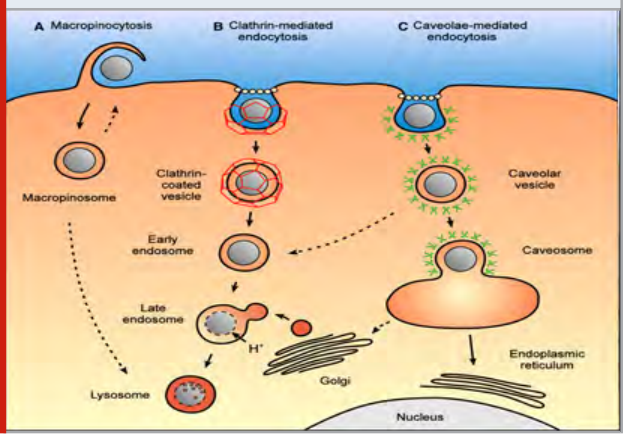
Figure 1: Different ways of crossing the BBB. Source: (Teleanu et al., 2019)



Applications of Nanotechnology in CNS Disorders: In Alzheimer's disease, polyethylene glycol (PEG) stabilized nanomicelles made up of phospholipids inhibit Ab-aggregation and attenuate Ab-induced neurotoxicity in SHSY-5Y human neuroblastoma cell line in vitro. Microemulsion nanoparticles loaded with copper chelator d-penicillamine were found to have capability of crossing the BBB and dissolving the pre-existing Ab aggregates in vitro (Vinogradow et al., 2004). The nanoliposomes made up of curcumin not only inhibits Ab aggregation but also enhanced its bioavailability. Besides, Fullerene has a neuroprotective action, has an ability to inhibit Ab peptide fibrillization and prevention of Ab-induced cognitive impairments after intraventricular administration (Taylor et al., 2011). In Parkinson's disease, the PEG and polyethylenimine nano gels complexes with antisense oligonucleotides can efficiently cross BBB *in vitro*. When injected intravenously, the oligonucleotides supply the brain more efficiently, particularly when the gels were functionalized with insulin (Mohanraj et al., 2013). The nerve growth factor (NGF) bound polybutylcyanoacrylate (PBCA) nanoparticles and L-Dopa encapsulated nanoparticles cross blood brain barrier (Siddiqi et al, 2018).

In Huntington's disease, fullerenols have ability to clear free radicals and reduce oxidative stress to cell.

Figure 2: Macropinocytosis and phagocytosis pathways for drug delivery into brain. Source: (Hillaireau and Couvreur, 2009)



Nitrendipine encapsulated in SLNs showed higher uptake of drug in comparison of bulk drug. Short-interfering RNA (siRNA) encapsulated cyclodextrin nanoparticles reduce expression of Huntingtin (HTT) mRNA both in vivo as well as in vitro (Huang et al., 2012). In multiple sclerosis, the interaction of carbon nanotubes along with stem cell is a way for tissue engineering to explore and add to cell behaviour. In a preclinical study, ciliary neurotrophic factor (CNTF) loaded microcapsules demonstrated in situ sustained delivery of CNTF upon encapsulation into polymers. This does not cause any immune response and cytotoxic effect (Godinho et al., 2013). In amyotrophic lateral sclerosis, a superoxide dismutase (SOD)-coated gold nanoparticle along with SOD1 aggregates is used as colorimetric detection system for ALS diagnosis. Sometimes carboxyfullerenenanotubes with SOD can be used. Carbon NPs may be used to effectively and precisely deliver riluzole, a glutamate inhibitor, to the affected sites (Klyachko et al., 2013; Alexander et al., 2019).

In brain tumour, nanoformulations like PBCA nanoparticles filled with methotrexate and temozolamide have resulted in increased intracerebral drug concentration as compared with free drugs. Solid lipid nanoparticles (SLNs) of etoposide and paclitaxel, in vitro, then enhanced inhibitory effect on glioma cell line proliferation was shown to be more effective than the free drug alone (Kohane et al., 2002). SLNs filled with carbamazepine and PLGA nanoparticles loaded with b-carotene are effective in epilepsy. In rat model, liposomal muscimol formulation is found to suppress focal seizures while producing minimal histological alterations (Brioschi et al., 2012). Xenon gas loaded liposomes were found to be successful in rat models administered for up to 5 h after the onset of stroke with an acceptable dosage range of 7-14 mg/kg (Peng et al., 2013). In neuro-AIDS, enhanced targeted delivery of Azidothymidine (AZT) to macrophages is possible using poly (hexylcyanoacrylate) NPs. Poly (hexylcyanoacrylate) NPs can also be used to deliver Saquinavir in human monocytes or macrophages (Chhabra et al., 2015; Alexander et al., 2019).

Nanotechnology Based Delivery of Neuroprotective Drugs: The biologically active and key phenolic constituent of turmeric, Curcumin (diferuloylmethane), obtained from the rhizomes of *Curcuma longa*, has shown considerable therapeutic efficacy in several diseases (Chattopadhyay et al., 2008). Being a natural antioxidant, curcumin has been found to possess many pharmacological activities including anti-inflammatory, antimicrobial, anticancer, the neuroprotective effect in neurodegenerative disorders, in both preclinical and clinical studies. Despite the wide medicinal applications of curcumin, due to low solubility, physico-chemical instability, poor bioavailability and quick metabolism, its clinical implications are hindered (Chattopadhyay et al., 2008). However, these problems can be resolved by developing efficient delivery systems with the help of nanotechnological approach. Compared to bulk curcumin, curcumin loaded PLGA-PEG nanoparticles, curcumin-loaded polysorbate 80 modified with some (CPC) nanoparticles showed better stability, longer circulation period and higher permeation of curcumin nanoformulation (Naksuriya et al., 2014; Alexander et al., 2019).

Among growth factors, Nerve Growth factors (NGFs) have great therapeutic potential for various CNS disorders. Vascular endothelial growth factor (VEGF) has been shown to participate in the process of post-ischemic brain repair via promoting neurogenesis and cerebral angiogenesis. Successful neuroprotection and promotion of vascular regeneration in the ischemic brain have therefore been achieved by treatment with modified liposomes with VEGF loaded transferrin. Edaravone (EDR), a well-known lipophilic drug, is used as a free radical scavenger for not only neurodegenerative disease, but also cardiovascular disease and cancer (Hudson et al., 2013). In preclinical studies, EDR has shown great efficiency against AD and cerebral aneurysm via oral administration, although oral bioavailability of EDR is very limited (Cruz, 2018). The lipid-based nanosystem (LNS) loaded with EDR was developed to promote its successful oral delivery by increasing the oral bioavailability (Alexander et al., 2019).

Neurotoxicity of Nanomaterials (Nano-Neurotoxicity): While invading the barriers within neural networks, doors are open not only for drug delivery but also to toxicity. The scope and size of toxic events is a part of the challenge in determining nanotoxicology. They interact heavily with components and pathways in both the biochemical environment of the cell and physiological system (Karmakar et al., 2014). Metal oxide NPs are highly useful in various fields such as medicine and engineering.

However, these NPs have high chemical reactivity and toxicity as a consequence of their small size and large surface area. These NPs can accumulate in structures of the brain, such as the cerebellum and cortex (Valavanidis and Vlachogianni, 2016). For the use of multi walled carbon nanotubes (MWCNTs) as scaffolds, studies have inferred a substantial degree of genotoxicity (DNA interference) that is symptomatic of a broader problem posed by the use of nanomaterials. Hence, nanotoxicology profiling is a critical component of studies of nanomaterials (Kumar et al., 2017; Alexander et al., 2019).

CONCLUSION

In last few recent years brain-targeted drug delivery systems have been developed and gained large attention. Applications of nanotechnology have been developed in many fields in the last decade such as method of drug delivery, biological sensing, biomedical imaging, targeted anticancer drugs and antibiotic carriers. Within the realm of medicine, nanotechnology has found its way not only in improving drug delivery, but also in improving the required surgical procedures as seen in case of brain tumours. Compared to conventional implants that may cause neuroinflammation due to rigidity of the material, new polymeric implants are advantageous as they provide increased bioavailability with minimal or no neuroinflammation.

Though several nanoformulations have shown great efficacy in preclinical and clinical studies, there are several basic concerns which should be addressed in the future to achieve the successful clinical translation of nanoformulations. The nanomaterials should be effective and safe in brain-targeted drug delivery systems, as well as they must be easily biodegradable. The approaches for the development of nanomaterials should be eco-friendly. The physico-chemical properties attached to nanomaterials must be evaluated carefully for the development of effective brain targeted drug delivery systems. To avoid complications associated with invasive routes a non- invasive alternative method for drug delivery should be developed. More studies are required on the basic level to increase the possibility of the use of nanoparticles in clinical settings.

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Performance Assessment of Different Machine Learning Algorithms in Predicting Diabetes Mellitus

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ABSTRACT

Diabetes Mellitus (DM) is considered as a heretical metabolic disorder and widely spread long standing slow poison which poses a great threat to human health. Faster and accurate prediction of diabetes is a dire need and Machine Learning (ML) can play a pivotal role in terms of enhancing medical health technology and develop an e-healthcare system. In this regard, ten ML algorithms have been studied comprehensively and they are implemented by Jupyter Notebook. Hence, the ML models are trained with the dataset of Kaggle machine learning data repository of Frankfurt hospital, Germany. Effective data processing method is proposed using 5-fold cross validation method to achieve stable accuracy. However, hyper-parameter tuning technique is employed with a view to achieving better performance from the ML models. After rigorous simulation, Gaussian Process (GP) emerged as the best performing algorithm which is proposed as the most efficient classifier with an accuracy of 98.25%. However, Random Forest (RF) and Artificial Neural Network (ANN) displayed accuracy of 97.25% and 96.5% respectively which are quite satisfactory. Hence, the performances of the ML models are assessed with different metrics like Accuracy, Sensitivity, Precision, F1-score, Specificity and ROC_AUC and thus, a comparative analysis among all the ML models are portrayed graphically. Efficient prediction of Diabetes by ML algorithms can significantly contribute in decreasing the annual mortality rate specially in developing countries like Bangladesh. Therefore, this study can meaningfully assist the healthcare professionals in the process of proper and faster treatment of Diabetes Mellitus and thus, an efficient e-healthcare system can be established in future.

KEY WORDS: DIABETES MELLITUS, MACHINE LEARNING (ML), CROSS VALIDATION, HYPER-PARAMETER TUNING, E-HEALTHCARE.

INTRODUCTION

Diabetes mellitus (DM) is considered as a cluster of metabolic disorders and appears to be a common disease among mass people nowadays imposing a lot of complications in human body. Insulin Dependent Diabetes Mellitus (IDDM) or Type-1 diabetes is witnessed among children because of the genetic disorders where the body fails to produce adequate insulin. However, 'Type 2' diabetes is normally observed in middle-aged people for most cases where body is not able to use the insulin produced inside or it fails to produce adequate insulin or both and it is termed as "Non-Insulin-Dependent Diabetes Mellitus" (NIDDM). On the other hand, gestational diabetes is commonly seen among 2-10% pregnant women where they may not have diabetes prior to pregnancy or can develop 'Type 2' diabetes after the pregnancy (Lee et al., 2018).

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However, Diabetes Mellitus (DM) is affected by various factors like pregnancies, blood pressure, glucose, skin thickness, BMI, diabetes pedigree function, age but amongst all, the prime reason is blood sugar level. If diabetes remains untreated and unidentified many complications occur, for instance, various organs like eyes, teeth, legs, tiny blood vessels, kidneys, liver, heart and nerves get affected which results in various acute and chronic diseases in course of time (Yoon et al., 2017). In 2019, WHO estimates that worldwide 463 million people have diabetes with 'Type 2' diabetes making up about 90% of the cases and the number of cases may increase to 642 million by 2040. Rates are alike in women and men and this disease leads to a person's risk of early death. The WHO also states that approximately 4.2 million deaths occurred in 2019 due to diabetes and globally it is the 7th leading cause of death (Saeedi et al., 2020).

In Bangladesh, most of the people are not aware of deadly clutch of diabetes which becomes rampant in course of time. Therefore, early detection can pave the way to ensure better treatment for the patients and assist the healthcare professionals in this regard.Machine learning algorithms are being employed in different times by many researchers in predicting diabetes. A hybrid Neural Network System was developed by implementing Artificial Neural Network (ANN) and Fuzzy Neural Network (FNN) for diabetes diagnosis and an accuracy of 84.2% was attained (Kahramanli and Allahverdi, 2008). On the other hand, an approach was proposed that combining the Least Square Support Vector Machine (LS-SVM) classifier with Generalized Discriminant Analysis (GDA) improves the accuracy of diabetes classification, (Polat et al., 2008).

Here, the GDA technique was used for feature reduction and then LS-SVM was applied for modeling. Using 10-fold cross validation, this combination of two methods depicted 82.05% accuracy. However, a classification system is manufactured for diabetes using the Bayes' network, obtaining an accuracy rate of 72.3% (Guo et al., 2012). Again, a research is conducted on diabetes prediction exploiting real-time dataset and different ML models are implemented (Meng et al., 2013). This study achieved its highest accuracy of 77.87% for the decision tree model (C5.0), 76.13% of accuracy in regression model and 73.23% in ANN model. Furthermore, a hybrid method was implemented utilizing NSGA-II technique for diabetes detection and 86.13% accuracy was obtained (Zangooei et al., 2014). On the other hand, a unique system is designed for diabetes classification employing an adaptive network-based fuzzy inference system and 82.3% accuracy was obtained (Sagir and Sathasivam, 2017).

It is observed that the above-mentioned researches contributed in employing various ML techniques in predicting diabetes but none of them came up with an accuracy more than 90%. So, with the advancement of modern technology, ML algorithms hold the promise to assist in providing more accurate predictions and satisfactory results than current practices which leverage the healthcare system and save healthcare expenditures. The key objective of this study is to develop ML models and investigate their performances to predict diabetes with promising outcomes. As it is seen that achieving higher accuracy is always a challenge for the ML researchers. In this regard, a good diabetes dataset is explored and promising outcomes are observed with the assistance of ML algorithms.

Hence, several machine learning algorithms are studied extensively such as Logistics Regression (LR), K-Nearest Neighbours (KNN), Support Vector Machine (SVM), Naive Bayes (NB), Adaboost (AdB), Random Forest (RF), Stochastic Gradient Descent (SGD), Gradient Boosting (GB), Gaussian Process (GP) and Artificial Neural Network (ANN) and an investigative and comparative analysis is portrayed in the forthcoming sections. The performance metrics of different algorithms were explored by various standards, such as accuracy, sensitivity, precision, F1-score, specificity and ROC_AUC. Therefore, this kind of comprehensive analysis among all the ML models with diabetes dataset will shape the way of developing a computer-aided healthcare system which is direly needed specially in developing countries like Bangladesh.

MATERIAL AND METHODS

Data Preprocessing: The proposed approach consists of three basic steps. Firstly, the Kaggle dataset was loaded into pandas for data preprocessing (Kaggle Diabetes Dataset, Frankfurt hospital, Germany). However, further data preprocessing is accomplished on the proposed dataset with 5-fold cross validation. Secondly, the preprocessed dataset is fitted into our proposed ten different machine learning models with hyper parameter tuning. Lastly, the models are tested and various performance metrics like accuracy, precision, sensitivity, specificity, F1 score and ROC_AUC are evaluated and overall comparative analysis is carried out. In this work, this dataset of diabetes has been taken from the hospital Frankfurt, Germany. The data set contains 2000 instances of observations of patients consisting of 9 attributes with no missing values. In this work, 1600 samples are selected as training set and 400 samples chosen for test set. The details of the attributes of the dataset is depicted in Table 1.

From the dataset, it is observed that some attributes like glucose, blood pressure, skin thickness, insulin and BMI have zero value but this is not possible practically. So, those are treated as missing data and they are replaced by the mean value of the specific attribute column having the missing value. From the Table 1, it is evident that some of the values of attributes of the dataset are not on the same scale which might have caused some issues in the machine learning models. As lots of the machine learning models are based on Euclidean distance, the higher range attributes dominated the lower range attributes. Therefore, the entire attribute should be in same scale. Some observations of scaled attributes are shown in Table 2.

Study of Machine Learning Algorithms: The machine-learning algorithms used in this paper are briefly described below:

Logistics Regression (LR): Logistic Regression (LR), a widely used model in machine learning, utilizes a logistic function to classify a binary dependent variable over one or more independent variables or features (Maniruzzaman et al., 2019). The main advantages of this type of supervised machine learning algorithms are that it can handle nonlinearity and it is easy to implement and very efficient to train. Combining linear regression line and sigmoid function, the best fitting curve can be attained for dataset. The following equations are used in this process:

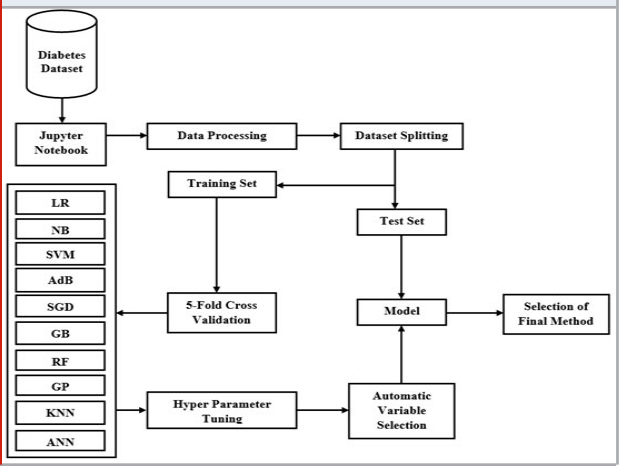
Table 1. The attributes of dataset

No	Attribute	Description	Range of value	Type
1	Pregnancies	How many times	0 - 17	Numeric
2	Glucose	Glucose value	0 - 199	Numeric
3	Blood Pressure	Blood Pressure level	0 - 122	Numeric
4	Skin Thickness	Skin Thickness value	0 - 110	Numeric
5	Insulin	Insulin Level	0 - 744	Numeric
6	BMI	Mass	0 – 80.6	Numeric
7	Diabetes Pedigree Function	Family History	0.08 – 2.42	Numeric
8	Age	Age of diabetic patient	21 - 81	Numeric
9	Outcome	Binary (Yes or No)	0 - 1	Nominal

Table 2. Observation of preprocessed scaled dataset

No	Pregnancies	Glucose	Blood Pressure	Skin Thickness	Insulin	BMI	Diabetes	Age Pedigree Function
1	2.540	-0.014	0.466	-0.233	-0.715	-0.701	-0.650	2.507
2	4.065	1.293	0.160	1.243	0.294	1.044	1.043	1.211
3	-0.814	-0.606	0.262	-1.280	-0.715	0.874	-0.547	0.780
4	-0.204	-0.201	-0.144	1.120	0.525	0.704	-0.981	-0.428
5	1.625	1.324	0.466	-1.280	-0.715	0.062	-0.987	1.039

Figure 1: Proposed workflow diagram



Linear Regression Function: $y = b_0 + b_1 * x$

Sigmoid/Logistic Function: $p = \frac{1}{1+e^{-y}}$

Logistic Regression Function: $\log it(p) = \ln\left(\frac{p}{1-p}\right) = b_0 + b_1 * x$

Where, p is the dichotomous (binary) output which is the result of weighted sum of input features x. If the probabilistic output is more than 0.5 line, the output is 1 otherwise the output is 0.

Naive Bayes (NB): Based on the Bayes' Theorem, Naïve Bayes is appointed extensively in various classification problems (Balaji et al., 2020). This classifier is a probabilistic machine learning algorithm that can be implemented simply and the predictions made in real-time are quick and space efficient.
Bayes' theorem:

$$P(A \mid B) = \frac{P(B \mid A)P(A)}{P(B)}$$

Where,
P(A|B) = Probability of B occurring given event A has already occurred.

P(B|A) = Probability of A occurring given event B has already occurred.

P(A) = Probability of event A occurring.

P(B) = Probability of event B occurring.

Let, 'X' is a new data point, found P(A|X) and P(B|X). Then our classifier compares those two and decides X belongs to 'A' or 'B'.

Support Vector Machine (SVM): Support Vector Machine, a commonly used classification technique which aims to classify data points by an appropriate hyper plane in a multidimensional space. The decision boundary line or the hyper plane is drawn, maintaining the maximum margin from the support vectors. SVM works proficiently as there is a margin of separation between classes and also more effective in high dimensional spaces. When dataset is not linearly separable mapping to a higher dimension to make the dataset linearly separable, nonlinear functions are used as kernel. So, polynomial kernel is applied as the hyper plane to get more accuracy and less over fitting than linear kernel (Djelloul and Amir, 2019).

For degree-d polynomials, the polynomial kernel is defined as,

$$K(x,y) = \left(x^T y + c\right)^d$$

Where x and y are points in our dataset and c stands for the homogeneity of our function.

Adaboost (AdB): Amongst Machine Learning algorithms, Boosting is an ensemble learning method used to improve the prediction power. AdaBoost (Adaptive Boosting) is a sequential learning process where multiple decision tree models are used as weak learners (Li et al., 2019). All the models do not have equal weight for the final model. The hypothesis is obtained for each subset of the dataset and then combined to get a single better hypothesis. The compensation is done by varying the weights of data. AdaBoost is sensitive to noisy data. The final equation for classification can be represented as,

$$F(x) = sign\left(\sum_{m=1}^{M} \theta_m f_m(x)\right)$$

Where, f_m stands for the m^{th} weak classifier and θ_m is the corresponding weight.

Stochastic Gradient Descent (SGD): Stochastic Gradient Descent (SGD) refers to descending of a slope to reach the lowest point called global minima on the structure by minimizing the cost function to update the weights (Talo et al., 2019). The equations used for Gradient descent:

Updated weights, $\theta_j := \theta_j - \alpha \frac{1}{m} \sum_{i=1}^{m} \left(h_\theta(x^{(i)}) - y^{(i)}\right) x_j^{(i)}$

Cost function, $j(\theta) = \frac{1}{2m} \sum_{i=1}^{m} \left(h_\theta(x^{(i)}) - y^{(i)}\right)^2$

m = number of examples

$h_\theta(x^{(i)})$ = hypothesis function

$y^{(i)}$ =Actual output

$x_j^{(i)}$ = Input

α = Learning rate; Range (0 to 1)

The main difference between Gradient Descent (GD) and Stochastic Gradient Descent is that the whole training data per epoch is used in GD whereas, in SGD, only single training example per epoch are employed to fine-tune the weight. Stochastic Gradient Descent helps to find overall global minima which is a faster process than Gradient Descent.

Gradient Boosting (GB): Gradient Boosting technique refers to technique where a prediction model is constructed in the form of an ensemble of weak prediction models, typically decision trees. The Gradient Boosting, an ensemble learning algorithm works on the principle of gradient decent (Chen et al., 2018). A base (weak) model is created and learned by optimizing the loss function. We are boosting base model with the help of sequentially adding several DT models, where we took last model's residual value as next models predicted value to reduce the overall error. And with the help of learning rate (α) we reduce over fitting.

Random Forest (RF): In Machine Learning Bagging is an Ensemble Learning used to improve the prediction power (Javeed et al., 2019). Random Forest method which combines a lot of Decision Tree method and combines the idea of bagging and the random selection of features for each one of the trees from our dataset as a subset together. Taking the majority vote from the trees and deciding the classification based on that. And that power of numbers can help get rid of certain errors and certain uncertainties in our algorithm and make it more precise and one of the best learning algorithms. One of the major pros is that it can handle a huge amount of data proficiently.

Gaussian Process (GP): Gaussian process (GP), a nonparametric classification method is founded on Laplace approximation and Bayesian' methodology (Lang et al., 2019). For approximating the non-Gaussian posterior by a Gaussian, Laplace approximation is used. Bayesian' methodology undertakes some preceding distribution on the basic probability densities that promises some smoothness properties. Gaussian Process are a type of Kernel method, like SVMs, although they are able to predict highly calibrated probabilities, unlike SVMs. Hence it is a very effective classifier.

K-Nearest Neighbors (KNN): K-nearest neighbors (KNN) is one of the simplest supervised machine learning algorithms that can be deployed for both classification and regression analysis. KNN assumes the nearest data points in the feature space. It is based on feature similarity and classifies a data point based on how its neighbors are classified (Hossain et al., 2019). It uses Euclidean distance calculation to find the nearest data point (neighbor). The K-nearest neighbors of the new data point, according to the straight-line distance (also called the Euclidean distance) is a popular and familiar choice.

Where, Euclidean Distance= $\sqrt{\sum_{i=1}^{k}(x_i - y_i)^2}$

Artificial Neural Network (ANN): Artificial Neural Network (ANN) is an advanced method that mimics the human brain holding a notable promise in pattern recognition of huge datasets (Nasser and Abu-Naser, 2019). Here, layers of neurons are constituted which acts as the fundamental processing unit. Firstly, input layer is placed that takes the inputs from the dataset. Then, the output layer forecasts final outcome. However, the hidden layers stay between these two layers, which accomplishes most of the calculation compulsory for the network. Hence, the forward and backpropagation method are implemented iteratively and the cost functions are evaluated each time. The output of this layer is fed to the next layer and by this manner the data is propagated through the network and this is called Forward Propagation.

Then the cost function is calculated by actual output and the predicted output and back propagated through the network. This cycle of forward propagation and back propagation is iteratively performed with multiple inputs. This process continues until our weights are adjusted such that the network can predict the classification correctly. Some prime application of ANN is facial recognition, forecasting, music composition etc. In this paper, ANN is used as binary classifier. So, the same hypothesis function used in Logistic Regression is brought into action.

$h_\theta(x) = \frac{1}{1+e^{-\theta^T x}}$ Where, θ^T = Transpose of weight matrix for a layer.

The network actually learns through Back propagation algorithm as,

$\partial_j^{(l)} = \frac{\partial(\cos t(i))}{\partial z_j^{(l)}}$ for, $j \geq 0$

Where, for i^{th} sample,

$\cos t(i) = y^{(i)} \log h_\theta(x^{(i)}) + (1-y^{(i)}) \log h_\theta(x^{(i)})$

RESULTS AND DISCUSSION

After studying ten supervised machine learning techniques, they are implemented for the classification of diabetes disease samples and satisfactory performances are witnessed from the ML models. The corresponding confusion matrices are presented in Table 3.

Table 3. Confusion Matrices of all ML models

Confusion Matrix (LR)		Predicted True	Predicted False
Actual	True	243	29
	False	59	69

Confusion Matrix (NB)		Predicted True	Predicted False
Actual	True	230	42
	False	55	73

Confusion Matrix (SVM)		Predicted True	Predicted False
Actual	True	271	1
	False	62	66

Confusion Matrix (AdB)		Predicted True	Predicted False
Actual	True	237	35
	False	50	78

Confusion Matrix (SGD)		Predicted True	Predicted False
Actual	True	210	62
	False	44	84

Confusion Matrix (GB)		Predicted True	Predicted False
Actual	True	266	6
	False	8	120

Confusion Matrix (RF)		Predicted True	Predicted False
Actual	True	266	6
	False	5	123

Confusion Matrix (GP)		Predicted True	Predicted False
Actual	True	269	3
	False	4	124

Confusion Matrix (KNN)		Predicted True	Predicted False
Actual	True	257	15
	False	33	95

Confusion Matrix (ANN)		Predicted True	Predicted False
Actual	True	265	7
	False	7	121

The actual comparison among the studied ML models is evident from Table 4 based on various performance metrics like Accuracy, Sensitivity, Specificity, F1 score and ROC_AUC. All these metrics can be achieved with the assistance of confusion matrix. With the tuned configuration, Gaussian Process (GP) depicted the highest accuracy (98.25%) whereas the overall accuracy is above 75%. Besides Gaussian Process (GP), satisfactory accuracy is also witnessed in other algorithms like RF (97.25%), ANN (96.75%) and GB (96.50%).

Figure 2: ROC of all the ML models

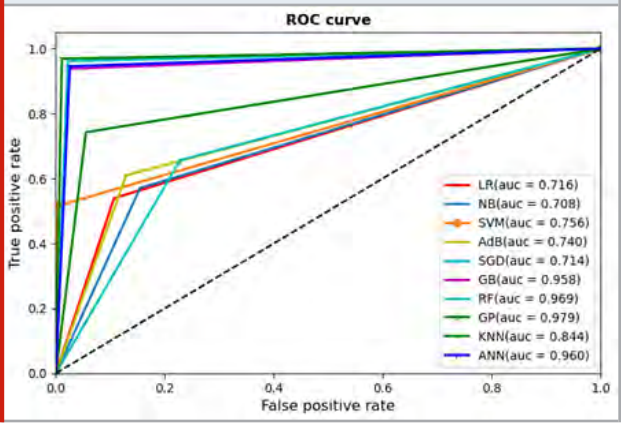


Table 4. Performance metrics of all ML models

Name of the algorithm	Accuracy (%)	Precision	Sensitivity	Specificity	F1 Score	ROC_-AUC
LR	78.00	0.704	0.539	0.893	0.610	0.716
NB	75.75	0.634	0.570	0.845	0.600	0.708
SVM	84.25	0.985	0.515	0.996	0.676	0.756
AdB	78.75	0.690	0.609	0.871	0.647	0.740
SGD	76.50	0.713	0.445	0.915	0.548	0.710
GB	96.49	0.952	0.937	0.976	0.944	0.958
RF	97.25	0.953	0.960	0.977	0.957	0.969
GP	98.25	0.976	0.968	0.988	0.972	0.979
KNN	88.00	0.863	0.742	0.944	0.798	0.844
ANN	96.75	0.932	0.969	0.967	0.950	0.968

Table 5. Performance Assessment of ML Models

Serial No.	Accuracy	Precision	Sensitivity	Specificity	F1 Score	ROC-AUC
1.	GP (98.25%)	SVM (0.985)	ANN (0.969)	SVM (0.996)	GP (0.972)	GP (0.979)
2.	RF (97.25%)	GP (0.976)	GP (0.968)	GP (0.988)	RF (0.957)	RF (0.969)
3.	ANN (96.75%)	RF (0.953)	RF (0.960)	RF (0.977)	ANN (0.950)	ANN (0.968)

Figure 3: Comparison of accuracy among all the ML models

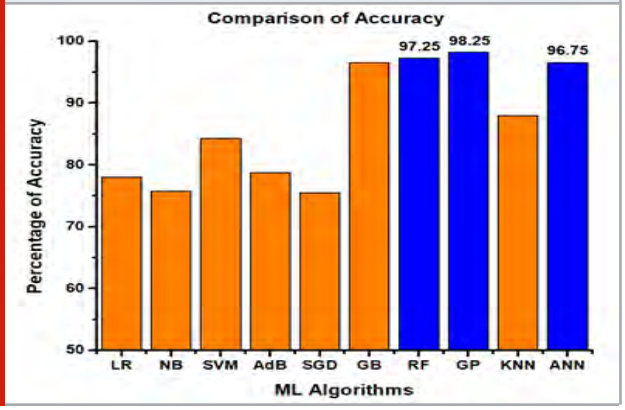


Figure 4: Comparison of precision among all the ML models

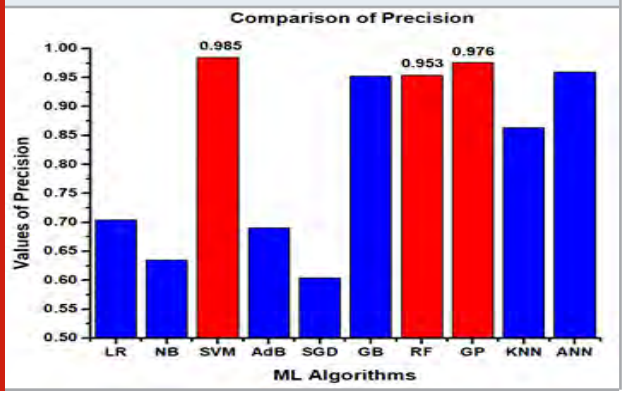


Figure 5: Comparison of sensitivity among all the ML models

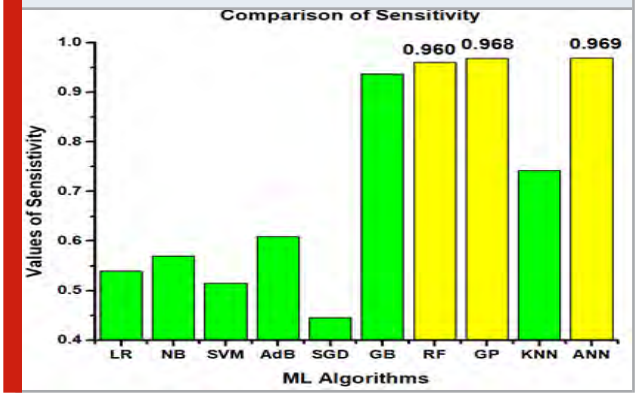


Figure 6: Comparison of specificity among all the ML models

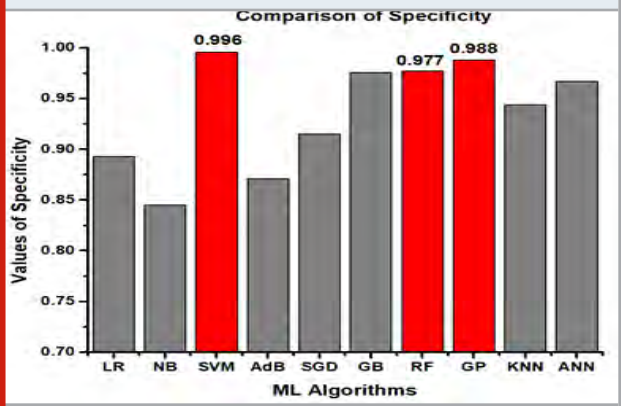


Figure 7: Comparison of F1 Score among all the ML models

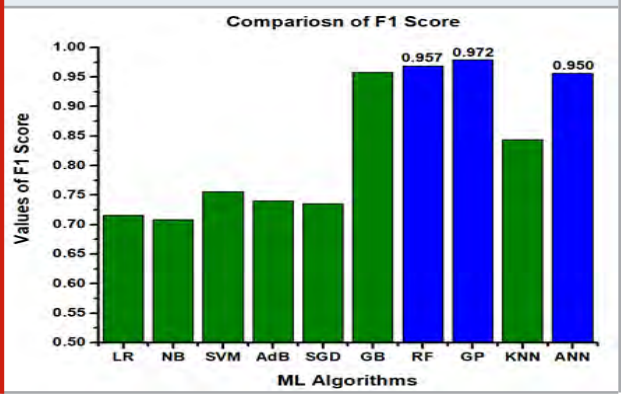
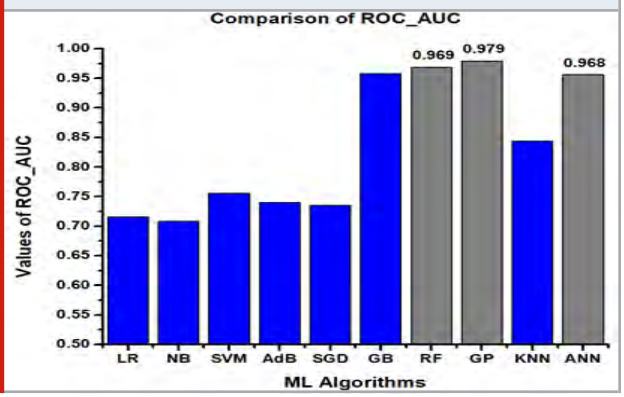


Figure 8: Comparison of ROC_AUC among all the ML models



The dataset, being imbalanced, some machine learning algorithms can get biased and still gives higher accuracy. So, different performance metrics like accuracy, sensitivity, precision, specificity, F1-Score and ROC_AUC are investigated so that the ML models can be evaluated more comprehensively. Table 5 represents the best performing algorithms considering different performance parameters. Amongst them, the best ML model is Gaussian Process (GP) as it contains least amount of over fitting, fast and accurate prediction. The other nearly performed models are GB, RF and ANN. It is also observed that these algorithms showcased better accuracy in comparison with other literature studied.

The comparative analyses among all the ML models in terms of accuracy, precision, sensitivity, specificity, F1 score and ROC_AUC are presented graphically in Figure 3, Figure 4, Figure 5, Figure 6, Figure 7 and Figure 8 respectively.

CONCLUSION

In our proposed work, different machine learning algorithms are compared and analyzed based on various performance evaluation techniques like accuracy, sensitivity, precision, F1-score, Specificity and ROC_AUC. The obtained classification results demonstrate that the machine learning method Gaussian Process (GP) gives more accurate prediction and better performance than other methods discussed in this study. Still, some of the other methods used in this study such as Gradient Boosting (GB), Random Forest (RF) and Artificial Neural Network (ANN) provide exemplary results compared to other studies available in the existing literature. The primary goal of this study is to be a supportive element for doctors to arrive at a precise treatment routine for their patients suffering from diabetes. Because of great accuracy and fast processing time, this study can open a window in developing e-healthcare system for the diabetic patients. In future, more algorithms will be explored in different datasets so that more insights can be achieved and more information can be stored which will enable the healthcare professionals to utilize computer-aided diagnosis as an efficient tool in the process of faster and proper treatment for diabetic patients.

Conflict of Interest: Authors have no conflict of interest.

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Preparation of Iron Nanoparticles and Composites for Arsenic Removal: An Updated Review

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ABSTRACT

Due to advancement and increasing development in industrialization, heavy metals especially Arsenic (As) may cause an environmental threat because of continuous release of effluents in ground water. Metallic As is hazardous in nature and have severe harmful outcomes on human, aquatic animals, plants and environment. As cause severe lethal impacts on the healthy human as well as environment after appearing into the chain of food. As is one of major cancer causing agent in humans. Though, development of new technology like nanotechnology gives hope for better techniques for As removal from waste water. Preparation of unique, novel and low cost of nanomaterials for environmental applications, detection of pollutant and other uses has drew attention for further considerable. On this note, zero valent iron and iron oxide nanoparticles are observed as the suitable materials for the As adsorption from waste water or ground water. Electrical, ionic interaction, mechanical and physiochemical properties play key role in nanoparticle fabrication and control in desirable morphology. Iron oxide nanoparticles can also be used as catalyst, drug delivery carriers and contrasting agent. Different categories of iron oxide nanoparticles desired shape or topography, and size can be prepared by using different methodology such as sol-gel, co-precipitation, solvothermal reactions and iron oxide composites. Iron oxide nanoparticles have previously shown its efficiency, diversity and reusability in several areas including bio-imaging, drug or gene delivery, catalytic properties, immobilization of industrial important enzymes and removal of dye, phenol and toxic compounds. Present review is dedicated on the preparation of iron oxide nanoparticles and its composites for As metal removal.

KEY WORDS: NANOPARTICLES, IRON, IRON OXIDE, COMPOSITES, ARSENIC, ENVIRONMENT.

INTRODUCTION

Arsenic occurs as in oxides form within dirt, dregs, aqueous solution, and ground water in several part of the globe (Nurmesniemi et al., 2010, Park et al., 2011). Naturally, arsenic occurs more than different 200 distinctive mineral structures. Arsenic appears around in the form of arsenates (60%), sulphides (20%) and sulphosalts, and consequently arsenide, arsenite, silicates and elemental arsenic are the left over 20% (McCarty et al., 2011, Chiban et al., 2012). The well-known Aresenic compounds that naturally occur are arsenite (As(III)) and arsenate (As(V)) (Drewniak et al., 2012). As(V) is the prevalent type of As existing under oxidized surroundings and present as oxyanions of arsenic acid, while As(III) occurs as arsenious acid under mild reducing environments (Fig.1) (Sinha et al., 2013, Campbell et al., 2014, Podgorski, et al., 2020).

Arsenic and related compounds have been recognized as potent carcinogen as per guideline of the International Agency for Cancer Research (IARC) (Karagas et al., 2015).

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Hydrothermal activity associated with granitic magma intrusion and orogenesis shapes the primary arsenic containing mineral arsenopyrite (Schindler et al.,2016). Due to the natural geological distribution in the bedrock, the presence of As in natural source water may appear (Bondu et al., 2016). It primarily presents as an inorganic as well as organic type in drinking H_2O, particularly in surface water. If there is a higher concentration of sulphide ions is present in water, and it forms precipitate with arsenic in a reduction state (Bibi et al., 2017). The permissible limit of As in drinking water is 10 mg/L as per recommendation of World Health Organization (WHO) (Chen et al.,2017, Adimalla, et al.,2020).

Figure 1: Graphical representation of Geochemical Cycle of As in Environment (Hao et al., 2018)

Natural phenomena like minerals dissolution due to weathering, activity of different types of microbes, and complexing with organic materials may release arsenic into the aquatic environments (Tabelin et al., 2018). On the other hand, arsenic pollution in soils and surface water may result from human caused activities such as mineral mining and metallurgy productions, fuel burning and, the use of arsenic based pesticides (Zhang et al., 2018, Rahman et al.,2019). There are several techniques including process of oxidation, co-precipitation with other materials, ion-exchange, membrane filtration processes and adsorption via using matrix have been practiced to treating polluted groundwater or surface water (Karthikeyan et al., 2019).

Adsorption on matrix is a purely surface phenomenon which relied on the interaction among the adsorbent and adsorbate (Ravi et al., 2019, Sirajudheen et al., 2020). Robust particle such as nanomaterials with smaller size, high volume to surface area ratio, and enormous number of pores, can significantly decrease the concentration of arsenic from aqueous solution (Ahmad et al., 2020). The matrix applied for the removal of arsenic must be cost effective, simple to formulate, and abundant availability in nature, (Hasan et al., 2020). This review highlights the removal of As (III) and As (V) from H_2O by adsorption process using iron nanoparticles and its composites.

Synthesis of Iron Oxides NPs and Their composites: Currently, Iron NPs and their composites of different shapes and sizes is already applied successfully in several areas including living tissue imaging, farming applications and environmental applications such as removal of dyes, toxic compounds and toxic metals like mercury, lead, arsenic etc. (Tucek et al., 2014, Gangadhar, et al., 2020). The importance of magnetic nanoparticles for several of applications is because of their significant properties including stability in different conditions, biological compatibility, easy to prepare and process and reusability (Bohara et al.,2016). Usually, there are two oxidation states of iron i.e. Fe^{2+} and Fe^{3+} present in iron oxides, possessing four and five unpaired electrons in 3d sub shell, respectively. Nassar (2012) claimed several categories of iron oxides for example hematite, maghemite, magnetite, and wustite, oxides/hydroxides of Fe such as goethite, akagneite, lepidocrocite, feroxyhyte, and hydroxides of iron viz. ferrihydrite and bernalite (Figure 2.). A brief outline of the different approaches used for the synthesis of iron nanoparticles or composite for arsenic removal is given below:

Sol–gel deposition: Sol- gel deposition is a one of the most used process for the preparation of nanostructured porous membranes, nanoscaled layers and coatings can be prepared via the production of Sol-gel (Nisticò et al., 2017). The initivial step involves a polymerization process that creates a suspension of colloid, or "sol," of separate, homogenous dispersion of fine particles which is held in suspension after adding by the surfactant (Alehosseini et al., 2020). Further, the sol may be treated to remove the suspended particles, like casting or spin-coating on a substrate. It is changed into a gel by chemical reactions so as to restrict the surfactant from making network of bound particles in the solution, which may lead to produce a class of superpolymer, a huge molecule in the form of 3D or, on the surface, a 2D complex i.e. the "gel." Sol-gel thin film deposition process offers numerous benefits including processing at low temperature and effortless processing. Many researchers like Puscasu et al., (2016), Demirci et al., (2018), Yilmaz, et al., (2020) synthesized difference (Fe_2O_3) particles by sol-gel method.

Co–precipitation: The co-precipitation technique is most probable method for synthesizing magnetic nanoparticles due to easy to prepare and proficient chemical method (Bhateria et al., 2019). Fe3O4 is typically prepared by stoichiometric ratio of mixing of Fe2+ and Fe3+ solution in water (Sundar et al., 2020). The Fe3O4 is precipitated and predicted range of pH between 8 to 14. The structure and topography of the nanoparticles can be controlled by changing the concentration of respective salts, pH of aqueous solution, strength of ions and temperature (Díaz-Amaya et al., 2020).

Hydrothermal and Solvo–thermal synthesis: Hydrothermal process enables the solvents to heat up in a tightly packed container (bomb, autoclave, etc.) to reach a temperature beyond their boiling point (BP) (Biswas et al., 2017). When reaction takes place under definite

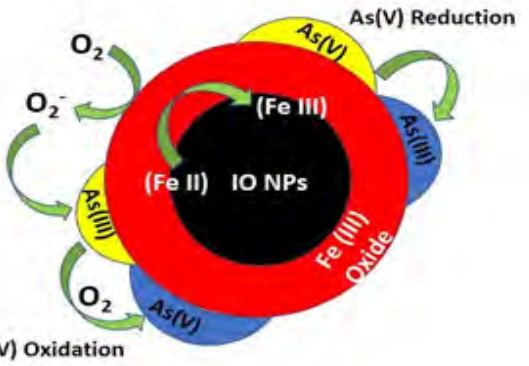
conditions of temperature and pressure called as Solvo-thermal treatment and when H_2O is applied as solvent acknowledged as hydrothermal (Guo et al., 2019). When water attained above the critical temperature and pressure is stated as supercritical and, as a liquid, exhibits the properties of both liquid and gas. In addition, to obtain hollow iron oxide NPs, the hydrothermal and solvo thermal way is a simple and traditional process, (Ounacer et al., 2020). In a standard process, reagents are mixed together and continuous mixing by the help of stirrer with ferric salt as the iron supply, which gives homogeneous mixture is further moved to a steel autoclave lined with Teflon and heat sealed for 8-24 h at about 200°C. Organic solvent is used in a reaction mixture in its place of water in the solvothermal phase. Normally, hydrothermal process for synthesizing Fe3O4 using salt of ferrous, ferric and sodium hydroxide with a molar ratio of 1:2:8 to an autoclave and heat treatment at elevated temperatures (Bhateria et al., 2019).

Magnetic Iron nanocomposites: Several substances such as Ag, Si or Au may be applied to coat the external surface of Fe_3O_4 nanoparticles (Salem et al., 2019). These coatings on the external surface of the magnetic nanoparticle provide covalent binding positions for a specific ligand and also enhance the stability of the nanocomposites. The composition of magnetic nanoparticles is such a way where they have an outer shell which possess inorganic material while iron oxide is composing the inside center. For example, the alteration of electrolytes and pH is occupied by silica coating and therefore leads to a superior degree of robustness of nanoparticles (Hurtado et al., 2020). Magnetic nanoparticles are doped with other metal(s) and other polymer(s) to form nanocomposites. Doping of iron oxides can be achieved by using oxidizing metallic agent such as manganese leading to precipitation and hydrolysis-precipitation processes. Bimetallic such as Fe-Mn, Fe-Ce and Fe-Cu and its oxides are insufficient patterns of metal doped iron oxides (Ma et al.,2020). These composites are very significant to prevent the step carried out in the pre-oxidation of arsenic using oxidants. The improved composites efficiently removed arsenic via oxidation and adsorption process (Priyadarshni et al., 2020).

Figure 2: Illustration of Synthesis of IO NPs and its Composites using different methods (Ravi et al., 2020)

Removal of As (III) and As (V): Iron based compounds for example hematite (α-Fe_2O_3), goethite (Mineral of the diaspore group, consisting of Fe^{3+} oxide-hydroxide), iron oxide coated nanomaterials and ferric hydroxide the ideal set of materials for As removal due to the minimum leaching of adsorbed As from the use adsorbent (Ghanizadeh et al., 2010). Iron nanoparticles possess magnetic properties which helps in smooth isolation of iron nanoparticles from H_2O using powerful magnet (Gadad et al., 2014). Furthermore, Iron nanoparticles, zero-valent nanoparticles have also shown in different research papers for removal of metallic arsenic from H2O and industrial wastes or discharge to prove its efficacy for purification of water and environmental sustainability (Figure 3.) (Mosaferi et al., 2014).

Figure 3: Mechanistic representation of Arsenic removal using IO NPs under aerobic condition (Hao et al., 2018)



Interaction of arsenate with iron oxides nanoparticles are established by the establishment of inner part of sphere and to a lower degree by weaker ionic exchange reactions phenomena. Luther et al., (2012) synthesized the Nanophase IO and shown its efficiency for the removal of As(III) and As(V). Maximum binding observed by the IO (Fe2O3 and Fe_3O_4) NPs were found 1.25 (mg/g), 8.19 (mg/g) for As(III) and 4.6 (mg/g), 6.7 (mg/g) for As(V) respectively at incubation period 1 hr. Graphene carbon nanotube-IO have shown significant absorption capacity for removal from As from dirty water because of high surface area to volume ratio and open porous structure (Vadahanambi et al., 2013, Mamaril et al., 2020).

Aredes et al., (2013) revealed that all natural IO NPs adsorbed arsenic at pH ranges from 4-11. Raul et al., (2014) synthesized IOH nanoflower and application in removal of As(III) (475 µg/g) from water. Bhowmick et al., (2014) fabricated the Mt-nZVI which shown very impressive result at pH 7.0 on both As(III) and As(V) 59.9 and 45.5 mg/g for As(III) and As(V) respectively. Devi et al., (2014) used IO coated sand for the As (III) removal from drinking water. Qi et al., (2015) reported that bimetallic oxide NPs like (Fe-Mn) remove 39.1 mg/g and 54.2 mg/g of As(V) and As (III) respectively. Composite γ-Fe_2O_3@CTF (Leus et al., 2018) shows excellent removal for both form of arsenic (As(III) (198.0 mg g^{-1}) and As(V) (102.3 mg g^{-1}). Mishra et al., (2019) formulated aero gel

based cerium doped IO for the As(III) removal and the maximum efficacy was 263 mg/g. Recently Dong et al., (2020) have formulated cellulosed nanocrystal IO and used this for the removal As(III) and As(V) adsorption. Which shows at pH levels of 7 and 3 CN/IO removed 13.866 mg/g and 15.712 mg/g of As(III) and As (V) from H_2O respectively. Table I. shows synthesis of iron nanoparticles and its composites for As removal.

Table I. Removal of Arsenic As (III) and As (V) by Iron NPs and its composite

S.N.	Nanoparticle Matrix	Isotherm	pH	Removal of Arsenic		Ref.
				As (III) (mg/g)	As (V) (mg/g)	
1	Bare NZVI	Freundlich	7	3.5	-	Kanel et al.,2005
2	Bare NZVI	Langumir	7	-	38.2	Yuan et al., 2006
3	γ-Fe_2O_3	Langumir	7	-	2.9	Park et al., 2009
4	α-Fe_2O_3	-	7	95	47	Tang et al.,2011
5	Fe_2O_3– γ-Fe_2O_3 nanoparticles	Langumir	2.0	3.69	3.71	Chowdhury et al.,2011
6	γ-Fe_2O_3	Langumir	3-11	74.83	105.25	Lin et al. 2012
7	γ-Fe_2O_3	Langumir	6.6	47	95	Prucek et al., 2013
8	B-FeOOH/GONs	Langumir	-	77.50	45.70	Ming et al. 2015
9	Fe-NN/BFs	-	7	70.22	93.94	Wei et al., 2019
10	IO gel	Langumir	7	35.75	-	Otero-González et al., 2020
11	RH+IO	Langmuir	7	82	-	Pillai et al., 2020
12	BT-FeN	Langmuir	7	-	18.98	
13	OL-FeNP	Langmuir	7	-	32.05	
14	GT-FeNP	Langmuir	7	-	13.70	Kamat et al., 2020
15	PL-FeNP	Langmuir	7	-	11.65	
16	EL-FeNP	Langmuir	7	-	39.84	
17	HFOR	Langmuir	5-7	41.6	71.5	Liu et al., 2020
18	Iron Oxide Composite	Langmuir	6	83.84	-	Ramasubbu et al., 2020

CONCLUSION

The present scenario of nanotechnology related with preparation and application of iron nanoparticle in the light of As removal has been outlined and reviewed. Iron oxide have exclusive magnetic and physiochemical characteristics which could be harness to use in environmental applications. Nanostructure iron oxide materials have outstanding capacity for the get rid of arsenic contaminants from water. Presently, this is similarly significant to find new ideas for enhancing the stability and biocompatibility of iron nanoparticles and composites to serve the purpose of environmental applications. In addition, iron oxide nanoparticles and its composites are observed to be the very good absorbents for arsenic removal.

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Nutritional Benefits and Role of Probiotics in the Modulation of Human Health

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ABSTRACT

Manuscript aims to describe the nutritional benefits of probiotics and their role in human health benefits. Probiotics are live microorganisms which are considered as non-pathogenic flora and provide benefits to the health of human. Probiotics are mainly used to hold the bacteria balance in the intestine. Probiotic bacteria help to reduce the development of harmful bacteria which may cause disease. Recently, probiotics are used in the food supplements to increase their nutritional value which also plays a role in the management of disease caused due to harmful bacteria. It can also modulate the immune function of the body. Probiotics are the latest products that lead to contribute to future health through the prevention and reduction of disease risk. This manuscript describes the properties, function and advantages of probiotics. The manuscript focuses on the types of probiotics and their role in the management of a disease. It also describes the probiotics mode of action in the treatment of disease. The list of marketed products related to probiotics is also summarized. The probiotics which are known as good bacteria are used to increase the nutritional value of the food which helps to manage the health and in the treatment of diseases related to the gastrointestinal tract.

KEY WORDS: PROBIOTICS; NUTRITIONAL BENEFIT; LACTOBACILLUS; BIFIDOBACTERIA; EUKARYOTIC; INFLAMMATORY BOWEL SYNDROME; H. PYLORI.

INTRODUCTION

The Probiotic word generally comes from the Greek words pro and bios which means 'for life'. In 1965, probiotics were first introduced by Stillwell and Lilly. Probiotics are the living microorganisms and microbial feed supplements. A probiotic is a live microbial feed that enhances the intestinal health of the host animal. The word probiotics are often referred as the microbial direct-fed. The terms microbial direct-fed and probiotics are used exchangeably. (Markowiak and Slizewska, 2018).

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Probiotics act as additives to feed which contains microbial species and known as normal non-pathogenic flora. The probiotics benefit health by improving the bacterial balance in the intestines when it is taken orally. Probiotics consist mainly bacteria, but they also include other types of organisms like yeast (Markowiak and Slizewska, 2018). Probiotics are identical or same as the "healthy bacteria" already exist in the body, mainly in guts. The intestinal tract of a normal human encloses 300-1,000 various species of bacteria. The digestive tract of a normal human contains approximately 400 types of probiotic bacteria which decrease the harmful bacteria growth and indorse a healthy digestive system (Markowiak and Slizewska, 2018;1 and Kerry et al., 2018,2).

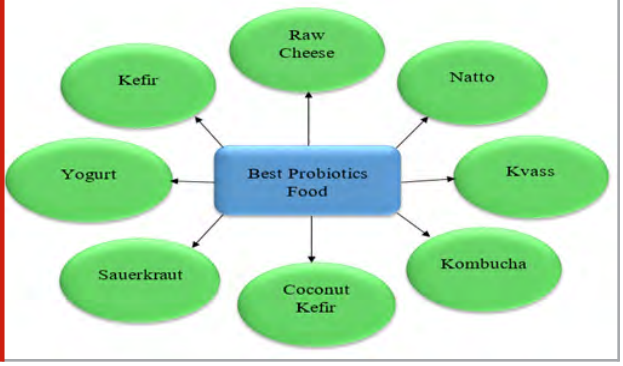
Probiotics can avoid or decrease the risk of disease that is also preferable for the treatment of the disease. Probiotics are developed as the main nutritional factor affecting the physiology and function of the gastrointestinal system. Intestinal microflora uses the probiotic microorganisms to improve well being (Markowiak and Slizewska,; 2018, Oak ,and Jha, 2019).

There is sufficient clinical evidence exists for the use of specific probiotics which help in the prevention and treatment of a few types of diarrhoea. Probiotics are also used to create new nutritional products. There is also scientific evidence that exists for specific forms of probiotic benefits in alleviating the symptoms of lactose intolerance, controlling the movements of the intestines and decreasing harmful enzyme activities in the intestine. The modification and binding of mycotoxin in the uncultivable portion of the human intestinal microflora help to prevent and treat food allergy (Oak and Jha 2019; Fenster et al., 2019).

Probiotics are the compound that is mainly isolated from the intestinal tracts of humans and animals. The products derived from end products of bacte¬rial growth or dead bacteria may also provide some advantages, but these derivatives are not used as probiotics because during administration they are not alive. The bacteria when not isolated, purified and proved for benefit of health after administration are called native bacteria (Cassani, Gomez-Zavaglia, Simal-Gandara, 2020). Probiotics are used in nutritional supplements and food like tablets, capsules, powders and other forms. Other sources of probiotics foods include yogurt, fermented and unfermented milk, juice, miso, soy beverages. Probiotics are similar to those bacteria which found naturally in humans guts, particularly in breastfed infants. The probiotics other than bacteria is yeast such as *Saccharomyces boulardii* (Focco et al., 2020). The best probiotics food are enlisted in figure 1.

Some Good Bacteria Examples: *Lactobacillus* (abbreviated L.) *acidophilus* (produces natural antibodies), *L. reuteri* (may protect against food poisoning caused due to *Salmonella* and *E. coli*), *L. salivarius, L. casei, L. plantarum, L. rhamnosus, L. paracasei, L. brevis, L. intantis* and *Lactobacillus* GG; *Bifidobacterium* (*abbreviated* B.) *bifidum, B. lactis, B. longum, Enterococcus faecium, Streptococcus thermophilus, Saccharomyces boulardii* (Santo et al., 2020)[7]. This manuscript describes the properties, function and advantages of probiotics. The manuscript describes the role of probiotics and their role in the health management. It also summarizes the list of products related to probiotics selled in market (Santo et al., 2020).

Figure 1: Schmeatic diagram to show best probiotics



Function Of Probiotics: Probiotics help in diarrhoea treatment. It can treat and prevent infections of the urinary tract and the genital organ of females. Probiotics treat the disease of irritable bowel syndrome and decrease the chance of bladder cancer (Parvez et al., 2006). Long intestinal infection and atopic dermatitis may be treated with probiotics. Probiotics can play a major role in the treatment of lactose intolerance and to maintain cholesterol levels (Kerry et al., 2018).

Advantage of Probiotics: Probiotics are dietary supplements or food products that contain beneficial elements to the host body. It maintains the optimal health and wellness of human beings. It can provide a natural defense or immune system to the body. Probiotics can prevent the growth of harmful bacteria. Probiotics make the immune system to fight against allergies and other autoimmune diseases. It helps the body to produce vitamins and support healthy digestion (Verschuere et al., 2000). Probiotic increase defecation and reduce constipation. It can control the illness-caused by bacteria present in the intestinal tract. It reduces the effects of Candida infection.

Probiotics improve the digestion of lactose, especially for the lactose-intolerant individual. It reduces cholesterol levels and blood pressure. It improves the absorption of minerals, especially calcium from the body (Verschuere et al., 2000). It decreases the dental-caries caused by microbes present in the mouth. Probiotics used to cure vaginal yeast infections and in urinary tract infections treatment. It manages the signs and symptoms of irritable bowel syndrome. Reduces the amount of cancer causing substances in the intestine. Reduce the development of allergy in children and also reduces the infections and inflammation (Kerry et al., 2018, Verschuere et al., 2000; Kerry et al., 2018).

Probiotics provide bile acid tolerance which is difficult to maintain during oral administration. Adherence to epithelial and mucosal surfaces is a crucial factor for successful immune modulation, competitive exclusion of pathogens and pathogen adherence and colonization prevention. It has antimicrobial activity against pathogenic bacteria and also act as bile salt hydrolase (Ziemer and Gibson,1998; Kerry et al., 2018).

Types of Probiotics: Different types of probiotics are described below:

1. Lactobacillus: *Lactobacilli* have more than 50 species. They are found naturally in the urinary, genital and digestive systems. Fermented food like yogurt is used as dietary supplements. *Lactobacillus* has been used to treat and prevent a wide variety of diseases and conditions. Different *Lactobacilli* species are found in foods supplements such as *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *L.acidophilus* DDS-1, *Lactobacillus* rhamnosus GG, *Lactobacillus reuteri*, *Lactobacillus salivarius, Lactobacillus plantarium, Lactobacillus johnsonii, Lactobacillus casei* etc. *Lactobacillus* can prevent and treat bacterial vaginosis, yeast infections, urinary tract infection, antibiotic-related diarrhoea, irritable bowel syndrome, travelers diarrhoea, diarrhoea resulted from Clostridium difficile, skin disorders, treating lactose intolerance and respiratory infections prevention (Ziemer and Gibson, 1998; Amara and Shibl, 2015).

2. Bifidobacteria: *Bifidobacteria* have more than 25 species. They are used to make up healthy bacteria in the colon. It exists in the intestinal tract of breastfed infants from the day of birth. *Bifidobacteria* species are used as probiotics such as *Bifidobacterium bifidum*, *Bifidobacterium longum, Bifidobacterium lactis*, *Bifidobacterium breve*, *Bifidobacterium thermophilum, Bifidobacterium infants* and *Bifidobacterium pseudolongum. Bifidobacteria* help to increase glucose tolerance and blood lipids levels. *Bifidobacteria* improve symptoms of the IBS such as pain, discomfort, bloating distension, disorders of digestion (Amara and Shibl, 2015).

3. Saccharomyces boulardii: This bacteria is the only yeast probiotic called as *S. boulardii*. It may prevent and treat traveler's diarrhoea and diarrhoea associated with the use of antibiotics. It has been also used to prevent *C. difficile* reoccurrence which helps to treat acne and reduce side effects of H. pylori treatment (Kerry et al., 2018,; Amara and Shibl, 2015.

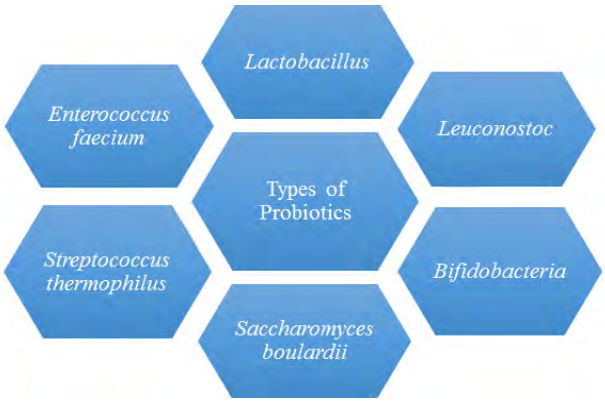
4. Streptococcus thermophilus: It produces a large amount of the lactase enzyme and helps to prevent the intolerance of lactose.

5. Enterococcus faecium: It is commonly present in both the human and animal intestinal tract.

6. Leuconostoc: It is used in food processing from a very earlier time. The foods containing metabolites of microorganisms, live bacteria and dead bacteria are ingested from a long time (Amara and Shibl, 2015).

Different types of probiotics are shown in figure 2.

Figure 2: Schematic diagram of different types of probiotics



Probiotics In Helicobacter pylori Infections: *H. Pylori* induces multiple gastrointestinal diseases like chronic gastritis and peptic ulcer. The latest treatment choices are antibiotics and proton pump inhibitors. Probiotics have been used to supplement infection control by using different *Lactobacillus* species which is demonstrated in both in vitro and in vivo studies. From in vitro studies, it was suggested that direct antimicrobial activity of *Lactobacillus* species occurs by competition with *H. pylori*, thereby demonstrating the clinical progress in patients treated with probiotics (Markowiak and Slizewska, 2018).

However, the results are positive, yet probiotics can not be proposed as a valid substitute for *H. Pylori* infections standard treatment. *Helicobacter pylori* is a bacterium in small curved to spiral rod shape. It is strongly associated with duodenal peptic ulceration and used as the main etiologic agent for chronic gastritis, gastric cancer and other gastric malignancies (Tripathi and Giri, 2014). Today, the therapy based on a combination of antibiotics and proton pump inhibitors are used to kill this bacterium. Probiotics tend to have a direct antimicrobial effect which was demonstrated through in vitro studies, competing with *H. pylori*, adherence inhibition, metabolites production and antimicrobial molecules. (Markowiak and Slizewska, 2018, Tripathi and Giri, 2014).

In a study, 60 participants were treated with triple antibiotic therapy on days 1-7 and *Lactobacillus* GG on days 1-14 in a double blind, randomized, placebo-controlled trial. Probiotics considerably improved the symptoms of taste disturbance, nausea and diarrhoea. However, epigastric pain during eradication treatment did not significantly improve. The eradication rates between the groups did not differ significantly (83.3% vs 80%) (Tripathi and Giri, 2014; Markowiak and Slizewska, 2018).

In another placebo-controlled, randomized, double blind trial the asymptomatic 85 patients of *H. pylori* were randomized to receive treatment from days 1-7 with placebo from days 1-14. Probiotics show significantly improved symptoms during treatment of diarrhoea and taste disturbance; but epigastric pain and nausea did not improve significantly during *H. pylori* treatment (Granato et al. 2010). During the study all the differences between the probiotics and placebo were noted. None of the probiotics has been better than another. Eradication rates among the 4 groups that received probiotics were not significantly different. A randomized double-blind placebo-control study was conducted on 47 patients using a milk based fruit drink containing *Propioni bacterium*, *Lactobacillus* GG, *Bifidobacterium*, or placebo from day 1-28 and triple antibiotic therapy from days 1-7 (Granato et al., 2010).

Probiotics did not improve symptoms significantly, including taste disturbance, nausea, epigastric pain and diarrhoea (Ziemer and Gibson, 1998,; Granato et al., 2010). Recently a meta-analysis showed that supplementation with *S. boulardii* significantly raised the eradication rate and reduced the overall risk for *H. pylori* related adverse reactions, particularly in diarrheoa. However, the products used in such trials are not usually marketed in the US, making it difficult to support evidence related to probiotic. Although a specific strain of *Lactobacillus* supported by the US may not be available in the market, because it may not be fair to extrapolate the results of strain to other types of *Lactobacillus* so the product selections are limited (Butel, 2014).

Probiotics In Irritable Bowel Disease: Gastrointestinal problem includes irritable bowel syndrome (IBS), abdominal pain and excessive flatulence. Motility disorders and psychological mechanisms have been suggested to differentiate the intestinal microflora in people with IBS and with healthy peoples. In comparison with healthy people, these patients have low numbers of *Llactobacilli, Bbifidobacteria* and higher numbers of facultative microbes. Probiotics are used as therapy but the results are unclear. A preventive strategy may have more benefit for *Llacto bacillus* than when it is used in the IBS treatment, although this has not been confirmed. (Amara and Shibl, 2015).

The role of intestinal bacteria in the IBS pathogenesis has been suggested by physiological, epidemiological and clinical trials. Some earlier studies indicate that gastroenteritis is the main cause of IBS (Butel, 2014)[14]. A cohort study in Canada, an epidemic of gastroenteritis showed an increased IBS patient in 2 years, which lasted for 8 years. In another study, the incidence of gastroenteritis was associated with approximately a four-fold rise in the probability of developing IBS in the previous 2 years. Physiological research on animals and humans demonstrated a profound impact of alterations in the intestinal microbiota composition of the normal and IBS patients intestine (Amara and Shibl, 2015). The developing IBS increases the risk of dysbiosis, gastroenteritis and increases the production of luminous gas and immune activation suggests that the gastrointestinal microbiota may be a therapeutic target for IBS (Butel, 2014; Amara and Shibl, 2015, Butel, 2014).

While numerous probiotics efficacy Randomized Clinical Trials have been assessed with IBS patients, they often suffer from severe methodological flaws. Brenner and colleagues reported in a recent systematic review that 16 RCTs were assessed as effective probiotics in the treatment of IBS, *Bifidobacterium infants* 35624 was the only probiotic that offered substantial improvements in IBS symptoms. VSL#3 has demonstrated a greater improvement in abdominal pain and bloating symptoms globally. A randomized cross-over trials was done with 59 children having IBS. Some meta-analysis indicates that probiotics have a more beneficial effect on abdominal pain and flatulence. *Bifidio bacterium* is available on the market in combination with Align capsules or other probiotic organisms as OWP probiotic capsules, and VSL#3 packets for the treatment of IBS. More evidence is needed before IBS is used as probiotics for control symptoms (Rivera-Espinoza and Gallardo-Navarro, 2010).

Probiotics and Bacterial Translocations: Many studies have been shown that patients who are unable to feed enternally after severe gastrointestinal surgery or liver transplantation also have a high risk of septicemia from the intestinal tract triggered by bacterial organisms. A study describes various ways in which probiotics can decrease bacterial translocation. It seems possible to eliminate postoperative infections by altering the luminal bacterial milieu. The research results are promising but need confirmation in larger prospective studies. In mesenteric lymph nodes (MLN), the detection of viable bacteria represents bacterial translocation in the intestine lumen. (Millette et al., 2013). Each rats lymph nodes were aseptically removed from the ileocaecal and left colonic regions and dissected (Millette et al., 2013).

Nodes were then homogenized for the cultivation of aerobic and anaerobic bacteria in 1 ml of sterile phosphate buffer saline or thioglycollate broth respectively. At 37°C, a 0.1 ml aliquot of each homogeneous was placed on blood agar and incubated and the number of colonies was counted on all plates. Bacterial translocation data are defined as medians and ranges of the total colony forming unit (CFU) (both aerobic and anaerobic) will be calculated from the cultured plate after 48 hours of incubation from MLN of each rat (Cousin et al., 2012; Millette et al., 2013, Cousin et al., 2012).

Probiotics and Safety: Over the last few decades the use of probiotics has increased, especially in dairy products. The studies focus on infection risk, toxicity, deleterious metabolic activity and antibiotic resistance with increasing probiotic strain in dairy products (Ozyurt and Otles, 2014). In safety assessment, children and infants are especially found to be vulnerable at a period when the intestinal environment and the immune system are under development. However numerous studies have

not shown any adverse results even on preterm infants. It seems like most people do not suffer from probiotics side effects or have just mild gastrointestinal side effects including gas. But there have been several case reports of serious adverse effects (Kent and Doherty, 2014).

A review on probiotics safety suggested that *Lactobacillus rhamnosus* GG was widely studied for a variety of conditions in clinical trials and found to be generally safe. Nevertheless, a recent review of *Lactobacillus* and *Bifidobacterium* noted the long-term, cumulative effects of probiotics use, especially in children and also indicates the evidence that probiotics should not be used in patients with a critical illness (Saxelin et al., 2010). Similarly, a 2011 Agency for Healthcare Research and Quality Assessment on the safety of the probiotic, partly funded by National Center for Complementary Alternative Medicine (NCCAM), concluded that the current evidence does not suggest a widespread risk for probiotic related side effects. However, safety data, especially long-term protection are limited and the risk of serious side effects in people may be greater with underlying health conditions (Garanto et al., 2010; Saxelin et al., 2010;19,20 Kent and Doherty, 2014).

Eukaryotic Probiotics: Eukaryotic microorganisms are very useful as probiotics for animal health. There are several eukaryotes grade of food/feed, like as algae (e.g. Spirulina, Chlorella species), fungi (e.g. Penicillium, Aspergillus species), yeasts (e.g. Candida, Saccharomyces, Pichia, Kluyveromyces, Torulopsis species), which are being consumed by human and animals throughout the world since a very long time. These organisms are mostly used as single cell protein and as food starters components. However, certain eukaryotes are found to be executing probiotics like beneficial effects in the host when supplemented in living conditions through diet (Hennequin et al., 2000) (Hirimuthugoda, Chi and Wu, 2007).

Therefore, the development of new candidate species beyond prokaryotic origin is believed to be a very crucial event in the field of probiotics. Significant interest in eukaryotic probiotics is growing nowadays and in most cases their efficacy and usefulness have been proven by strong scientific evidence. Most of the eukaryotic probiotics used in human and animal practices belong to the dominant group of fungi, yeasts and mould. Pichia, Candida, Saccharomyces, Yarrowia, Metschnikowia, Isaatchenkia, Debaryomyces, Aspergillus and Kluyveromyces are common examples of eukaryotic microorganisms with probiotic properties (Holubarova, Muller and Svoboda, 2000). From 1,550 BC, yeast has historically been used for fermentation purposes. Nowadays, yeasts are a part of dietary supplements and healthy food realms because of their proven beneficial probiotic effects. Saccharomyces genus of yeast has commonly used probiotics in humans and animals worldwide (Hottiger, Boller and Wiemken, 1987; Holubarova, Muller and Svoboda, 2000).

Mode of Action of Probiotics: Several studies have demonstrated several types of probiotic action in the aquatic environment. Selected strains were determined to produce digestive enzymes, thus facilitating the utilization and digestion of the feed. The enzymatic properties of intestinal anaerobic bacteria isolated from three species of fish, showing the potential role as a probiotic. In the research, the addition of the two intestinal fish Bacillus spp. was done. Increased performance as assessed by several factors including growth, feed conversion and protein efficiency ratio (Gomez-Gil, Rogue and Velasco-Blanco, 2002). The bacteria attributed the result to the production of the extracellular cellulolytic and amylolytic enzymes. While competition has been widely suggested as a mode of action for adhesion sites, there is little evidence in the literature to prove this fact. Studies report adhesion of certain bacteria to in vitro intestinal mucus and the attachment ability of potential probiotics seen *in vitro* can not be assumed to demonstrate the real *in vivo* effect (Gomez-Gil, Rogue and Velasco-Blanco, 2002).

Additionally, studies have shown the ability of some bacteria to adhere with in vitro intestinal mucus they have failed to assess a competitive exclusion effect. More recently, it has been shown that five probiotics versus two pathogens on fish intestinal mucus exhibited a competitive exclusion effect. The presence of one of the probiotics on the mucus was found to inhibit the attachment of one of the tested pathogens. Interestingly, pre-colonization with the other probiotics prompted the two pathogens to attach themselves. However, the general trend of their research has shown that the pathogen was displaced after treatment with probiotics (Holubarova, Muller and Svoboda, 2000,; Gomez-Gil, Rogue and Velasco-Blanco, 2002).

Although not directly related to attachment competition, it was shown that two seaweed-associated Bacillus spp. produced antibiotic substances. It was dependent on bacteria forming biofilms. This study highlighted a factor i.e. surface attachment, that could be essential for some bacteria to be successful probiotics. This observation concurred with the definition of a probiotic, i.e. the colonization requirement for GIT. (Rogue and Velasco-Blanco, 2002).

It was suggested that the competitive exclusion mechanism for attachment sites could be given a distinct advantage through the addition of probiotic bacteria during the larviculture initial egg fertilization steps, thus "getting in there first". This concept was not supported because when these bacteria were administered at hatching and two days after hatching, no difference was observed between the concentrations of two bacteria in the gut of turbot larvae. Several studies have attributed a probiotic effect to an energy source competition. Artemia sp. was found beneficial for growth and survival.

It was pre-exposed to nine bacterial strain before challenging with *V. proteolytic*. It was concluded that the extracellular products do not cause any effect, but the live bacterial cell was required. Although not

specifically tested, they hypothesized that the protective effect was probably the result of competition for energy sources and sites of adhesion. Competition for iron has been reported as an important factor in marine bacteria. Iron is required for the growth of most of the bacteria but is generally limited in the animal tissues and body fluids and the insoluble ferric Fe^{3+} type iron-binding agents, siderophores, enable iron acquisition suitable for microbial growth (Gram et al., 1999).

Siderophore production is a noted mechanism of virulence in some pathogens equally, a siderophore producing probiotic could deprive potential pathogens of iron under iron limiting conditions. This was shown by a supernatant culture of Pseudomonas fluoresces, grown under limited conditions of iron, inhibited *V. anguillarum* growth, while the supernatant from iron-available cultures did not inhibit the growth (Gram et al., 2001). It was found that the addition of *Bifidobacterium thermophilum* derived peptidoglycan increased significantly their survival when they were challenged with V. penaeicida. It was attributed that an immune stimulatory effect, as the phagocytic activity of shrimp granulocytes was significantly higher in the treated shrimp compared with those of the control animals. Research differentiated slightly to approach towards immune-stimulating probiotic (Gullian, Thompson and Rodriguez, 2004).

Instead of analysing bacterial derivatives such as glycans or lipopolysaccharides, they tested live *Vibrio* sp. (P62) for immune stimulation and Bacillus sp. (P64) and *V. alginolyticus* used as a positive control. They concluded the immune stimulants activity of P64 and *V. alginolyticus* (Gram et al., 2001; Gullian, Thompson and Rodriguez, 2004).

Probiotic Products: The most popular approach to consume probiotic cells are through food products. The global market for functional foods and beverages has grown from $33 billion in 2000 to $176.7 billion in 2013, representing 5% of the food market as a whole. Probiotic foods are comprised between 60%-70% of the total functional food market. Probiotic microorganisms are typically available as dried or deep-freeze culture concentrates to be added to a food matrix. Lactic acid bacteria of the genera *Lactobacillus* and *Bifidobacterium*, are the most common genera and species, as they are widely recognized as safe (Hagi et al., 2004,; Granato et al., 2020).

The species *Lactobacillus* and *Bifidobacterium* are also predominate in the human intestine (*Bifidobacterium* in the large intestine and Lactobacillus in the small intestine). However, bacterial species of the genera *Lactococcus, Enterococcus* and *Propionibacterium*, yeasts (e.g. *Saccharomyces boulardii* and *Saccharomyces cerevisiae*) and filamentous fungi (e.g. *Aspergillus oryzae*) are also used as probiotics due to their beneficial effects on health (Satkori, 2019,; Min. et al[31,32]., 2019).

Also, some people suggest that multispecies supplementation of dairy probiotic products may have a more specifically targeted function in the human food tract. Maintaining the viability of probiotic cells during food-processing and gastro-intestinal transit is important for microorganisms to reach adequately the intended site of action (108 cells/gram). (Tarkhani et al., 2020, Barbosa et al., 2011). Due to passage through the low pH environment of the stomach and high bile salt conditions in the intestine, there is a significant loss of viable cells following the ingestion of a probiotic (Barbosa et al., 2011; Tarkhani et al., 2020).

One possible solution for this problem is microencapsulation. *Encapsulation* is a mechanical or physicochemical process that traps a material that is potentially sensitive and provides a protective barrier between it and the external conditions. The spray-drying, emulsion and extrusion techniques are well known methods of encapsulation for the processing of probiotics microcapsules (Taskin, 2020). The probiotic effect and survival are strain dependent, therefore it must be perfectly identified and characterized (phenotypic and genotypic identification). *Lactobacilli* are generally stronger than Bifidobacteria, in terms of robustness of probiotic species, more resistant to low pH and have a greater tolerance to milk and other food substrates. Probiotic products can be classified as dairy probiotic products and non-dairy probiotic products depending on the matrix that carries the probiotic bacteria. Dairy beverages are produced from milk or its derivatives, with or without the addition of other ingredients in which the milk base represents at least 51% v/v of the formulation and can be fermented using yogurt cultures (Taskin, 2020, Guimaraes et al., 2019; Taskin, 2020).

Fermented milks, ice cream, different kinds of cheese, milk powder and baby food, whey-based beverages, frozen dairy desserts, buttermilk, sour cream, normal and flavored liquid milk are the most common dairy probiotic products. Milk and dairy products are abundant minerals sources which play a variety of roles in the human body. However, becuae of the high content of saturated fatty acids the availability of minerals from cheeses and cheese-like products is lower than that from other dairy products (Saxelin et al., 2010). Alejewicz and Cichosz have determined the effect of the probiotic culture of *Lactobacillus rhamnosus* HN001 on the increase of magnesium, calcium, phosphorus, zinc and potassium in cheese. The addition of *Lactobacillus rhamnosus* HN001 increases the availability of divalent metal cations. Also, other technologies and methodologies can be applied to existing probiotic dairy products (Taskin, 2020).

Kent and Doherty (2014) used an isotherm differential scanning calorimetry method to identify the probiotic microbes in probiotic products (Kent and Doherty, 2014). The products were developed and now commercial in Hungry. Products are Probiotic kefir (Symbiofir), probiotic sour cream, probiotic butter cream, poultry meat products supplemented with calcium and bakery products complement with calcium. Demonstrated that

the optimal concentration of constituents such as whey in probiotic dairy beverages could be calculated by using mathematical models such as survival analysis, minimal significant difference and mean global acceptability. Because of the high prevalence of lactose intolerance, different non-dairy probiotic products such as vegetarian-based products, fruit juices, cereal-based products, oat-based desserts, soya-based products, breakfast cereals, confectionery products and baby foods have been developed in recent years (Saxelin et al., 2010;, Gonzalez-Sanchez, 2010; Kent and Doherty, 2014).

Technological developments have made it possible to alter certain structural characteristics of fruit and vegetable matrices by modification of food components in a controlled way. It could make them perfect substrates for the probiotics culture. Cereal grains are one of the most essential sources of carbohydrates, protein, vitamins, fiber and minerals; Lactobacillus strains are fastidious microorganisms that require these sources for growth. Moreover, cereals can serve as prebiotics because they can be used as sources of non-digestible carbohydrates, encouraging the growth of the colon's *Lactobacilli* and *Bifidobacteria* (Matias et al., 2014). Another good raw material to be used as an alternative for the nondairy probiotic carrier is soy, which has some sugars and amino acids in its composition that are used as substrates by lactic acid bacteria to produce aroma compounds. However, soy intake is limited due to its undesirable beany flavor and the presence of oligosaccharides frequently contributing to flatulence and discomfort in the stomach (Matias et al., 2014).

One way to improve the sensory consistency of soymilk and also to mask undesirable compounds is by fermenting the lactic acid which can be combined with supplemental glucose, sucrose and lactose. Bakery products like bread are stapled foods composed of many main components (complex carbohydrates, insoluble dietary fiber, lipids, proteins, vitamins and minerals) in varying amounts and with varying physical interactions and structures. Cespedes et al. (2013) Soukoulis et al. developed probiotic bread with addition of the bacteria *Lactobacillus rhamnosus* GG, using air dried probiotic edible films. Meat can be also provide another source of probiotic products. The buffering capacity of meat may be attributable to an elevated pH of the microenvironment for the living of bacteria on its surface. It is important to continue the research into new non-dairy probiotic products that could have a wide market because of the high prevalence of lactose intolerance and vegetarianism (Cespedes et al., 2013).

CONCLUSION

It can be concluded from the literature survey that probiotics play a vital role in the management of the health of human beings. Proper concentration and species of probiotics are necessary for the maintenance of the immunity of the organism. Probiotics are used in the food supplements which increase the nutritional value of the food which is beneficial for human health. Probiotic microorganisms are available as culture concentrates in dried or deep-freeze form which is added to a food matrix and marketed as a food product. The main products of probiotics developed in recent years are vegetarian-based, cereal-based products, fruit juices, soya-based products, oat-based desserts, confectionery products, breakfast cereals and baby foods. The probiotics are mainly used to maintain the level of good bacteria inside the gastrointestinal tract mainly in the intestine. It helps to decrease the chances of disease related to the gastrointestinal tract. It also proves their activity in the treatment of the various diseases related to humans. The manuscript describes the function, advantages, mode of action and marketed products of probiotics and their role in human health management.

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Computer-Aided-Design and Manufacturing of Full Mouth Restoration of a Male Patient with Gastroesophageal Reflux Disease: A Case Report

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ABSTRACT

Modern dentistry increasingly concentrates on digital procedures, including computer-aided-design and computer-aided-manufacturing (CAD/CAM) technology and the development of fixed and removable protheses based on millable materials. This case-report presents a case of a 67-year-old male having a chronic gastroesophageal reflux disease (GERD) with severe abrasion of upper anterior teeth and loss of bone in edentulous areas. Evaluating the possible modalities of treatment and the associated empirical evidence. In addition, the full mouth rehabilitation with a minimally invasive procedure using veneers on the lower anterior teeth, CAD/CAM restorations on the remaining teeth, and implant-supported fixed dental prostheses in the edentulous areas were chosen considering the patient's factors such as tooth prognosis, wishes and economic status. Accurate diagnosis, ideal occlusal design with a 3D virtual implant planning and use of contemporary restorative materials can ensure favorable functional and esthetic rehabilitation for long-term prognosis.

KEY WORDS: GASTROESOPHAGEAL REFLUX DISEASE (GERD), TOOTH LOSS, VERTICAL DIMENSION OF OCCLUSION (VDO), CONE-BEAM COMPUTER TOMOGRAPHY, CAD/CAM.

INTRODUCTION

The use and application of computer-aided design and computer-aided development (CAD/CAM) technology in dentistry has increasingly expanded over the last two decades (Nassani et al., 2021). Dental CAD/CAM systems have been used to produce dental protheses that are excellently and reliably marginally and internally fit and to promote the manufacture of protheses. CAD/CAM systems use highly exacting scanners and software to digitally design the complicated forms required in dentistry. These systems have allowed digital dental treatments to be developed. Untreated tooth wear may lead to several complications such as hypersensitivity, discoloration, loss of occlusal vertical dimension, and impaired function and esthetics (Castrillion et al., 2018; Olley et al., 2017). These complications not only affect teeth and the masticatory system but also influence the quality of life. The incidence and prevalence of tooth wear are increasing and representing a growing concern in the field of dentistry (Atalay et al., 2018; Wei et al., 2016, Son et al., 2021).

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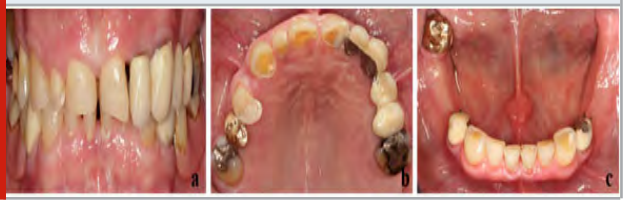
One of the most common causes of tooth wear is erosion, which is defined as the loss of tooth structure due to a chemical process. Extrinsic factors such as the consumption of acidic food or drinks are mainly responsible. While intrinsic factors such as gastroesophageal reflux disease (GERD) may equally be responsible. GERD is defined as a condition when the reflux of stomach contents causes troublesome symptoms and/or complications (Van Roekel et al., 2003 Picos et al., 2018). Several factors may intensify GERD such as dietary habits, smoking, physical exercise, and obstructive sleep apnea. Therefore, adequate diagnosis and accurate monitoring are necessary (Strub et al., 2011, Castrillion et al., 2018).

The restoration of worn teeth due to erosion is complex. Various treatments using different materials and techniques to treat patients with dental wear have been described in the literature (Mesko et al., 2016; Moretto et al., 2016). However, there is no strong evidence to help clinicians choose the most appropriate therapy involving aesthetic dental treatment for smile enhancement. This case presents the treatment modalities of a patient with chronic GERD who presented with tooth wear and required full mouth rehabilitation.

Case report: Ethical approval (PNU-011/2020) was obtained from Princes Norah University, Riyadh KSA at the IRB institute and consent form was signed by the patient who was involved in this study. A 67-year old male presented to the clinic with the complaint of impaired ability to masticate and dissatisfaction with his esthetics. The patient wished for a stable, preferably fixed and esthetic prosthetic rehabilitation. His medical history revealed no significant general conditions or allergies except hypertension. The patient was reported with a diagnosis of GERD a few years ago.

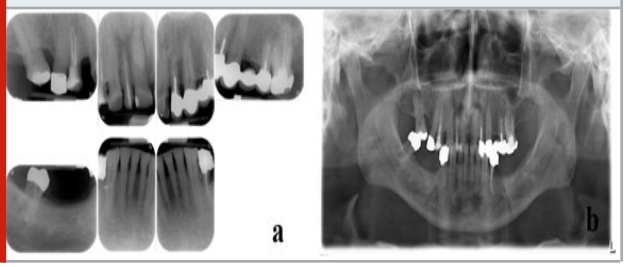
The patient's state of dental hygiene was average. Intraoral assessment of the patient exhibited inharmonic teeth forms and multiple diastemata. The upper central incisors and canines were massively short indicated severe abrasion and the vertical dimension of occlusion (VDO) was also affected (Fig. 1a). The horizontal and vertical bone loss was also diagnosed in edentulous areas. Several deficient crowns and fixed dental prostheses (FDPs) were identified as a result of poor marginal closure, as well as a deficient mandibular removable dental prosthesis (RDP) (Fig. 1b and 1c).

Figure 1: Patient's preoperative images: (a) close-up view of the smile; (b) intra oral maxillary view with erosion and attrition; and (c) intra oral mandibular view with erosion and attrition.



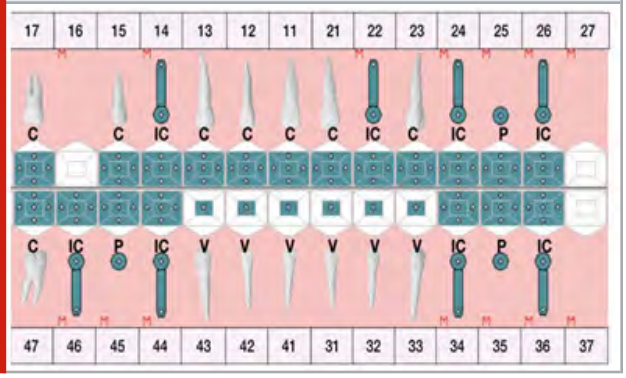
The radiographic assessment showed insufficient root canal fillings on teeth 5,7,10,13,19,21 and apicectomy on teeth 13, 14; and a transverse fracture in the cervical root part of tooth 21 and a mesiodens (Fig. 2a & 2b). The patient was diagnosed with the minor facial asymmetry toward left, multiple insufficient restorations, generalized gingivitis, multiple insufficient root fillings, horizontal root fracture on tooth 34 and compromised esthetics.

Figure 2: Patient's radiographic analyses: (a) periapical x-rays of maxillary and mandibular teeth showing dental wear in the anterior region and insufficient restorations; and (b) panoramic x-ray showing insufficient root canal fillings on teeth 14,12,22,25,36,34, apicectomy on teeth 25, 26, a transverse fracture in the cervical root part of tooth 34 and a mesiodens.



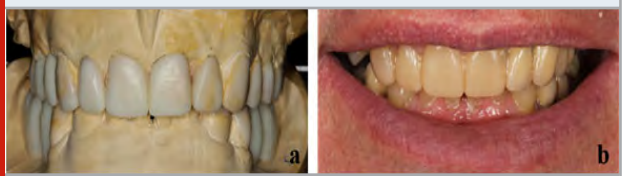
After evaluating the possible treatment modalities and the related scientific evidence, as well as considering the patient's factors such as tooth prognosis, wishes and economic status. The full mouth rehabilitation with a minimally invasive procedure using veneers on the lower anterior teeth, CAD/CAM restorations on the remaining teeth, and implant-supported fixed dental prostheses in the edentulous areas were selected (Fig. 3).

Figure 3: Final treatment plan with a minimally invasive procedure using veneers (V) on the lower anterior teeth, CAD/CAM restorations on the remaining teeth (C), and implant-supported fixed dental prostheses in the edentulous areas (IC: implant-supported crown, P: pontic, M: Missing).



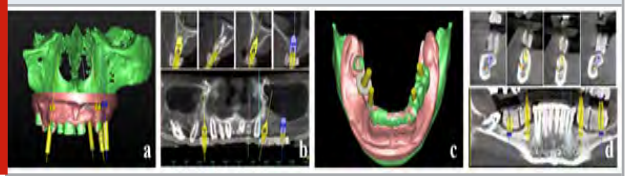
A full-arch wax-up was created to evaluate the predictability of the final esthetic and functional outcome. The wax-up was transferred into the mouth with the aid of a silicone index via a mock-up to evaluate the esthetic and phonetic parameters (Fig 4a & 4b).

Figure 4: (a) Diagnostic wax-up at proposed occlusal vertical dimension; (b) intraoral evaluation of wax-up via mock-ups.



Accordingly, CAD/CAM provisional restorations were fabricated. After removal of old restorations and extraction of hopeless teeth, teeth were prepared with care, since tooth structure is already lost due to erosion and bruxism, and provisional restorations were cemented. During this phase, two questionable teeth were given a poor prognosis due to thin dentin walls after post removal (tooth #10) and deep caries (tooth #28) and were therefore indicated for extraction. The crown of the anterior maxillary tooth (#7) was lengthened to improve the uneven gingival display. After 4-6 weeks, reevaluation of the pretreatment phase revealed a stable periodontal status with probing depths 2-3 mm and almost no bleeding on probing. The extraction sites healed successfully with no complications. The patient showed better compliance and improved oral hygiene. After successful pretreatment, preparations for implant placement took place. A 3D virtual implant planning was carried out based on data from cone-beam computer tomography (CBCT) using planning software Simplant®. Four implants in each of the maxilla and mandible were planned (Fig. 5a, b, c, d).

Figure 5: 3D virtual planning using planning software: (a & b) four implants in the maxilla; (c & d) four implants in mandible.



The data were uploaded to the production center, where the fabrication of the surgical guides took place. Using surgical guides, together with a guided surgery kit (Xive surgical kit GS, Dentsply Implant Manufacturing GmbH, Mannheim, Germany), the implants were placed in two separate surgical appointments (Fig. 6). Tooth 5 were placed with an internal sinus lift. Four months after placement, second-stage surgery was performed and healing abutments were fixed onto the implants. After definitive preparation of the abutment teeth, a double cord technique was used for the displacement of the gingival tissues. The implant transfer impression copings were mounted onto the implants and their position was controlled radiographically.

Final impressions were taken using custom trays with polyether impression material. Next, face bow transfer and jaw relation records. Next, fabrication of the individual implant abutments using computer aid design CAD (DentalDesigner™, 3shape dental system) was performed. All teeth received monolithic lithium disilicate crowns (e.max), which were adhesively cemented. Layered zirconia crowns were fabricated for the custom implant abutments, which were horizontally screwed. At the one-week follow-up, the patient was satisfied with his new restorations, and a protective night guard was delivered at that time. To improve long-term prognosis, the patient entered a 4-month recall maintenance program and had been followed for 2 years (Fig. 7).

Figure 6: Intraoral occlusal views immediately after implant placement in the mandible and in the maxilla.

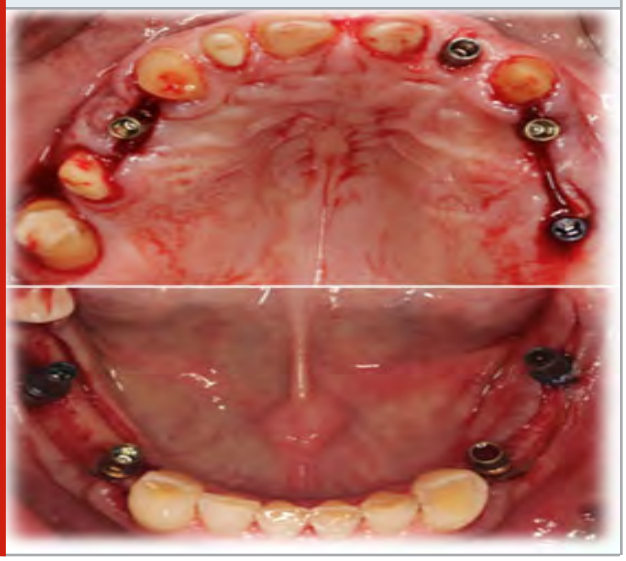
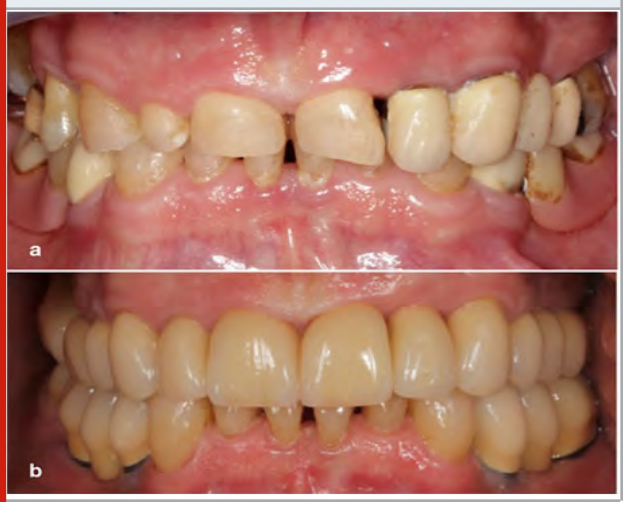


Figure 7: Intraoral frontal view: (a) Pre-treatment and (b) Post-treatment.



DISCUSSION

Tooth wear can be divided, based on its cause into abrasion, attrition and erosion (Warreth et al., 2020). The exact type of tooth wear can be identified by a

thorough medical history of the patient as well as clinical examination. However, in some cases, the synergy between the different factors causing wear may exist (Atalay et al., 2018). In this clinical case, wear was seen especially on the palatal and lingual surfaces of the anterior teeth, which indicated that the possible cause of wear in this patient had either a chemical or mechanical origin. A thorough review of the medical and dental history of the patient revealed that he suffered from severe reflux, which remained untreated until he retired. The patient showed also signs of bruxism, which was confirmed by his wife and further confirmed from the clinical examination showing stiff masticatory muscles. Therefore, it was diagnosed that the main reason for tooth wear in this patient was mechanical (bruxism) and chemical (acidic reflux).

It was essential to control all systemic and local etiological factors before starting the prosthodontic treatment. Due to the extensive tooth surface loss, restoring teeth with direct composite restorations was not an option. Moreover, severe occlusal wear was challenging in the rehabilitation of partially edentulous patients. Furthermore, to obtain the correct anatomical contour, it was important to increasing the VDO. Several studies have expressed concerns on VDO augmentation (Al-Zahrani et al., 2020; Abduo et al., 2012); however, evidence associating the procedure with pathological consequences are still lacking. In severe edentulous patients, increasing VDO assists in restorative treatment by aiding the practitioner in optimizing restorative material thickness.

Recent reports indicate an increase in VDO entails no significant adverse consequence (Viana et al., 2020; Fabbri et al., 2018). However, one has to consider with patients with TMD, where increasing the VDO should still be achieved using removable appliances to control TMD-associated symptoms before considering any form of irreversible procedure (Abduo et al., 2012). In this case, the increase of VDO was tested in the provisional stage as it is important to test the patient's adaptability to the new position. Another important aspect linked to the clinical outcome is implanting surgical technique. In a recent meta-analysis and comparative review on conventional and computer-aided surgery, technology-assisted implant placement exhibited improved accuracy in contrast to free-hand operation (Chen et al., 2019). Another systematic review, reported lower deviation or enhanced accuracy in fully-guided implant surgery as compared to half-guided surgery (Bover-Ramos et al., 2018).

In this context, a systematic review by Schnider et al., (2009) reported that implant survival rates were 91-100% in up to 5-months observation period using computer-aided template-based surgery (Schnider et al., 2009). Computer-aided implant surgery, besides, can streamline operation and optimize implant placement (Geng et al., 2015). In regards to the digital or conventional rehabilitation, CAD/CAM prostheses were within acceptable clinical marginal discrepancy range. However, the type of material used influences the performance of the CAD/CAM system. Lithium disilicate crowns fabricated using heat press gave equivalent or smaller marginal discrepancy compared to those done on a CAD/CAM platform. Crowns produced by slip-casting showed similar or, even, improved marginal accuracy than CAD/CAM fabrications.

Recently, in terms of marginal fit, Freire et al., (2019) described the marginal fit of CAD/CAM monolithic and metal-ceramic crowns were within the acceptable clinical range. Monolithic lithium disilicate restoration (IPS e.max CAD) possessed the lowest discrepancy value compared to monolithic zirconia and metal-ceramic crowns.For the edentulous regions in the mandible, implants with a length of <10mm were inserted due to the vertical loss of alveolar bone. In 2011, the survival rate of short dental implants was evaluated through a systematic review. Several factors such as smoking, implant location, surface characteristics of implants and the influence of bone augmentation were investigated. All factors showed no statistically significant difference in terms of implant survival rate except for smoking and implant location.

In another recent study investigating the relationship between tooth wear, GERD and bruxism, it was confirmed that severe tooth wear was highly related with patients with sleep bruxism (Li et al., 2018). Considering the relationship between GERD and bruxism, tooth wear in patients with sleep bruxism may be a consequence of attrition intensified by intrinsic acids rather than attrition alone, which was exactly the situation in the present case. This finding supports and advances the understanding that tooth wear is a multifarious condition involving multiple mechanisms. To ensure long-term outcomes of the restorations especially that the patient was examined with bruxism, a night guard was fabricated.

CONCLUSION

In conclusion, this case report describes the complete oral recovery of a GERD patient. The patient was successfully treated with traditional all-ceramic and implant-supported restorations to fulfill the patient's needs and desires for fixed dental prostheses. An individualized maintenance program was placed in place to ensure a favorable long-term prognosis for the patient.

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Patho-Psychological Impact of COVID-19 Outbreak in Patients with Comorbidities in Saudi Arabia

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ABSTRACT

COVID-19 outbreak affects patients with chronic diseases in several ways including psychological and pathological effects. Accordingly, the present study aimed to evaluate the pathopsychological impact of the COVID-19 outbreak in patients with comorbidities in Saudi Arabia. In this online questionnaire-based cross-sectional study, 161 Saudi patients with chronic diseases were conscripted during the country's complete lockdown due to the COVID-19 outbreak (May–June,2020). A purposeful electronic questionnaire was premeditated and circulated over different social media clusters irrespective of age or gender.On asking the patients "Does COVID-19 outbreak disturb your treatment", 70/161(43.5%) answered "Yes". Out of the 70 patients 31/88(35.2%) were males and 39/73(53.4%) were females, the risk with female gender RR (95%CI) = 1.5166 (1.0632 to 2.1633), P = 0.0216, z statistic = 2.298. On asking the patients "Does COVID-19 outbreak increased your illness", 81/161(50.5%) answered "Yes". On asking the patients "Did you get new pathologic symptoms due to irregularity of treatment", 73/161(45.3%) answered "Yes". Patients with chronic diseases were extremely influenced (pathologically and psychologically) by the COVID-19 outbreak procedures. The psychological influence was more common among women compared to men.

KEY WORDS: COVID-19, PSYCHOLOGICAL, PATHOLOGICAL, SAUDI ARABIA, COMORBIDITIES, CHRONIC DISEASES.

INTRODUCTION

Coronavirus diseases 19 (COVID-19) provided a massive challenge to the whole health care system and test their available facilities exclusively for the management of patients with chronic diseases. Intensive care unit (ICU) is required for almost 20% of COVID-19 patients with multiple comorbidities and hospitalization was concomitant with a case fatality rate (CFR) of >13%. As the virus is globally spreading, nations necessitate urgent preparation of all national resources in terms of infrastructure, personnel, and other facilities to reduce the disease fatality particularly among severe cases (Gutiérrez-Ocampo et al. 2020). Epidemiological and the clinical presentations of COVID-19 infected individuals have been described but risk factors for mortality and a full clinical course of illness, comprising viral shedding, have not been well designated (Du et al. 2020).

This novel COVID-19 has specifically high morbidity in the elderly and comorbid populations. Uremic patients on dialysis combine an intrinsic fragility and a very frequent burden of comorbidities with a specific setting in which many patients are repeatedly treated in the same area (hemodialysis centers) (Pizzarelli et al. 2020). Since the COVID-19 has broad clinical range starting from mild sickness to acute respiratory distress syndrome (ARDS) with a high fatality, there is a necessity for further exploration to recognize primary indicators of disease severity. New recommendations stressed the fact

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that older patients, patients with chronic illnesses, or dyspnea must be monitored particularly in the 1st to 2nd week after the appearance of the initial symptoms (Cheong et al., 2020). Furthermore, comorbidities can significantly influence the prognosis mode of COVID-19 fate, especially among patients with CVD metabolic diseases. In such patients, the infection may lead to heart damage (Zhao et al. 2020) . However, the present study aimed to assess the pathopsychological impact of the COVID-19 outbreak in patients with comorbidities in Saudi Arabia.

MATERIAL AND METHODS

In this online questionnaire-based cross-sectional study, 161 Saudi patients with chronic diseases were conscripted during the country's complete lockdown due to the COVID-19 outbreak (May – June 2020). The study was premeditated to assess the pathopsychological effects of the COVID-19 outbreak and its association with a lockdown on patients with chronic illnesses. A purposeful electronic questionnaire was premeditated and circulated over different social media clusters irrespective of age or gender. The pathopsychological influence of the COVID-19 outbreak on patients with chronic diseases was thereafter recognized with several pathopsychiatric measures.

Besides the demographical characteristics of the patients, the questionnaire involved the following cognitive measures; Types of chronic disease, Does COVID-19 outbreak prevent you from seeing your doctor, Does COVID-19 outbreak disturb your treatment, Does COVID-19 outbreak increased your illness, Did you get new pathologic symptoms due to irregularity of treatment, Feeling highly anxious after registering of the first case in my city, I have psychological effects from home lockdown, such as nervousness, depression, etc., During the lockdown, frequencies of being upset, because of the Corona's, During the lockdown, frequencies of being unable to control important things in your life?, During the lockdown, frequencies of felt tense and anxious about the new epidemic around you?, During the lockdown, frequencies of felt angry about things that happened outside your control?, Social isolation led to increased depression and anxiety.

Statistical Analysis: Following the initial representation of the data in Microsoft Excel, the obtained data were then sent to the SPSS program and analyzed obtained. Statistical significant values, such as relative risk were produced applying a 95% confidence interval. A Chi-square test was done (P-value <0.05 was considered statistically significant). **Ethical Consent:** The proposal for the present study was approved by the Ethical Committee at the College of Medicine, University Ha'il, Saudi Arabia. HREC 00123a/CM-UOH.04/20

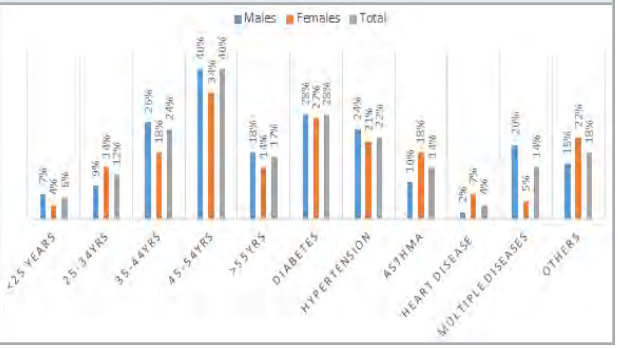
RESULTS AND DISCUSSION

About 161 patients with different chronic diseases were assessed their ages ranging from 19 to 70 years with a mean age of 45 years. Out of the 161 patients, 88/161(55%) were males and 73/161(45%) were females. The majority of patients were at the age range 45-54 years followed 35-44 years constituting 65/161(40.4%) and 39/161(24.2%), respectively (see Table 1, Fig 1.The most frequent chronic illness was diabetes 45/161(28%) followed by hypertension 36/161(22.4%) (See Table 1, Fig 1).

Table 1. Distribution of patients by age, chronic diseases, and sex

Variable	Males	Females	Total
Age			
<25 years	6	3	9
25-34	8	12	20
35-44	23	16	39
45-54	35	30	65
>55	16	12	28
Total	88	73	161
Types of chronic disease			
Diabetes	25	20	45
Hypertension	21	15	36
Asthma	9	13	22
Heart disease	2	5	7
Multiple diseases	18	4	22
Others	13	16	29
Total	88	73	161

Figure 1: Patients by age, chronic diseases, and sex



On asking the patients "Does COVID-19 outbreak prevent you from seeing your doctor", 99/161(61.5%) answered "Yes". Out of the 99 patients, 46/88(52.3%) were males and 53/73(72.6%) were females. The relative risk (RR) associated with females and the 95% confidence interval (95%CI) was 1.3889(1.0878 to 1.7734), P = 0.0084, z statistic = 2.635. On asking the patients "Does COVID-19 outbreak disturb your treatment", 70/161(43.5%) answered "Yes". Out of the 70 patients 31/88(35.2%) were males and 39/73(53.4%) were females, the risk with female gender RR (95%CI) = 1.5166 (1.0632 to 2.1633), P = 0.0216, z statistic = 2.298. On asking the patients "Does COVID-19 outbreak increased your illness", 81/161(50.5%) answered "Yes". Out of the 81 patients

43/88(49%) were males and 38/73(52%) were females, the risk with female gender RR (95%CI) = 1.0653 (0.7838 to 1.4479), P = 0.6861, z statistic = 0.404. On asking the patients "Did you get new pathologic symptoms due to irregularity of treatment", 73/161(45.3%) answered "Yes". Out of the 73 patients 43/88(49%) were males and 30/73(41%) were females, the risk with male gender RR (95%CI) = 1.1890 (0.8396 to 1.6839), P = 0.3295, z statistic = 0.975.

Table 2. COVID-19 outbreak effects on regular treatment of patients by sex

Variable	Males (n=88)	Females (n=73)	Total (n=161)
Does COVID-19 outbreak prevent you from seeing your doctor			
Yes	46	53	99
No	42	20	62
Does COVID-19 outbreak disturb your treatment			
Yes	31	39	70
No	57	34	91
Does COVID-19 outbreak increased your illness			
Yes	43	38	81
No	45	35	80
Did you get new pathologic symptoms due to irregularity of treatment			
Yes	43	30	73
No	45	43	88

Table 3, summarized the distribution of the COVID-19 outbreak effects on the regular treatment of patients by age. On asking the patients "Does COVID-19 outbreak prevent you from seeing your doctor", out of the 99 patients answered "Yes", 33/99(33.3%), 24/99(24.2%), and 21/99(21.2%) were aged 45-54 years, >55 years, and 35-44 years, correspondingly. On asking the patients "Does COVID-19 outbreak disturb your treatment", out of the 70 patients answered "Yes", 27/70(38.6%), 15/70(21.4%), and 14/70(20%) were aged 45-54 years, >55 years, and 35-44 years, one-to-one. On asking the patients "Does COVID-19 outbreak increased your illness", out of the 81 patients answered "Yes", 33/81(40.7%), and 20/81(24.7%),) were aged 45-54 years, and 35-44 years, respectively. On asking the patients "Did you get new pathologic symptoms due to irregularity of treatment", out of the 73 patients answered "Yes", 28/73(38.4%), and 17/73(23.3%),) were aged 45-54 years, and 35-44 years, respectively.

Table 4, Fig 2, summarized the distribution of COVID-19 outbreak by gender and psychological effects. On asking the patients "Feeling highly anxious after registering of the first case in my city", 116/161(72%) answered "Yes" of whom 54/88(61.4%) were males and 62/73(85%) were females. The risk associated with female gender was; RR (95%CI) = 1.3841(1.1424 to 1.6769), P = 0.0009, z statistics = 3.320. On asking the patients "I have psychological effects from home lockdown, such as nervousness, depression, etc.", 100/161(62%) responded "high" of whom 51/88(58%) were males and 49/73(67%) were females.

Table 3. COVID-19 outbreak effects on regular treatment of patients by age

Variable	<25years	26-34	35-44	45-54	>55	Total
Does COVID-19 outbreak prevent you from seeing your doctor						
Yes	5	16	21	33	24	99
No	4	4	18	32	4	62
Does COVID-19 outbreak disturb your treatment						
Yes	4	10	14	27	15	70
No	5	10	25	38	13	91
Does COVID-19 outbreak increased your illness						
Yes	6	10	20	33	12	81
No	3	10	19	32	16	80
Did you get new pathologic symptoms due to irregularity of treatment						
Yes	7	9	17	28	12	73
No	2	11	22	37	16	88

The risk associated with female gender was; RR (95%CI) = 1.1582 (0.9114 to 1.4719), P = 0.2297, z statistics = 1.201. On asking the patients "During the lockdown, frequencies of being upset, because of the Corona's", 41/161(25.5%) responded "high" of whom 12/88(13.6%) were males and 29/73(40%) were females. The risk associated with female gender was; RR (95%CI) = 2.9132 (1.6037 to 5.2920), P = 0.0004, z statistics = 3.511.On asking the patients "During the lockdown, frequencies of being unable to control important things in your life?", 15/161(9.3%) responded "high" of whom 9/88(10.2%) were males and 6/73(8.2%) were females.

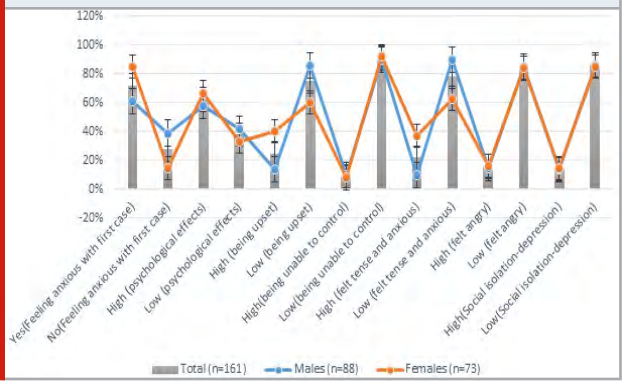
On asking the patients "During the lockdown, frequencies of felt tense and anxious about the new epidemic around you?", 36/161(22.4%) responded "high" of whom 9/88(10.2%) were males and 27/73(37%) were females. The risk associated with female gender was; RR (95%CI) = 3.6164 (1.8182 to 7.1931), P = 0.0002, z statistics = 3.664.On asking the patients "During the lockdown,

frequencies of felt angry about things that happened outside your control?", 25/161(15.5%) responded "high" of whom 13/88(14.8%) were males and 12/73(16.4%) were females. On asking the patients "Social isolation led to increased depression and anxiety", 23/161(14.3%) responded "high" of whom 12/88(13.6%) were males and 11/73(15%) were females.

Table 4. COVID-19 outbreak by gender and psychological effects

Variable	Males (n=88)	Females (n=73)	Total (n=161)
Feeling highly anxious after registering of the first case in my city			
Yes	54	62	116
No	34	11	45
I have psychological effects from home lockdown, such as nervousness, depression, etc.			
High	51	49	100
Low	37	24	61
During the lockdown, frequencies of being upset, because of the Corona's			
High	12	29	41
Low	76	44	120
During the lockdown, frequencies of being unable to control important things in your life?			
High	9	6	15
Low	79	67	146
During the lockdown, frequencies of felt tense and anxious about the new epidemic around you?			
High	9	27	36
Low	79	46	125
During the lockdown, frequencies of felt angry about things that happened outside your control?			
High	13	12	25
Low	75	61	136
Social isolation led to increased depression and anxiety			
High	12	11	23
Low	76	62	138

Figure 2: COVID-19 outbreak by gender and psychological effects



The highest COVD-19 related fatality was commonly occurring among individuals with comorbidities. COVID-19 outbreak affects patients with chronic diseases in several ways including psychological effects, chronic disease evolution due to interruption of follow up or loss of treatment optimization, loss of physical activity, increased consumption of avoidable nutrients which may be harmful with a particular illness, etc. Consequently, the present study aimed to evaluate the pathopsychological impact of the COVID-19 outbreak in patients with comorbidities in Saudi Arabia.

In the current study, about 61.5% of the patients claimed that COVID-19 associated procedures prevented them from seen their doctors, and women were significantly affected (P = 0.0084) compared to men. Although there limited data on follow up patients with chronic diseases during the COVID-19 outbreak, unfavorable outcomes involving disease progression and increased fatality risk were witnessed (Sala et al., 2020). About 43.5% of the patients indicated that the lockdown procedures disturb their treatment in one way or another. Females were also significantly (P = 0.0216) affected than males. Moreover, about 50.5% of the patients experienced increased illness liability during the lockdown. A recent investigation in this context has shown that COVID-19 is an independent risk factor for chronic comorbidity fatality upsurge (Green et al. 2020).

A recent data analysis in this context showed that considerable morbidity and mortality associated with chronic comorbidity among COVID-19 patients, particularly among those with cardiovascular (hypertension), metabolic (diabetes) diseases even greater than chronic pulmonary diseases, and the fatality increases with the severity of the preexistent chronic illness (Lu et al., 2020). Moreover, approximately 45.3% of the patients in this series of patients reported: "new pathologic symptoms due to the irregularity of treatment". Limited access to diagnostic services and

optimum treatment constituents represent the most important issues for patients with chronic diseases during the lockdown during the COVID-19 pandemic. Inadequate aptitude to control together disease severity and the existence of medication adversative special effects, and significantly affect the patient (Gutiérrez-Ocampo et al. 2020). The findings of the present study have shown that females and elderly people were more likely to be affected path psychologically by COVID-19 associated events. This necessitates future plans for this section of the population. Stress, and anxiety, and depression features were commonly observed indicators among patients with chronic diseases in the current series.

Similar findings were recently reported from Spain in a study assessed levels of stress anxiety and depression during the COVID-19 pandemic. Higher symptoms were more frequent among younger persons with chronic illnesses compared to the general population. The symptoms of stress, anxiety, and depression increased with the elevation of lockdown levels (Idoiaga-Mondragon et al., 2020). However, the findings in the present study seemed to be similar to those reported globally (Ventriglio et al., 2020). This necessitates future strategies for patients with comorbidities including psychological intervention and planned follow-up. Although the present study has some limitations including its cross-sectional setting, it provided valuable information, which may assist for future management of patients with chronic comorbidities in situations of pandemic diseases.

CONCLUSION

Patients with chronic diseases were extremely influenced (pathologically and psychologically) by the COVID-19 outbreak procedures. The psychological influence was more common among women compared to men. Future strategies for patients with comorbidities including psychological intervention and planned follow-up are deemed important.

Conflict of Interest: Author declares no conflicts of interests to disclose.

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Monomer Leakage Behavior of Conventional and CAD/CAM Denture Acrylic Materials Under Different pH Values

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ABSTRACT

Computer-aided design and Computer-aided manufacturing (CAD/CAM) has emerged as a new approach for the fabrication of removable prosthesis offering many advantages over the conventional fabrication methods. The pre-polymerized polymethyl Methacrylate (PMMA) pucks used for the fabrication of CAD/CAM removable prosthesis has a significantly enhanced physical and mechanical properties. This study aims to evaluate the effect of different salivary pH values on monomer leakage from heat-cured and CAD/CAM denture acrylic materials. Two groups of 60 discs were fabricated from heat-cured and CAD/CAM acrylic materials. These acrylic samples were subjected to mechanical brushing and thermocycling according to a standardized protocol. The discs of the two acrylic materials were immersed and incubated in three salivary solutions with different pH values (acidic, 5.7; neutral, 7; basic, 8.3) for 30 days, after which the amount of leaked monomer in the saliva solution in the two groups was determined using high-performance liquid chromatography (HPLC). Both the acrylic material type and salivary pH value had a significant effect on monomer leakage. An acidic salivary pH caused the most monomer leakage in both acrylic material groups ($P < 0.05$). The heat-cured acrylic material leaked less monomer than the CAD/CAM acrylic materials. The acidic salivary pH values were associated with higher amounts of monomer leakage in both heat-cured and CAD/CAM denture acrylic materials. In-laboratory immersion of newly fabricated heat-cured and CAD/CAM acrylic dentures in an acidic solution might be recommended to allow most unreacted monomers to leak before delivering the denture to the patient.

KEY WORDS: ACRYLIC, CAD/CAM, DENTURE, MONOMER LEAKAGE, SALIVARY PH.

INTRODUCTION

Despite advances in preventive dentistry, edentulism is still a major public health problem and is considered an important indicator of the oral health of elderly population where the loss of some or all remaining teeth has a negative impact on the health-related quality of life (Cunha-Cruz et al 2007; Emami et al 2013; Batista et al 2014; Silva-Junior et al 2017; Batista et al 2018).

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Accordingly, the use of removable dental prostheses has increased among older patients who are the primary wearers of dentures in the general population (Dye et al 2012 Kassebaum et al 2014; Kassebaum et al 2017). Several materials have been used for the construction of removable prosthesis and dentists have long been searching for ideal materials for the construction of dentures. Nowadays, Polymethyl methacrylate (PMMA) resin is considered the material of choice for the fabrication of removable prostheses. Despite its weak flexural and impact strength and low fatigue resistance, it has many advantageous properties, including good mechanical features, ease of fabrication and repair, aesthetic properties and stability in the oral cavity (Dogan et al 2007; Nakamura et al 2007; Mohamed 2008 ; Alla et al 2013; Gad et al 2017; Zafar 2020).

Similar to any other dental materials used inside the oral cavity, PMMA resin denture base materials are subjected to changing wet oral environment which is physiologically characterized by natural saliva and its components (Zidan et al 2020). Potential harmful effects may arise from pH changes due to cariogenic biofilms in the oral ecology, diet intake and different enzymes (Turssi et al 2003). These phenomena can lead to the leaching out of plasticizers and soluble components from the acrylic over extended periods (Mohamed 2008; Marsh and Zaura 2017; Du et al 2020). It is widely reported in the literature that substances leaching out from denture base acrylic resins can cause cytotoxic effects (Koutis and Freeman 2001; Gonçalves et al 2006; Mörmann et al 2013; Rashid et al 2015). Unreacted residual monomers are the main substances that leach out from acrylic resins by the process of diffusion, the quantity of which is highly related to the polymerization reaction of acrylic resins (Chaves et al 2012; Içа et al 2014; Nik et al 2014). Unreacted monomers may cause toxic effects, adverse allergic reactions and significant damage at the cellular level (Drozdz et al 2011; Goiato et al 2015; Çakırbay et al 2018; Degirmenci et al 2020).

Several studies have aimed to quantify the amount of diffusing monomer and other leachable components from acrylic-based materials into the saliva. One study found that the maximum concentration of residual monomer leaching into the saliva of patients wearing complete dentures in their post-insertion period peaked one day after the insertion and that despite this amount of released monomer being at a none toxic levels, it could still potentially sensitize complete denture patients and induce an allergic reaction (Singh et al 2013). Another study attempted to quantify the residual monomer elution of conventional and computer-aided design/computer-aided manufacturing (CAD/CAM) dental acrylic-based materials during artificial aging and it found that both CAD/CAM and conventional polymers eluted residual monomer within different aging time (Engler et al 2020). Another important factor to be considered in the diffusion of monomers from acrylic-based materials is the salivary pH value which is known to affect biodegradation of the material and it was found that the amount of monomer released from different denture base acrylic material processed by different polymerization methods and stored in different storage conditions is higher when stored in an acidic saliva environment in comparison to neutral saliva (Bettencourt et al 2010; Tuna et al 2013; Akay et al 2017; Sá et al 2020).

In recent years, CAD/CAM technology has become an alternative to conventional methods in the fabrication of removable prostheses. In 1994, the first scientific article discussing the use of CAD/CAM in the fabrication of complete dentures was published (Maeda et al 1994). Since then, numerous CAD/CAM denture systems have been introduced into the market (Kattadiyil et al 2013; Steinmassl et al 2017). CAD/CAM-fabricated complete dentures have several advantages over conventionally fabricated complete dentures, such as decreased porosity, enhanced predictability of the desired outcomes and excellent fitting accuracy (Bidra et al 2013; de Mendonça et al 2016). Because the acrylic used for the fabrication of dentures using CAD/CAM technology is pre-polymerized, the prosthesis seems to contain less residual monomer and is more hydrophobic than the conventionally processed one, resulting in a more bio-hygienic prosthesis (Masri and Driscoll 2015).

A recent research that studied CAD/CAM dentures and aimed at evaluating the color stability of it when immersed in different beverages found that milled denture blocks had greater resistance to stain accumulation in comparison to the conventional one (Al-Qarni et al 2020). However, limited data are available on the properties related to the monomer leakage of CAD/CAM processed denture material when the salivary pH values alternate between acidic and basic conditions. This study has aimed to evaluate the effect of different salivary pH values on monomer leakage from conventional and CAD/CAM acrylic denture base materials, with a null hypothesis that there is no difference between the two types of acrylic denture base materials in terms of the effect of the salivary pH values on monomer leakage.

MATERIAL AND METHODS

Two types of acrylic resin materials were used: a CAD/CAM-manufactured resin (IvoBase® CAD; Zenotec, Wieland Dental, Germany) and a heat-cured resin (SR Ivocap High Impact®; Ivoclar Vivadent AG, Liechtenstein). Two groups of 60 discs were fabricated. The dimensions of the discs were 10 mm (diameter) × 3 mm (thickness). Each of the two groups was divided into three subgroups, with 10 discs each.The CAD/CAM Acrylic discs were designed with predetermined dimensions using Zenotec® CAD software (Wieland Digital Denture; Ivoclar Vivadent, Schaan, Liechtenstein). PMMA blocks were used (Opera system, Principauté de Monaco, French), and the milling procedure was performed using Zenotec® selection (Wieland Digital Denture; Ivoclar Vivadent, Schaan, Liechtenstein). The discs were then finished and polished using a dental laboratory polishing machine with a vacuum cleaner (Aspyclean+ M2V®, Manfredi, Italy), pumice (Interdent, Slovenia) and a rag polishing wheel (Rag muslin wheel; Kerr, USA).

For the fabrication of Heat-Cured acrylic resin discs, a putty molds of the preferred disc dimensions were fabricated using a polyvinyl siloxane putty material (Express STD®; 3 M ESPE, United States). The silicone molds were filled with melted base plate wax. A Bantam flask was filled with a plaster mix with a powder : water ratio of 100 g:47 cc (Lab Plaster Fast Set®; Dentsply, Canada), and then the putty mold was immersed in the plaster mix so that the top of the mold was flushed with the top of the plaster mix. After the plaster was set, a thin layer of petroleum jelly (Vaseline) was applied to the top. The upper half of the flask was then fixed to the bottom half and filled with plaster mix, and then the lid of the flask was placed on the top. After that, the flask was placed in a wax elimination machine (Wapo-Ex®; Wassermann, Germany) for 30 minutes at 90 °F to 100 °F. The flask was then opened, and the melted wax was washed away using boiling water.

A thin layer of separating fluid (Ivoclar Vivadent; Schaan, Liechtenstein) was applied to the plaster surface. The heat-cured acrylic provided as a single capsule containing premeasured polymer and monomer (SR Ivocap High Impact®; Ivoclar Vivadent AG, Liechtenstein) was then mixed for 5 minutes using a cap vibrator (Cap vibrator®; Ivoclar Vivadent, Schaan, Liechtenstein). The mixture was poured into the putty mold and pressed using a pressure apparatus (OL 463, Manfredi, Italy). Next, the flask assembly was placed in a polymerization bath (100 °C water) for 35 minutes (Electronic Denture Curing System; Nevin Labs™; USA). The discs were finished and polished using a dental laboratory polishing machine with pumice (Interdent, Slovenia) and a rag polishing wheel (Rag Muslin wheel®; Kerr, USA).

A Mechanical brushing was performed according to the recommendations of the International Organization for Standardization (ISO). The specimens were brushed with soft toothbrushes mounted on a toothbrush simulator (ZM-3.12; SD Mechatromik GmbH, Germany) (Figure 1). The specimens were subjected to linear toothbrush abrasion movement at a rate of 356 brush strokes (back and forth) per minute. The machine provides a 200-g vertical load over each specimen and a 5-mm path starting from the center of each specimen and brushes six specimens simultaneously. The total brushing time was 50 minutes, with 17,800 cycles (representing one year). Brushing was performed in distilled water (23+3 °C) and dentifrice (Crest Cavity Protection Regular Paste; P&G, Germany) (Figure 2). Using an SD Mechatronik GmbH thermocycler (SD Mechatronik, Germany), all the specimens were stored in distilled water and subjected to thermocycling between 5 °C and 55 °C, with a dwell time of 30 seconds and a transfer time of 12 seconds for 1,000 cycles (Pusz et al 2010).

For the process of artificial saliva preparation and incubation of the samples, an artificial saliva was prepared at three different pH values (5.7, 7 and 8.3). An electrolyte composition similar to that of human saliva was used in this study, as shown in Table 1 (Kostic et al 2015):

Figure 1: Tooth brushing Simulator Machine

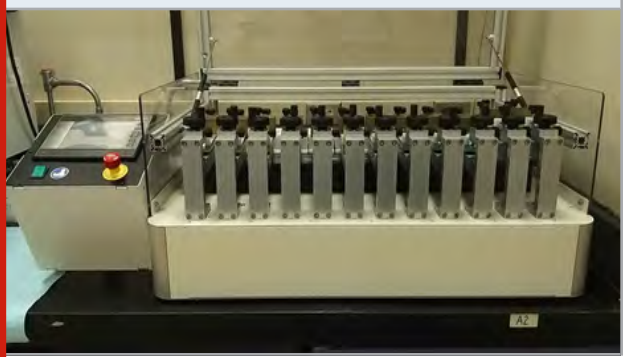

Figure 2: Brushing of the PMMA discs sample.

Table 1. Chemical Composition of Artificial Human Saliva

Na_2HPO_4	0.260 g/l
NaCl	0.700 g/l
KSCN	0.330 g/l
KH_2PO_4	0.200 g/l
$NaHCO_3$	1.500 g/l
KCl	1.200 g/l

A buffer solution comprising KH_2PO_4 and Na_2HPO_4 was prepared by dissolving each solution in 1 liter of deionized distilled water. Basic saliva was prepared by taking 500 ml of Na2HPO4 and adding KH_2PO_4 gradually until the desired pH was reached. Next, the other salts (NaCl, KSCN, $NaHCO_3$ and KCl) were added to the saliva, and the volume was completed to 1 liter using deionized distilled water. Neutral and acidic saliva solutions were prepared by taking 500 ml of KH2PO4 and adding Na_2HPO_4 gradually until the desired pH was reached. Next, the other salts (NaCl, KSCN, $NaHCO_3$ and KCl) were added in the same manner as described above. For neutral saliva, a greater amount of Na2HPO4 was added to reach the desired pH (Pusz et al 2010).The discs were assorted into 6 groups, with 10 discs in each group, and then were stored in artificial saliva in an incubator (Blanket warming cabinet; Malmet, Australia) at 37 °C for 30 days. High-Performance Liquid Chromatography (HPLC) was used to determine the quantity of residual methyl

methacrylate (MMA) monomer following the immersion of the two types of acrylic materials in artificial saliva at three different pH values.

A UV PerkinElmer Series 200 HPLC system (PerkinElmer, Shelton, USA) equipped with a C18 column was used to perform HPLC analysis. Ten milliliters (ml) of each sample solution was injected and analyzed at 40 °C and a flow rate of 1.0 ml/min (revolutions per minute) with acetonitrile in water (50/50). One reading was obtained from each milliliter of the 10 ml sample. Fifteen minutes after sample injection, the content of MMA was calculated from the area under the peak. The average of 10 readings for each sample was calculated (Mohamed 2008). Statistical Analysis: The data were analyzed using the SPSS statistical software (v16; SPSS Inc., Chicago, IL, USA). The effect of the acrylic material type and pH and their interaction on monomer leakage were analyzed by two-way ANOVA. Tukey's post hoc multiple comparison was used to evaluate the differences in monomer leakage among the three pH values under each type of acrylic material.

RESULTS AND DISCUSSION

At α = 0.05 and a sample size equal to 10 under each pH value used (acidic, neutral, basic), the power of the study was estimated to be 88%. Two-way ANOVA was performed to evaluate the effect of salivary pH on monomer leakage into the saliva. Both the material type and pH of the saliva significantly affected monomer leakage (P = 0.03 and P= 0.00, respectively) (Table 2). The mean and standard deviation (SD) of monomer leakage when the two acrylic material types were soaked in salivary solutions with different pH values are presented in table 3. The highest amount of monomer leaked from the CAD/CAM material when the material was soaked in an acidic salivary solution, while the least amount of monomer leakage occurred in the basic solution (table 3). Post-hoc multiple-comparison analysis revealed that the monomer leakage of the CAD/CAM material soaked in an acidic solution was significantly higher than that of the neutral and basic pH solutions (P = 0.01 and P = .00, respectively;(table 4). Similarly, heat-cured acrylic exhibited most of the monomer leakage when the material was soaked in an acidic salivary solution; however, the lowest monomer leakage was observed when the material was soaked in neutral pH solution (table 3). Post-hoc multiple-comparison analysis revealed that the monomer leakage of the heat-cured acrylic material soaked in an acidic solution was significantly higher than that of the two other salivary solutions. (P = 0.00; table 4).

Acrylic-based resins are frequently used in daily dental practice. These acrylic resins are used to replace lost tissue and transfer masticatory forces from the denture to the residual ridges because they can provide essential properties and have the necessary characteristics for use in diverse functions. Although acrylic resins have many desirable properties, one of their main drawbacks is that they contain residual monomers that may leach out and trigger undesirable side effects (Oliveira et al 2010; Ivkovic et al 2013; Kostis et al 2015). Diffusion is the mechanism that underlies residual monomer leakage from acrylic resins in which the constant contact of saliva with the material causes expansion of the openings present between the polymer chains, causing the unreacted monomer to diffuse out. Thus, the substances that are leached out from the denture bases into the saliva are transferred to the oral structures, causing adverse allergic reactions (Urban et al 2009; Kopperud et al 2011;Chaves et al 2012; Gautam et al 2012; Nik et al 2014; Choudhary et al 2020).

Table 2. Two-way ANOVA of the effect of two independent variables material type and pH value on monomer leakage (uV.sec).

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Disc Material	426398.26	1	426398.26	4.86	0.03
pH	2635911.05	2	1317955.52	15.03	0.00
Disc Material * pH	440815.82	2	220407.91	2.51	0.09
Error	4735157.61	54	87688.10		
Total	49121084.98	60			
Corrected Total	8238282.73	59			

Based on the results obtained from this study, the null hypothesis was rejected indicating that the variation in salivary pH values had a significant effect on the monomer leakage from the acrylic materials used in the study. The results demonstrated that, when different acrylic resin materials were soaked in saliva with different pH values, the greatest amount of monomer leakage occurred in the acidic salivary solution, a finding that was in agreement with other studies (Faltermeier et al 2007; Bettencourt et al 2010; Akay et al 2017; Sá et al 2020). One study evaluated the residual monomer using high performance liquid chromatography (HPLC) for microwave-cured, conventional heat and injection-technique acrylic materials that were stored in neutral and acidic artificial saliva for 24 hours and it was found that all three materials exhibited higher monomer release into the acidic saliva (Tuna et al 2013). The chemical structure of the monomers used to prepare the resins could directly affect the amount of eluted monomer. Lefebvre et al (1995) studied the pattern of release of

cytotoxic substances from four light-polymerized denture base resins and suggested that different components may leach out at different rates and that the release of cytotoxic resin components may continue for several days.

Table 3. Monomer leakage (uV.sec) from the three different acrylic materials (CAD/CAM and heat-cured) when soaked in three solutions with different pH values (acidic, neutral, basic).

Acrylic material	pH	Mean	Std. Deviation
CAD/CAM	Acid	1320.52	159.31
	Neutral	750.54	146.11
	Basic	658.21	122.36
Heat-Cured	Acid	922.57	65.43
	Neutral	628.57	138.56
	Basic	672.34	73.43

Table 4. Post hoc multiple-comparisons analysis to compare the effect of salivary pH value on monomer leakage for each acrylic material type.

Disc material	(I) pH	(J) pH	Mean Difference (I-J)	Sig.
CAD/CAM	Acidic	Neutral	569.98	0.01*
		Basic	662.31	0.00*
	Neutral	Acid	-569.98	0.01*
		Basic	92.33	0.87
	Basic	Acid	-662.31	0.00*
		Neutral	-92.33	0.87
Heat-Cured	Acidic	Neutral	294.00	0.00*
		Basic	250.23	0.00*
	Neutral	Acid	-294.00	0.00*
		Basic	-43.77	0.59
	Basic	Acid	-250.23	0.00*
		Neutral	43.77	0.59

* The mean difference is significant at the .05 level.

Heat-cured acrylic resin showed the least monomer leakage in both acidic and neutral solutions compared with the CAD/CAM material. Many studies were conducted to evaluate the amount of monomer leakage from heat-cured acrylic compared with that of other materials and all presented similar findings in which the heat-cured acrylic material showed less monomer leakage. This finding might be related to the high polymerization temperature needed to cure the acrylic material (Vallittu et al 1998; Shim and Watts 1999; Sideridou and Achilias 2005; Mohamed et al 2008; Chaves et al 2012; Nik et al 2014). In a recent study conducted to compare the residual monomer concentration and cytotoxic effect of three acrylic materials that were hot-cured or polymerized under pressure and at lower temperatures, the authors reported that the acrylic material polymerized at high temperatures has a lower residual monomer concentration, while self-curing materials polymerized at lower temperatures have a higher concentration of residual monomer, leading to a lower number of living cells that might trigger allergic reactions shortly after the new denture is delivered (Raszewski 2020).

CAD/CAM denture base acrylic resin is supplied as pre-polymerized blocks which are produced in industrially controlled conditions with standardized pressure and temperature and are known to have enhanced material-specific properties (McCabe and Walls 2013). As a result of the polymerization of PMMA blocks used for the milling of denture under high temperature and pressure, long polymer chains are formed leading to a higher degree of monomer conversion and lower values of residual monomer as well as minimal porosity (Kattadiyil et al 2013; Mörmann et al 2013; Murakami et al 2013 ; Nguyen et al 2014; Akin et al 2015; Kattadiyil et al 2015). In a recent study that aimed to evaluate the amount of monomer released from a CAD/CAM acrylic material when soaked in water, the results demonstrated that the CAD/CAM acrylic material released very little monomer. However, the amount released was not different from that released from conventionally heat-cured acrylic material (Steinmassl et al 2017). This finding agreed with ours when the two acrylic materials were soaked in neutral and basic salivary solutions. On the contrary, one study that instigated the mechanical properties including monomer leakage between heat cured and CAD/CAM denture base material found that CAD/CAD material leached lower amount of monomer compared to heat cure denture acrylic material and this variation was attributed to the method of polymerization under high pressure (Ayman 2017).

The presence of unreacted residual monomers in denture base acrylic resins is inevitable, and every effort should be applied in laboratory and clinical settings to reduce the exposure as much as possible (Rashid et al 2015). Generally, and regardless of the acrylic material type, lower pH values were associated with more monomer leakage. Because lower amounts of monomer leakage occurred from the heat-cured acrylic material in the acidic solution, this material might be the material of choice when treating patients who report a high intake of an acidic diet. Similarly, using acidic solutions as storage media for dentures before denture insertion might be warranted to eliminate larger amounts of monomer release.

The salivary pH value in the oral cavity changes continuously between acidic and basic based on the dietary intake of the patient. Consequently, it might be necessary to subject the same acrylic material to alter salivary pH values and study the effect of this parameter on monomer leakage. Similarly, acrylic materials are subjected to many other factors that might affect monomer leakage. These factors include enzymes in the oral cavity, cleanser agents, different

brushing techniques, polymerization techniques, surface treatments and chewing forces. Further investigation is needed to study the effects of the combination of these factors on acrylic materials, particularly the newly introduced CAD/CAM materials.

CONCLUSION

Within the limitations of this study, acidic salivary pH values were associated with higher amounts of monomer leakage in both heat-cured and CAD/CAM denture acrylic materials. It might be recommended to immerse newly fabricated heat-cured and CAD/CAM acrylic dentures in an acidic solution to allow most unreacted monomers to leak before delivering the denture to the patient.

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Marginal Integrity of Peri-Bracket Excess Adhesive. A Confocal Laser Scanning Microscopic Study

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ABSTRACT

In orthodontic bonding, it is crucial to obtain optimal marginal integrity between tooth surface and bonding adhesive. Undermining the intimate contact create gaps at the enamel-adhesive interface, these gaps may affect the bond strength and predispose the enamel to white spot lesions. This study evaluated the effect of leaving excess adhesive resin around orthodontic brackets on marginal integrity in vitro. In this in vitro experimental trial, 24 intact premolars were bonded with a stainless-steel orthodontic bracket using Transbond XT light cure adhesive composites mixed with Rhodamine B fluorescent dye. After positioning the bracket and before light curing, excess adhesive was removed according to the test groups. Group 1: the entire adhesive around the bracket was removed. Group 2, only 1-mm excess around the bracket was left. Group 3, only 2-mm excess around the bracket was left. The angle between enamel surface and bonding adhesive was measured using confocal laser scanning microscopy and data were analyzed by one way analysis of variance and post hoc Tukey test. The presence of excess adhesive significantly increased the angle ($p<0.05$), group 1 (0mm excess) presented a more favorable marginal integrity ($4.5°\pm 1.5°$) compared to groups 2 ($14.65°\pm2.5°$) and 3 ($19.44°\pm4°$). Excess adhesive around orthodontic brackets did not improve the marginal integrity.

KEY WORDS: CONFOCAL LASER SCANNING MICROSCOPY, EXCESS ADHESIVE, MARGINAL INTEGRITY, ORTHODONTIC BRACKETS.

INTRODUCTION

In orthodontic bonding, it is crucial to obtain optimal marginal integrity between tooth surface and bonding adhesive for both, bond strength (decreasing bond failure) and tight seal (minimizing passage of bacteria and oral fluid i.e. microleakage), thus, reducing white spot lesions (WSL) around orthodontic brackets. Efforts targeting these two problems have been developed, such as the introduction of new adhesive materials, the use of amorphous calcium phosphate and fluoride, minimizing the number of spots in the interface between the bracket base and the prepared enamel where adhesive might fail to continuously penetrate that space, creating tiny fracture-prone voids, through modifications to enamel etching procedures, or the use of sealants around orthodontic brackets,(Cucu et al., 2002; Daub et al., 2006; Yagci et al., 2010; Canbek et al., 2013; Bilal and Arjumand, 2019; Sonesson et al., 2020 Babanouri et al 2020).

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Despite these efforts, questionable marginal integrity, bond failure, and white spot lesions still occur, and each of the aforementioned methods has its own associated disadvantages. This include adding an extra step to bonding procedure, thus increases the complexity of an already technique-sensitive procedure. Additional costs are also added - both in terms of time and resources-, plus, the technical requirements for proper implementation of these procedures may raise issues with compatibility. As well, requiring patient cooperation, which often clearly inadequate (Karandish, 2016; Lee et al., 2020; Sonesson et al., 2020).

Thereby, unless a fundamental change in orthodontic bonding technology occurs, the presence of these deficiencies with the currently used bonding procedure may force us to accept a suboptimal bond between enamel and orthodontic brackets. This suggests that developing a material or a method that takes the patient's compliance out of the equation and requires no extra chair time or additional cost in the clinic would be promising in the field of preventive care during orthodontic treatment among selected patients. Typical orthodontic sealants work as mechanical barrier to protect the around orthodontic brackets, some have added antimicrobial agents or release fluoride. These sealants are deemed useful for preventing microleakage. However, their efficacy is limited by their antimicrobial activity, color stability, and ability to endure intraoral stresses such as thermal changes and abrasion. An effective sealant material would be one with high abrasion resistance and low thickness facilitating its flow and adaptation (Asefi et al., 2016; Singh et al., 2019; Lee et al., 2020; Linjawi, 2020).

Orthodontic resin bonding materials meets those requirements, but they are not being used for this purpose. The traditional orthodontic bonding procedure using resin composite consists of the application of a bonding agent, often an unfilled resin, to the etched enamel surface followed by a filled resin composite paste applied to the bracket. The clinician positions the bracket on the tooth and press firmly toward the tooth surface. As the bracket is positioned on the tooth surface, excess adhesive typically flows around the bracket base as pressure is applied on the bracket and this standard orthodontic bonding procedure involves a final step of removing the peri-bracket excess adhesives before light curing (Proffit et al., 2013). This excess adhesive is removed for two reasons; 1) preventive reasons, as plaque tends to accumulate on rough surface and 2) aesthetic reasons, excess adhesive may get stained over time. On the other hand, it was suggested that removing the excess adhesive following conventional acid etching and bonding might predispose the enamel to white spot lesions (WSL) due to the washout phenomena of bonding material with time (Farrow et al., 2007; Hilgert et al., 2008; Decha et al., 2019).

For this reason, leaving certain amount of resin around brackets could – theoretically- seal the gap between them and the enamel thus reducing the problems associated with these gaps (Palot et al., 1991; Joseph et al., 1994; Alencar et al., 2016). Therefore, the aim of this in vitro study is to assess the marginal integrity at the enamel adhesive interface by measuring the contact angle between the composite adhesive and enamel surface with and without leaving excess adhesive using confocal laser scanning microscopy (CFLSM).

MATERIAL AND METHODS

The study was approved by the Institutional Review Board (IRB) at the College of Dentistry, King Saud University [E-17-2369]. Twenty-four human premolars were extracted for orthodontic reasons and informed consent was obtained for their use in this study. The extracted teeth were visually examined to be devoid of caries, restorations, fluorosis or abrasion. The extracted teeth were stored in distilled water until use (maximum 6 months) as per the ISO document 11405 (ISO/TS 11405:2015(en), Dentistry – Testing of adhesion to tooth structure, 2015). The teeth were randomly assigned to three equal groups of 8 in each. Immediately before conditioning the enamel, the buccal surfaces were cleaned with a rubber cup and pumice slurry to remove plaque and extrinsic stains. The brackets were bonded on the buccal surfaces according to manufacturer's instructions.

The area where the bracket is to be bonded was etched in the same manner for all three study groups using 38% phosphoric acid (Pulpdent Corporation, Watertown, USA) for 30 seconds and then rinsed thoroughly with water. The teeth were dried with compressed oil-free air for 5 seconds until a frosted appearance was seen on the enamel surface. Next, A thin layer of unfilled bonding resin (Transbond XT primer; 3M Unitek, Marinova, USA) mixed with fluorescein dye was applied with a microbrush applicator and the surface was lightly blown with air to ensure a uniform layer of primer remains before light curing for 20 seconds. brackets were then bonded using Transbond XT light cure adhesive adhesive (3M Unitek, Marinova, USA) mixed with Rhodamine B dye at concentration of 0.1 mmol/L, Rhodamine B is an inert dye that is used to facilitate visualization of resin under confocal microscope (Kumar et al., 2011). Transbond XT paste was applied to the bracket base and pressed firmly onto the tooth (Farrow et al., 2007).

The teeth in group 1: all excess adhesive around the bracket was removed using an explorer before light curing.
The teeth in group 2: the adhesive around the bracket was removed allowing only 1 mm excess contoured around the bracket.
The teeth in group 3: the adhesive around the bracket was removed allowing only 2 mm excess contoured around the bracket.

The bonding site and amount of the excess resin was controlled using a customized puncher to create a window in a piece of adhesive tape of the corresponding size, which was attached to the specimen prior to bonding

procedure. After seating the bracket into position, each bracket was then cured with light-emitting diode curing light (3M Unitek, Monrovia, USA) for a total of 40 seconds, 20 seconds each on mesial and distal aspects to achieve optimal curing of bracket adhesive (Oesterle et al., 1995; Farrow et al., 2007).

The teeth were then sectioned labiolingually parallel to the long axis of crown by using a low speed cutting machine (IsoMet, Buehler, Lake Bluff, USA), with a 4-inch circular diamond wheel (MetLab Technologies Limited, UK) under water coolant/lubrication to produce 2 sectioned slabs (Kumar et al., 2011). Prior to examination, each slab was hand polished using 180, 400 and 600 grit Silicone Carbide (SiC) papers and ultrasonicated between each paper grade for 3 minutes. The slabs were examined using a confocal laser scanning microscope (Nikon Instruments Inc., Melville, USA) with a 20×/1.4 air objective lens to assess the enamel–adhesive interface, a double labelling technique was used. For detecting rhodamine B dye fluorescence, the slabs were excited with a 561-nm laser, and the fluorescence signal was detected using 600–630 nm emission filters. Fluorescein was excited at 488 nm and the emission was detected using a 500- to 520-nm filter. The resultant angle between the enamel surface and adhesive around the bracket was calculated using ImageJ software (Wayne Rasband, NIH, USA) and numerical value was scored accordingly. For the statistical analysis, one-way analysis of variance (ANOVA) was used to analyze the angle formed between enamel surface and composite adhesive in the three groups. The ANOVA test results were significant, therefore, pairwise comparison between the groups was done by post-hoc Tukey test. The significance level (i.e., α value) was 0.05.

RESULTS AND DISCUSSION

There was a significant increase in contact angle value when excess adhesive was left on the tooth. Group 1 had significantly lower contact angle (4.5°± 1.5) compared to groups 2 (14.65°±2.5) and 3 (19.44°±4) $P<0.05$. there was also significant difference between groups 2 and 3 (Figure 1). Representative CLSM scans for each group are in Figure 2.

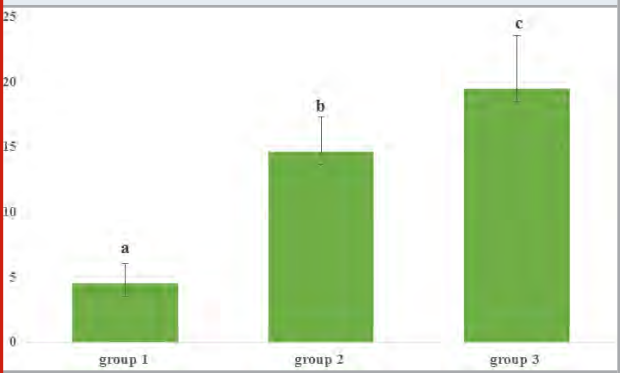
In orthodontic bonding, microleakage at enamel-adhesive-bracket interfaces decrease the bond strength causing bracket failure, and microleakage between enamel surface and adhesive layers can cause white spot lesions and enamel demineralization (Bilen et al.,2020).

These white spot lesions are usually found around the periphery of orthodontic bracket, under loosened bands and at the areas that are inaccessible by brush or undetectable by the patient. These lesions are considered the most common iatrogenic effect of fixed orthodontic treatment (Bishara and Ostby, 2008; Babanouri et al., 2020; Kamarudin et al., 2020). Poor oral hygiene leading to plaque accumulation is the primary cause of demineralization, however, enamel etching and bonding procedure, in terms of sealant and composite resin selection, also plays a role on the exacerbation of demineralization (Hedayati and Farjood, 2018).

Previous in vitro studies evaluated sealing the enamel margins around orthodontic brackets and reported successful results and reduction in demineralization without affecting the shear bond strength (Behnan et al., 2010; Knösel et al., 2012). However, most of these materials are technique sensitive and they have thin, weak films with low abrasion resistance that may compromise their longevity. From this perspective, our study aimed to investigate the adaption and seal of one of the most commonly used orthodontic bonding adhesive when all excess adhesive is removed as opposed to intentionally leaving 1mm or 2mm excess acting as the sealant material around orthodontic brackets.

Resin composites adaptation is determined by its behavior during polymerization, the efficacy of adhesive agent, and the viscosity of resin (Asmussen, 1975, Tay et al., 1995). Accurate evaluation of marginal seal in vitro is done either by tracer penetration tests, where the penetration of different markers along the interface between the adhesive resin and dental hard tissues of extracted teeth resembles the in vivo bacterial, fluids and other liquids penetration, or by quantitative marginal analysis with a microscope (with or without the use of dyes), where the gaps appearing at the interface resemble the in vivo bacterial, fluids and other liquids penetration (Heintze, 2013). In orthodontic literature, most studies measured these microgaps by dye penetration to reflect microleakage.

Figure 1: Mean contact angle (in degrees) for the three groups. Vertical bar indicates standard deviation. Dissimilar letters indicate significant difference.

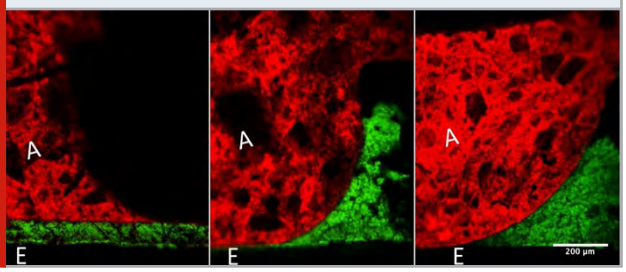


Microleakage of resin-based adhesives is evident, a study by Sukontapatipark et al. (2001) evaluated conventionally bonded premolars after extraction and reported the presence of gap approximately 10 µm in width at the enamel-composite interface. these gaps are thought to be a result of polymerization shrinkage commonly reported with resin-based materials and were considered a predisposing factor for bacterial accumulation and subsequent white spot lesion development. For this reason, Buyuk et al. (2013) investigated low-shrinking composites and reported lower microleakage with these

composites compared to conventional composites, although they produced insufficient shear bond strength and adhesive remnant scores. Another study did not find significant difference in leakage between the flash-free adhesive and color-change coated adhesive system using ceramic brackets (Kim et al., 2016). Similarly, Arruda et al (2016) tested the bond condition of conventional and flash-free adhesives and reported no statistically significant differences in microleakage between the two, the presence or absence of excess adhesive did not affect the microleakage results.

In our study, the adaptation between the bonding adhesive and enamel surface measured by means of an angle, the exact gap width i.e. microleakage was not measured, therefore only prediction of adhesive clinical behavior can be obtained. The angle we measured is the one formed between adhesive and enamel, an angle closer to 0° reflects better adaptation. Based on our results, group 1 (0mm excess) exhibited a more favorable marginal adaptation, followed by group 2 (1mm excess) and lastly, group 3 (2mm excess). Leaving excess material could provide sealing for the previously etched enamel initially, but according to our results, it may not be sufficient because subsequent failure may occur due to predicted wash out of bonding agent leaving an exposed gap that can provide a passage for bacteria and oral fluids, ending up affecting both the bond strength and enamel integrity.

Figure 2: Representative confocal laser scanning microscopy scans (20×/1.4 air objective lens) for each group. The angle between enamel surface and bonding adhesive was measured for each group. The letter (A) indicate the adhesive resin, and the letter (E) indicate the enamel surface.



The currently available orthodontic sealants provide protection against demineralization. Both Pro Seal and Opal Seal, which are fluoride releasing sealants, have been effective against WSL (Premaraj et al., 2017; Bartzela, 2018). Other non-fluoride releasing sealants reported to be of similar effect against WSL, thereby questioning the time and expense of using fluoride releasing materials (Leizer et al., 2010). Unfortunately, all the currently available sealants have sub-optimal longevity and re-application is often required with their use. No clear evidence of the long-term protection function of these materials available, as few information available regarding its integrity and durability which play crucial part in its function to protect against WSL and caries (Sen et al., 2020).

A randomized clinical trial by Sen.et al (2020) investigated the durability and integrity of different orthodontic surface sealants by means of optical coherence tomography. The layer thickness of opal seal and Pro seal significantly reduced after few months of treatment. Loss of integrity, up to 50%, was also reported after only three months.Interestingly, all teeth in group 1 had some excess adhesive, although efforts were taken to remove all excess, some excess remained around the brackets. This was also seen in previous studies (Sukontapatipark et al., 2001; Armstrong et al., 2007). We also noted that the excess adhesive in all groups showed irregular transition from adhesive to enamel surface creating a less smooth surface which may create areas that favor plaque accumulation.

Although excess adhesive was contoured before light curing in our study, the contact between the adhesive and enamel surface was not optimal. These findings could be related to the characteristics of the Transbond XT adhesives used in this study. This adhesive has a relatively large molecular weight and high filler concentration (77% quartz- silica hybrid fillers) which increase the viscosity of the material and although the flow characteristics of Transbond XT is considered acceptable when used for orthodontic bonding in traditional fashion, this might not be the case when excess adhesive is present (Bishara 2004; Vasudevan 2014). In addition, other desirable clinical handling characteristics such as nonstickness might deficient when compared to other composites with thinner viscosities.

CONCLUSION

Our study only gives a general idea of excess adhesive behavior, leaving excess adhesive around orthodontic brackets does not improve the marginal seal of Transbond XT adhesive and provide no benefit of sealing around the periphery of orthodontic brackets. Further researches are necessary to determine the exact effect of excess adhesive on plaque accumulation, white spot lesions formation and bond strength. Enhancement of composite bonding materials and application techniques is needed to overcome problems related to microleakage and gap formation.

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Authors Contribution: All authors have equal contribution in bringing out this research Work.

Conflict of Interest: None

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Effect of Cortactin and MMP-9 Expression Inhibited by Norcantharidin on Invasion of PC-3 Cells

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ABSTRACT

The objective of the present study was to determine the effects of norcantharidin on the invasion of PC-3 cells. In this study, norcantharidin was applied to PC-3 cells, after which the real-time fluorescence quantitative PCR and Western-blot were used to detect the expression level of cortactin and MMP-9, scratch test and invasion tests were also used to detect the invasion of PC-3 cells. Transmission electron microscopy was used to observe the morphological changes of pseudopods of PC-3 cells. We found the mRNA expression of cortactin and MMP-9 in PC-3 cells of norcantharidin group was significantly lower than that in PC-3 cells of blank control group, The protein expression of cortactin and MMP-9 in PC-3 cells of the norcantharidin group was also significantly lower than that of the blank control group ($P < 0.05$); the scratch test results showed that the scratch healing degree of PC-3 cells in the norcantharidin group was significantly lower than that of the blank control group ($P < 0.05$). Similarly, the invasion of PC-3 cells in the norcantharidin group was also significantly lower than that of the blank control group ($P < 0.05$), and observation of transmission electron microscope showed that the invasion of pseudopodia of PC-3 cells in the norcantharidin group was significantly less than that in the blank control group. The results of this study indicate that when norcantharidin acts on PC-3 cells, the invasion of PC-3 cells decreases, which may be related to the decrease of the expression level of cortactin and MMP-9. The results also demonstrate that norcantharidinmay may be a new treatment for prostate cancer.

KEY WORDS: NORCANTHARIDIN; PROSTATE CARCINOMA; CORTACTIN; MMP-9; MIGRATION; INVASION.

INTRODUCTION

A recent study has indicated that norcantharidin (NCTD) can inhibit PC-3 cell proliferation and induce PC-3 cells apoptosis (Luo, 2020). As well, few studies have also shown that NCTD can inhibit the invasiveness of some tumor cells (Fan, 2020; Yang, 2020 and Gao,2020). Cortactin and MMP-9 have been shown to be associated with prostate carcinoma cell invasion (Ma, 2016 and Liu, 2016). This study aims to confirm if NCTD can inhibit the expression of cortactin and MMP-9 and then reduce the invasion of PC-3 cells. Prostate carcinoma is the most common malignant tumor of the male reproductive system, which is more common in the elderly men over 60 years. In recent 10 years, the incidence rate of prostate carcinoma in China has been increasing year by year. Compared with developed countries, the incidence of prostate carcinoma in China is quite higher than before, (Li,2021).

The incidence rate of advanced carcinoma is also higher in China than in developed countries, (Xu,2020), and

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many patients lose the chance of radical operation. The vast majority of patients with advanced prostate carcinoma are initially sensitive to androgen inhibition. However, after an average of 2.5 years of androgen suppression endocrine therapy, all of these patients will be transformed into hormone resistant prostate carcinoma (HRPC), that is, it has no response to the endocrine therapy of inhibiting androgen.

The latter has been developed rapidly, with a median survival of no more than 18 months. Cortactin, a protein that interacts directly with cytoplasmic microfilaments, served as the tyrosine protein kinase SRC 80 in the early 1990s.The direct substrate of 85-kDa (Wu,1991), encoded by CTTN gene located in 11q13 region of chromosome, contains four structural regions: N-terminal acid region, tandem repeat region, carboxyl proline rich region and SH3 region, which are located in invasive pseudopodia and play an important role in the function of invasive pseudopodia (Ren,2018).

At the same time, matrix metalloproteinases (MMPs) have been confirmed to be involved in the procedure of invasion and distant metastasis of various tumors. At present, 26 different kinds of MMPs have been found, in which MMP-9 can degrade and destroy tumor extracellular matrix, and promote the formation of invasive pseudopodia, (Murphy and Courtneidge 2011). Norcantharidin (NCTD) is a demethylated analogue of cantharidin extracted from cantharidin(Tang,2010). NCTD has strong anti-cancer activity and has been used in the clinical treatment of a variety of malignant tumors. This experiment focuses on whether NCTD can down regulate the expression of cortactin and MMP-9 protein in PC-3 cells, and then inhibit the invasion of PC-3 cells.

MATERIAL AND METHODS

Cell line PC-3 was purchased from the National Cell Bank of Shanghai Institute of Cell Technology. Main reagents used were: NCTD (Aladdin company, batch number d131603-1g), cisplatin (MCE company, batch number hy-17394) cytoplasmic nucleoprotein Extraction Kit (Nanjing Kaiji biological company, batch number kgp150),BCA protein concentration determination kit(biyuntian biological technology company, batch number p0010), mouse monoclonal antibody β-actin(Wuhan PhD Bioengineering Co.,Ltd, batch number bm0627), rabbit monoclonal antibody C ortactin (Abcam, batch number: ab81208), rat monoclonal antibody MMP-9 (Abcam, batch number: ab58803), Transwell culture dish (Corning, batch number: 658042)Instruments used were: Real-time fluorescent quantitative PCR instrument (ABI company of America), CO_2 constant temperature incubator (Sanyo company of Japan), inverted microscope (Olympus company of Japan), 3001 enzyme labeling instrument (Thermo Fisher Scientific Company of America).

Cell culture PC-3 cells were resuspended in a complete medium containing 10% fetal bovine serum and placed at 37□ and 5% CO_2 Culture in cell incubator. Take the PC-3 cells in logarithmic growth period and add the culture medium containing NCTD with different concentrations prepared in advance,The final concentration of NCTD was 12.5 μg/ml, 25 μg/ml and 50 μg/ml respectively, the blank control group and positive control(cisplatin concentration 2.5 μmol/L).

The total RNA of PC-3 cells in each group was extracted by Trizol reagent in real-time PCR experiment, and then the operation instructions of real-time PCR kit were followed. PCR primers were synthesized by Xi'an Kehao Bioengineering Co., Ltd. the upstream primer sequence of cortactin was 5 '-CGATGAGTACGAGAACGAT-3', the downstream primer sequence was 5 '-GCAACACGAACACAAGAGA-3', the upstream primer sequence of MMP-9 was 5 '-GCTACCACCTCGAACTTTGAC -3', and the downstream primer sequence was 5 '- TCAGTGAAGCGGTACATAGGG -3', the upstream primer sequence of β-actin was 5' - agcgagcatcccaaagtt-3', and the downstream primer sequence was 5' - gggcacaggcatcatcat3 '. PCR reaction conditions: 50 °C 2min, 95 °C 10min; 95 °C 30 sec, 60 °C 30sec, 40cycles. In the experiment, each sample was tested three times, and the dissolution curve was drawn. The final data was analyzed with 2-ΔCt.

Western-blot was used to collect PC-3 cells from five groups, add the lysate and place it on ice for 30min, 4, 12000rpm for 5min, and use BCA protein concentration test kit to determine the protein content in the supernatant. Take 40μg of total protein for SDS-PAGE electrophoresis, transfer membrane, 5% skimmed milk powder, add anti-cortactin rabbit monoclonal antibody and mouse monoclonal antibody MMP-9 4 overnight, take-actin mouse monoclonal antibody as reference, and then add corresponding anti IgG (HRP labeled Sheep anti rabbit and HRP labeled Sheep anti mouse) to incubate at room temperature for 1h. ECL exposure imaging, chemiluminescence system analysis results.

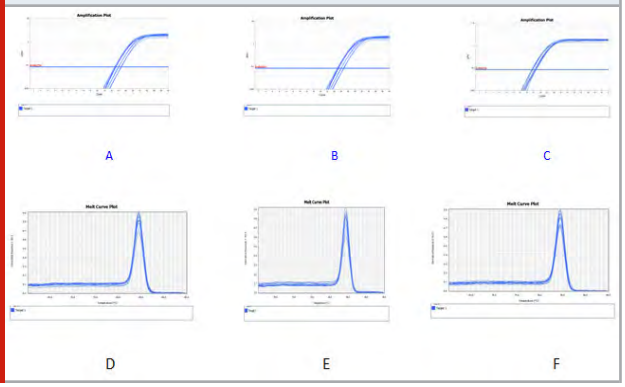
In the cell scratch test, PC-3 cells of blank control group, NCTD 12.5 μg/ml group, NCTD 25 μg/ml group, NCTD 50 μg/ml group and cisplatin 2.5 μmol/l group were taken respectively, and the cell density was adjusted to 2.5×105 cells/ml with 1640 medium, and 6-well plates were connected with 2ml cell suspension of each hole, which was cultured overnight at 37 □ ; cells were washed with PBS for three times to remove the scratched cells, and serum-free medium was added. After 24 hours of treatment, samples were taken for photos.

Transwell experiment, 2.5×105 cells were taken from the blank control group, 12.5 μg/ml, 25 μg/ml, 50 μg/ml NCTD group and positive control group (2.5 μmol /L cisplatin) PC-3 cells respectively. 100μl Matrigel (final concentration was 1 mg/ml) was added vertically to the center of the upper chamber bottom of the Transwell, chamber of Matrigel was incubated at 37□ for 4-5h to make it dry and gelatinous. After Matrigel dry and gelatinous, 200 μL cell suspensions of the above groups were respectively connected into the upper chamber of Transwell, cultured in 5% CO_2 incubator at 37□ for 24h and fixed with 70% ice ethanol solution h. Staining with

0.5% crystal violet dye solution, cleaning with PBS once, wiping the non-migrated cells on one side of the upper chamber with clean cotton ball, observing and taking photos under the microscope.

Transmission electron microscope was used to observe 10 µl of PC-3 cell suspension in blank control group, 12.5 µg/ml, 25 µg/ml, 50 µg/ml NCTD group and 2.5 µmol/L cisplatin positive control group. After 50 times dilution with 1×PBS, 10 µl of suspension was added to copper mesh, dried at 25□ for 12 hours, and then re-stained with uranyl acetate. The morphology of pseudopodia was observed and photographed under transmission electron microscope,under accelerating voltage 200kV, ×5000. Statistical analysis: The measurement data was expressed as mean±standard error . The difference between the two groups of independent samples was compared by t-test. The difference between the multi-factor groups was compared by single factor analysis of variance. P<0.05 was statistically significant. All the statistical analyses were performed using Statistical Package forthe Social Sciences 16.0 (IBM, New York, USA).

Figure 1: The curves of amplification and dissolution
A-C:amplification curves of cortactin ,MMP-9 and β-actin
D-F: dissolution curves of cortactin,MMP-9 and β-actin

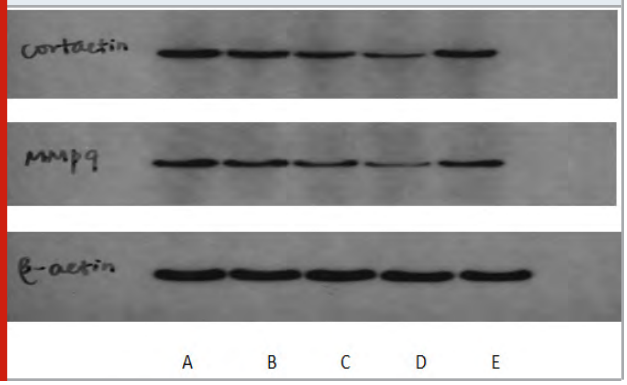


RESULTS AND DISCUSSION

Real time fluorescent quantitative PCR was used to detect the content of cortacin and MMP-9 mRNA in PC-3 cells of each group. The results showed that the content of cortactin mRNA in12.5 µ g/ml,25 µg/ml and 50 µg/ml NCTD decreased by 20%, 38% and 59% respectively, P<0.05, and the content of cortacin mRNA in 50 µg/ml NCTD group decreased by 37% compared with that in the positive control group ,P<0.05, The content of MMP-9 mRNA decreased by 13%, 39% and 58% respectively compared with the blank control group,P<0.05.and the content of MMP-9 mRNA decreased by 45% in the 50 µg/ml NCTD group compared with the positive control group ,P<0.05. Real-time quantitative PCR detect the amplification curve and dissolution curve of cortactin, MMP-9, and β-actin internal parameter, the amplification curves of cortactin, MMP-9 and β-actin internal parameter were smooth s-shaped with clear inflection points;dissolution of cortactin, MMP-9 and β-actin internal parameter showed a single peak.Indicating that the specificity of amplification was good and the results were reliable.See Figure 1.

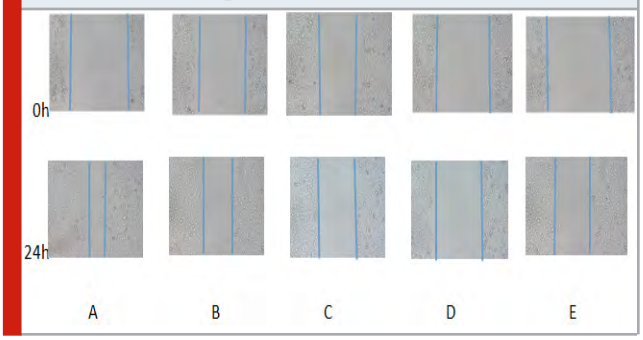
Western-blot showed that the expression levels of cortactin and MMP-9 in 12.5 µ g /ml, 25 µg/ml and 50 µg/ml NCTD group were lower than those in the blank control group, and the expression levels of cortactin and MMP-9 in the 50 µg/ml NCTD group were lower than those in the blank control group with the increasing of NCTD concentration ,P<0.05. see Figure.2.

Figure 2: Electrophoresis of NCTD effect on expressions of cortactin and MMP-9 in PC-3



A:blank control group ;B-D:NCTD group(12.5,25,50 µg/mL);E:positive control group (cisplatin 2.5 µmol/L) The effect of NCTD on the migration and invasion of PC-3 cells showed that the scratch healing area of 12.5 µg /ml, 25 µg/ml and 50 µg/ml NCTD group was smaller than that of the blank control group, as shown in Figure 3. At the same time, Transwell chamber method also found that the number of invasive cells in 12.5 µg / ml, 25 µg/ml and 50µg/ml NCTD groups were (66.4 ± 12.4), (53.4 ± 8.1), (31.6 ± 4.6) respectively, which were significantly lower than that in the blank control group (84.2 ± 7.3),P<0.05, as shown in Figure.4.

Figure 3: Effect of NCTD on migration of PC-3 cells (inverted microscope,× 100)



A:blank control group ;B-D:NCTD group(12.5,25,50 µg/mL))E:positive control group(cisplatin 2.5 µmol/L).

A:blank control group ;B-D:NCTD group(12.5,25,50 µg/mL))E:positive control group(cisplatin 2.5 µmol/L).

Transmission electron microscopy showed that the diameter of PC-3 cells was about 4 μ m, the nucleus was large, the nucleolus was obvious, and there were many pseudopodoid processes on the cell surface. After 24 hours of incubation with 12.5 μ g / ml, 25 μ g / ml and 50 μ g / ml NCTD, the number of pseudopodia on the surface of PC-3 cells decreased. Compared with negative control, with the increase of NCTD concentration,the number of pseudopodia of PC-3 cells decreased gradually.What's more,the number of pseudopodia decreased obviously in 50 μ g / ml NCTD group compared with positive control group. See Figure.5.

Figure 4: Effect of NCTD on invasion of PC-3 cells (crystal violet (×200)

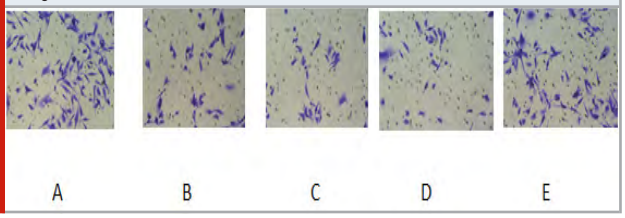
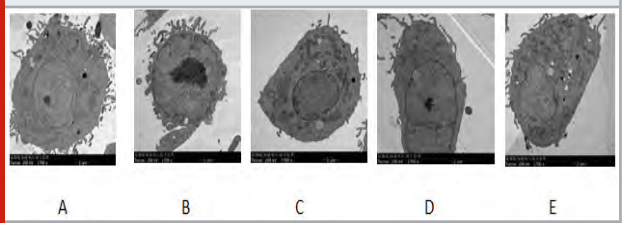


Figure 5: Effect of NCTD on pseudopodia morphology of PC-3 cells (transmission electron microscopy, accelerating voltage 200kV, ×5000)



A:blank control group ;B-D:NCTD group(12.5,25,50 μg/mL))E:positive control group(cisplatin 2.5 μmol/L).

The existing evidence (Marioni,2018;Zhao,2016;Horn,2018;Wen,2019) shows that the overexpression of cortactin may increase the formation of pseudopodia of various cancer cells, accelerate the degradation of peripheral matrix components of cancer cells, and facilitate the invasion and diffusion of cancer cells. Recent studies (Ma,2016;Qi,2020) have also shown that the expression of cortactin in prostate cancer is higher than that in benign prostatic hyperplasia, and the expression of cortactin gradually increases with the development of prostate cancer, and is related to the distant metastasis of prostate cancer. After knockout of the expression of cortactin in PC-3 cells, the invasion ability and extracellular matrix degradation ability of PC-3 cells were significantly reduced, (Horn,2018).

Recent studies of Ren (2019) have shown that the expression of MMP-9 mRNA in prostate cancer tissue is significantly higher than that in benign prostate tissue, and the expression of mm-9 mRNA in invasion and metastasis prostate cancer tissue is significantly higher than that in non invasion and metastasis prostate cancer tissue, indicating that MMP-9 may play an important role in invasion and metastasis of prostate cancer. MMP-9 can degrade and destroy tumor extracellular matrix and promote the formation of invasive pseudopodia of cancer cells, which suggests that MMP-9 may have synergistic effect with cortactin protein in the development of prostate cancer. There are few studies of NCTD in the treatment of prostate cancer. Recent clinical studies of scholars have found (Song,2018)that NCTD combined with paclitaxel is better than paclitaxel alone in the treatment of HRPC patients, and may reduce the adverse reactions caused by paclitaxel, but the specific mechanism is not clear. A recent study indicated that norcantharidin (NCTD) can inhibit PC-3 cells proliferation and induce PC-3 cell apoptosis (LUO,2020).

In this study, after the PC-3 cells were treated with different concentrations of NCTD, real-time fluorescence quantitative PCR and Western blot were used to detect the content of cortacin and MMP-9 mRNA and protein expression. The results showed that the content of cortacin and MMP-9 mRNA and protein expression of PC-3 cells in each concentration group of NCTD were lower than those in the blank control group. At the same time, with the increase of NCTD concentration, the mRNA content and protein expression of cortin and MMP-9 in PC-3 cells decreased gradually, and there was a dose-response relationship. The results of scratch test and Transwell test indicate that NCTD can inhibit the peripheral migration and invasiveness of PC-3 cells, and there is a dose-response relationship between the concentration of NCTD and the invasiveness of PC-3 cells. At last, the ultrastructural observation of PC-3 cells was carried out by means of transmission electron microscopy. Compared with the blank control group, the number of pseudopodoid protrusions on the surface of PC-3 cells decreased in the NCTD group. With the increase of NCTD concentration, the number of pseudopods decreased significantly. It indicates that NCTD would inhibit the formation of pseudopodia on the surface of PC-3 cells.

CONCLUSION

NCTD can inhibit the peripheral migration and invasiveness of PC-3 cells, and its mechanism may be related to the down-regulation of mRNA content and protein expression of cortactin and MMP-9 in PC-3 cells and the inhibition of the formation of pseudopods on the surface of PC-3 cells. At the same time, there may be some synergistic effect between cortactin and MMP-9 in promoting the invasion and metastasis of prostate cancer.

Author contributions: W.J. conceived the study. K. and J.J. and H.H. performed the experiments and analyzed the data. X.N. wrote the manuscript.

Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Effect of Growth Conditions on Biosurfactant Production by Pseudomonas balearica Isolated from Oil Contaminated Sea Waters from Jeddah Saudi Arabia

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ABSTRACT

The biosurfactant produced by Pseudomonas balearica isolated from oil-contaminated sea water was studied in the present research. A range of different growth conditions, such as temperature, pH, incubation period, inoculum size and different carbon/nitrogen sources and different C/N ratios were investigated to determine the optimum conditions for maximum production of a biosurfactant by the selected bacterial strain using a mineral salt medium. The best carbon andnitrogen sources were olive oil and urea, giving biosurfactant yields of 6.23±0.06 and 6.33±0.10 mg/ml, respectively. The maximum rhamnolipid production was 6.40±0.14 mg/ml at C/N (olive oil/ urea) of 30. The highest biosurfactant yield was at pH 7 (6.37±0.06 mg/ml), with inoculum size 2% (6.29±0.16 mg/ml), and incubation temperature 30°C (6.18±0.14 mg/ml) and after a 312 hrs incubation period (6.30±0.09). The previous investigated nutritional and environmental factors showed the highest emulsification activity. The produced rhamnolipid biosurfactant reduced the surface tension of water from 72 to 34 mN/m. In conclusion, the findings herein demonstrated that the rhamnolipid biosurfactant production by *P. balearica* can be used during oil hydrolysis.

KEY WORDS: BIOSURFACTANT, PSEUDOMONAS BALEARICA, CONTAMINATION, DEGRADATION, OIL HYDROLYSIS.

INTRODUCTION

Biosurfactants are important materials produced by microorganisms and are used in various industries. The production of biosurfactants not only depends on the producer's strain but also on the culture conditions. Additionally, many parameters affect not only the amount of biosurfactant but also the type of product produced (Salihu et al., 2009). These parameters include environmental (pH, temperature, and aeration), and nutritional factors (carbon source and nitrogen source). It is important to know the suitable nutrients and cultural conditions required to achieve higher productivity (Reddy et al., 2011, El-sersy, 2012). First of all, the carbon source plays a vital role in the growth and production of biosurfactants by microorganisms.

The substrates required for biosurfactant generations are typically formed of either carbohydrates or hydrocarbons, though they can also be used consecutively. Sarubbo et al. (2007) reported that the maximum yield of sophorolipids was obtained from *Candida lipolytica* when canola oil and glucose were used as carbon sources at concentrations of 10% for each. The type of carbon substrate used for

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production has been reported to influence both the quality and quantity of biosurfactants (Abouseoud et al., 2008). Waste cooking oil and coffee wastewater have also been used as a carbon source for biosurfactant production (Yañez-Ocampo et al., 2017, Nejad et al., 2020).

Nitrogen is the second most important supplement required to produce biosurfactants in microorganisms. Low nitrogen levels limit bacterial growth, pushing cell metabolism towards the production of metabolites. In contrast, excessive nitrogen leads to the synthesis of cellular material and limits the build-up of products (Robert et al., 1989, Fakruddin, 2012). It has been reported that nitrogen limitation enhances biosurfactant production (Kim et al., 2006). Different nitrogen compounds have been used to produce biosurfactants including urea, peptone, yeast extract, ammonium sulphate, ammonium nitrate, sodium nitrate, meat extract, and malt extracts. Additionally, environmental parameters such as pH, temperature, and incubation period all influence both microbial growth and biosurfactant production.

In a low pH culture medium, bacteria cannot efficiently synthesise biosurfactants (Saikia et al., 2012). Likewise, different microbial processes are affected by even a small change in temperature. Most biosurfactant producers are reported to perform in a temperature range of 25–30°C (Desai and Banat, 1997). However, Hmidet et al. (2017) found that the production of biosurfactants called surfactin and fengycin by *Bacillus mojavensis* A21 was at 20–30oC. As microorganism production rates are themselves a function of time, adjustments to the length of incubation can radically affect the resultant products. The optimum condition for maximum biosurfactant productivity in *B. brevis* was 10 days (Mouafi et al., 2016). The growth and biosurfactant formation by *B. subtilis* and *B. tequilensis* were increased with the incubation period which was associated with reduction in surface tension (Marajan et al., 2018).

The current study estimates the effects of different environmental parameters (pH value, temperature, inoculum size, and incubation period) and also nutritional factors (carbon source, nitrogen source, carbon to nitrogen ratio) on biosurfactant production by the selected bacterial strain, isolated from oil-contaminated water from the southern sea-shores of Jeddah, Saudi Arabia. The emulsification ability and decrease in surface tension were also evaluated.

MATERIAL AND METHODS

Isolation and Screening method for biosurfactant producing bacteria: The mineral salt medium contained 1% model hydrocarbon compounds (diesel oil) as the sole source of carbon was used for bacterial isolation using the method described by Motwali et al., (2020). After four subcultures, samples were serially diluted using sterile saline solution (0.85% NaCl) and spread ontonutritional agar plates (Kumar et al., 2016). After 24 hours' incubation at 37 °C, the chosen isolates were repeatedly purified before preservation in nutrient agar slants.The isolated bacterial strains were then screened for biosurfactant production using an oil displacement test, drop collapse, CTAB assay and surface tension measurements, applying the same procedure as described by Motwali et al. (2020).

Characterisation and Identification of the Selected Isolates of Bacteria: The purified isolated bacterial cells' morphological shapes were observed with Gram staining under a microscope (oil immersion, 100×). The bacterial isolate was identified at Macrogen, Korea. Genomic DNA of the selected isolate was extracted according to the method described by Asubel et al. (1987). Universal bacterial primers corresponding to Escherichia coli positions 27F (5' (AGA GTT TGA TCM TGG CTC AG) 3) and 1492R (5' (TAC GGY TAC CTT GTT ACG ACT T) 3') were used for PCR amplification of the 16S rRNA gene.

Microorganism, inocula and production medium: The inoculum was prepared by the transferal of1% of the fresh bacterial subculture to the production medium. The production medium was a mineral salt medium that reported before for bacterial isolation containing 1% model hydrocarbon compounds (diesel oil) as the sole carbon and energy source.

Effects of carbon and nitrogen sources and C/N ratio on biosurfactant production: Different carbon sources were tested to determine the one most suitable for maximum biosurfactant production (Table 1). The carbon source was added separately to the mineral salt medium at approximately 1% concentration. Also, the effect of different nitrogen sources was also studied (Table 1) and each source was added to the medium at 0.1%. In addition, different carbon to nitrogen concentrations were investigated (Table1). The C/N ratio was varied from 10 to 50 (Kiran et al., 2009; Khopade et al., 2012). The effect of different pH of the medium and different incubation temperature was determined.

Table 1. Factors considered for the optimisation of biosurfactant production by the selected bacterial isolate.

Factor	Range
Carbon source	Glucose, olive oil, corn oil, sunflower oil, mustard oil, sesame oil, lubricant oil, xylene, toluene, and diesel.
Nitrogen source	Urea, peptone, yeast extract, $NaNO_3$, KNO_3, and NH_4NO_3.
C/N ratio	10, 20, 30, 40, 50.
pH value	3, 5, 7, 9, 11.
Temperature	20, 25, 30, 35, 37, 40, 45, 50°C.
Inoculum size	0.5, 1, 2, 3, 4, 5, 6, 7, 8 %.
Incubation period	96, 168, 240, 312hrs

Each flask was inoculated with 1% of the pre-culture of the selected bacterial isolate (5x106 CFU/ml) and incubated at 120 rpm for 7 days. The impact of changing inoculum volume and optimum incubation period on biosurfactant production were studied (Table 1). At the end of the growth period, emulsification index, bacterial dry weight and biosurfactant production were determined.

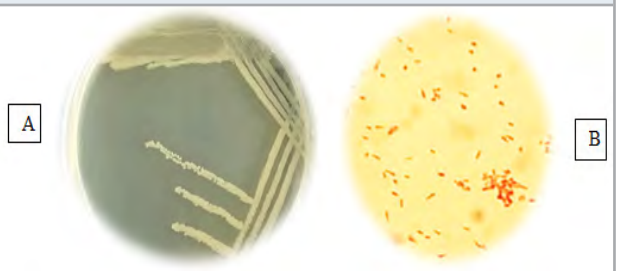
Detection of biosurfactant concentration: At the end of the incubation period, the bacterial culture was centrifuged at 4500 rpm for 30 min at 4°C. The filtrate was used to measure biosurfactant (Rhamnolipid) concentration using an orcinol assay as the method described by Pathaka and Nakhate, (2015). A standard curve for 10 mg/ml L-rhamnose (Sigma) was prepared. Measurement of bacterial dry weight: For bacterial dry weight measurement, 50 ml of each sample was centrifuged at 4500 rpm for 30 min at 4°C and the cell pellet was dried in an oven at 70°C for 24 h or until recording constant weight.

Detection of biosurfactant activity: The emulsification index (EI24) was calculated using the method described by Gagelidze et al., (2016). The EI24 of culture samples was determined by adding 2 ml diesel oil to 2 ml of the cell free broth in a test tube (15×15 mm) and vortexed at high speed for 2 minutes. Then, it was calculated after 24 hours at room temperature using the following equation:

EI24= (height of emulsion formed (cm)/ total height of solution (cm)) × 100

Measurement of the surface tension: The surface tension of the culture supernatant is the most upfront screening method of biosurfactant producing microbes. This measurement was performed using the Du-Nouy-Ring assay (Carrillo, 1996). The surface tension of the bacterial supernatant was then measured at room temperature using a tensiometer (Kruss Force K6).

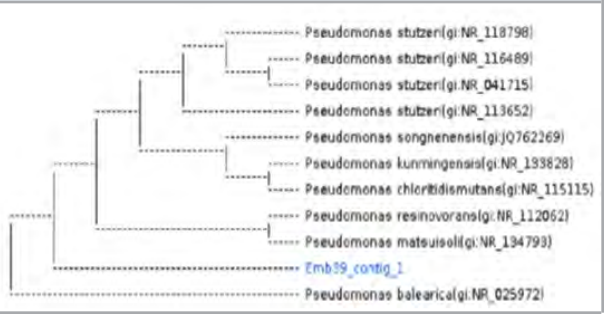
Figure 1 A: Bacterial culture on nutrient agar plate, B: Cells of P. balearica under light microscope X1000



RESULTS AND DISCUSSION

Screening the selected bacteria strain for biosurfactant production: The selected bacterial strain Emb39 was screened for its biosurfactant production ability. It showed positive activity on both the CTAB assay and drop collapse test. In addition, it was able to spread the oil in an oil spreading test by 3.05 ± 0.4 cm and to reduce surface tension to 42 mN/m Characterisation and molecular identification of the bacterial strain selected: The selected bacterial strain was characterised as an aerobic gram negative non spore forming bacteria (Figure1). Molecular identification of the selected isolate was performed using the GenBank BLAST tool on the 16S rRNA gene sequences. It was found that the selected bacterial strain was closely related (99%) to *P. balearica* (Figure 2). The bacteria accession number is NR_025972.1.

Figure 2: The phylogenetic tree of the bacterial strain *P. balearica*. Emb39.



Effect of different carbon sources on biosurfactant production: The bacterial strain *P. balearica* was selected to determine the effects of different carbon sources on biosurfactant production. Olive oil provides the greatest level of emulsification and greatest quantities of both biosurfactant and dry weight of *P. balearica* cells (Table 2 and Figure 3).

Table 2. Effect of different carbon sources on EI 24, biosurfactant concentration and bacterial dry weight (DW).

Carbon source	*P. balearica*		
	EI 24%	Rhamnose con. mg/ml	DW g/ 50ml
Glucose	25±3	3.58±0.4	0.21±0.02
Glycerol	0	3.40±0.4	0.17±0.03
Olive oil	64±2	6.23±0.06	0.21±0.04
Corn oil	35±2	3.35±0.05	0.19±0.04
Sunflower oil	0.0	1.56±0.11	0.18±0.03
Sesame oil	0.0	1.40±0.23	0.20±0.03
Mustard oil	9.5±2	1.90±0.21	0.14±0.03
Xylene	0.0	1.64±0.24	0.10±0.01
Diesel	21±2	3.35±0.06	0.20±0.02
Toluene	0.0	1.64±0.04	0.11±0.01
Lubricating oil	0.0	1.52±0.14	0.12±0.02

Effect of different nitrogen sources on biosurfactant production: The effect of changing the nitrogen source on biosurfactant production was also investigated. It is clear from Table 3 that using urea as the medium's

nitrogen source provided optimum results in all the three measured parameters. Likewise, ammonium nitrate (NH_4NO_3) was similarly suitable for biosurfactant production (Table 3 and Figure 4). Urea as a nitrogen source gave anemulsification index of 57±2.3%.

Figure 3: Effect of different carbon source on biosurfactantproduction

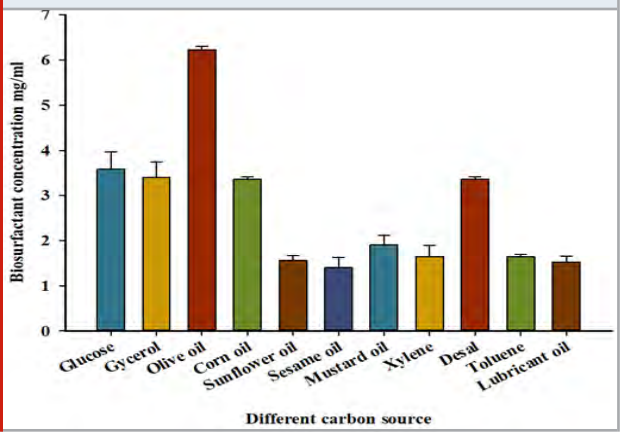
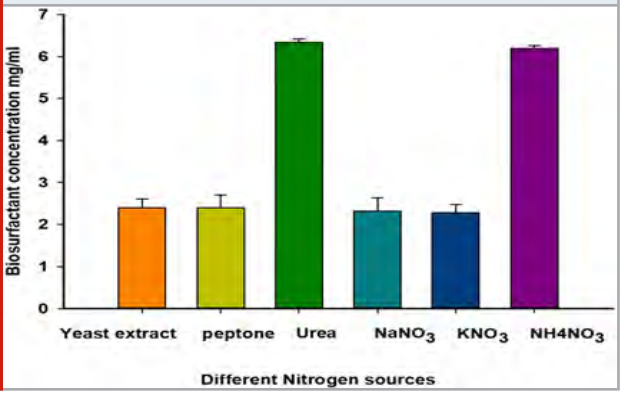


Table 3. Impact of different nitrogen sources on biosurfactant concentration, EI24, and bacterial dry weight (DW).

Nitrogen source	*P. balearica*		
	EI 24%	Rhamnose con. mg/ml	DW g/ 50ml
Yeast extract	0	2.40±0.22	0.23±0.02
Peptone	0	2.40±0.31	0.27±0.04
Urea	57±2.3	6.33±0.10	0.27±0.03
$NaNO_3$	0	2.7±0.31	0.19±0.02
KNO_3	0	2.30±0.20	0.17±0.05
NH_4NO_3	51±2.3	6.20±0.07	0.22±0.04

Figure 4: Effect of different nitrogen source on biosurfactant production.



Effect of different C/N ratios on biosurfactant production: A carbon source (olive oil) suitable for the tested strain was added to the production medium with different concentrations, along with aconstant concentration of a nitrogen source (Urea). The ultimate EI24 for diesel oil by *P.balearica* was recorded when the C/N ratio was 30 (Table 4). Likewise, the highest biosurfactant (rhamnolipid) concentration produced by this strain also occurred at a C/N ratio of 30 (Table 4 and Figure 5). A strong negative correlation (-0.82) apparent between C/N ratio and biosurfactant concentration.

Table 4. Outcome of different carbon/ nitrogen ratios on biosurfactant concentration, EI24, and bacterial dry weight (DW).

C/Nratio	*P. balearica*		
	EI 24%	Rhamnose con. mg/ml	Bacterial DW g/50ml
10	43±5	6.05±0.09	0.24±0.04
20	44±7	5.93±0.12	0.27±0.02
30	60±4	6.40±0.14	0.25±0.05
40	46±5	4.79±0.23	0.25±0.02
50	14±2	2.97±0.12	0.17±0.04

Figure 5: The effect of different C/N ratio on biosurfactant concentration.

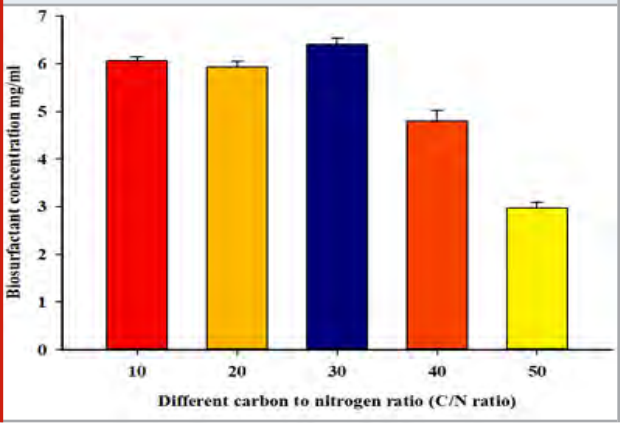


Table 5. Effect of different pH values on biosurfactant concentration, EI24, and bacterial dry weight (DW).

pH	*P. balearica*		
	EI 24%	Rhamnose con. mg/ml	Bacterial DW g/50ml
3	0.0	0.75±0.08	0.02±0.01
5	0.0	1.25±0.04	0.08±0.03
7	57±6	6.37±0.06	0.27±0.04
9	0.0	1.81±0.20	0.19±0.04
11	0.0	0.80±0.10	0.08±0.04

Effect of initial pH value on biosurfactant production: The pH of the production media for the tested strain was adjusted to different values, with amaximumrecorded emulsification index for diesel oil at pH 7 (Table5). Similarly, the highest biosurfactant production (6.37±0.06

mg/ml) as showed in Figure 6, and highest bacterial cell dry weight (0.27±0.04 g/50 ml) were recorded at pH 7 (Table 5). The correlation between biosurfactant concentration and pH is only weakly positive (+0.04).

Figure 6: Effect of different pH values on biosurfactant production by *P. balearica*.

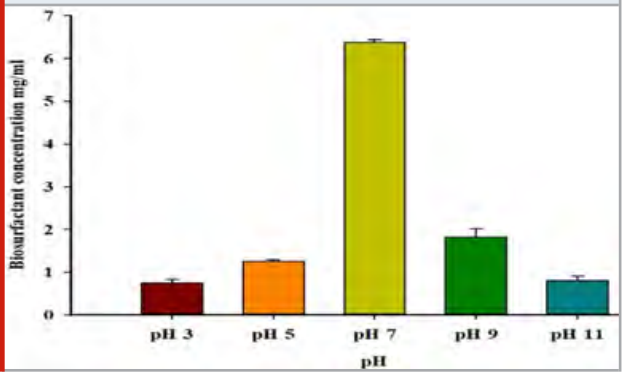
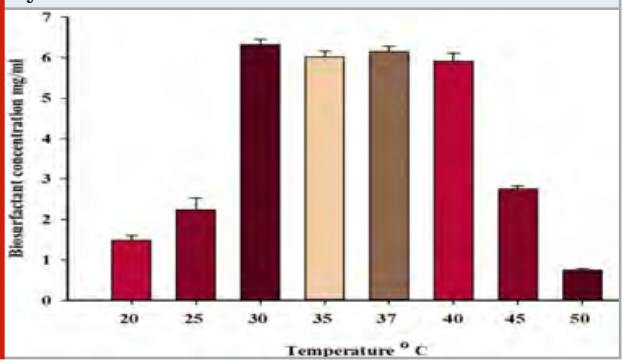


Table 6. Results of different incubation temperatures on biosurfactant concentration, EI24 and bacterial dry weight (DW).

Temperature (°C)	*P. balearica*		
	EI 24%	Rhamnose con. mg/ml	DW g/ 50ml
20	0	1.52±0.12	0.13±0.02
25	13±2	2.17±0.30	0.18±0.04
30	67±6	6.35±0.14	0.22±0.06
35	53±2	6.00±0.17	0.24±0.04
37	61±6	6.18±0.14	0.27±0.04
40	59±5	5.96±0.20	0.21±0.03
45	0	2.75±0.08	0.13±0.03
50	0	0.75±0.03	0.11±0.02

Figure 7: Effect of temperature on biosurfactantproduction by *P. balearica*.



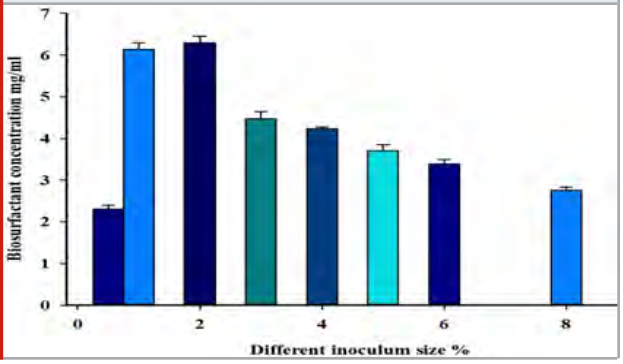
Effects of different incubation temperatures on biosurfactant production: The effect of incubation temperature on biosurfactant production was determined. The maximum emulsification index for diesel oil (67±6 %) and the highest biosurfactant production (6.35±0.14 mg/ml) with P.balearica were occurred at 30°C (Figure 7 and Table 6). For bacterial cell dry weight, the highest value was at 37°C (Table 6). Based on the correlation results, there appears to be a low negative correlation (-0.02) between incubation temperature and biosurfactant concentration.

Effects of changing the inoculum size on biosurfactant production: Effect of various inoculum sizes on biosurfactant concentration was studied. The results in table (7) indicate that the greatest emulsification index for diesel oil, bacterial cell dry weight and the highest biosurfactant production occurred with an inoculum size of 2% (Figure 8). Statistical analysis showed weak negative correlation (-0.45) between inoculum size and biosurfactant production.

Table 7. Effect of inoculum size on biosurfactant concentration, EI24, and bacterial dry weight.

Inoculum size%	*P. balearica*		
	EI 24%	Rhamnose con. mg/ml	Dry weight g/50ml
0.5	0	2.31±0.08	0.13±0.02
1	46±6	6.14±0.15	0.19±0.02
2	62±2	6.29±0.16	0.17±0.02
3	52±4	4.47±0.17	0.19±0.03
4	42±5	4.23±0.04	0.14±0.03
5	37±5	3.71±0.14	0.13±0.02
6	32±4	3.39±0.09	0.15±0.03
8	18±2	2.75±0.07	0.15±0.04

Figure 8: Effect of different inoculum size on biosurfactant concentration.



Effects of different incubation periods on biosurfactant production: *P. balearica* was incubated in the production medium with suitable selected nutritional factors for different time periods. The best diesel oil emulsification index, highest biosurfactant production, and highest bacterial cell dry weight were recorded after 312 hours (Table 8, Figure 9). The results show a strongly positive correlation (0.78) between incubation and biosurfactant production.

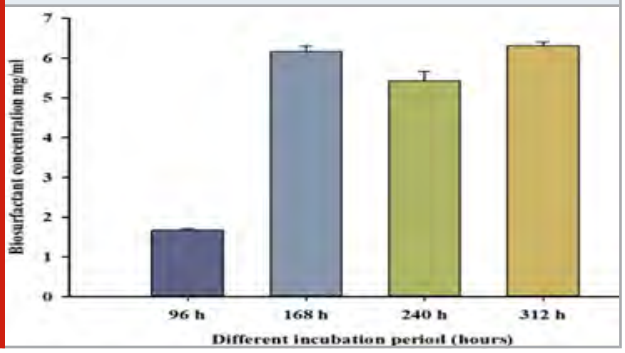
The optimum carbon and nitrogen sources were Olive oilandUrea, respectively. The best C/N ratio was 30 and

optimummedium pH was 7. The used inoculum size was 2%, incubation temperaturewas 30°C and incubation time was 312 hrs. At the previous optimum temperature, the supernatant surface tension was reducedto 34 mN/m in comparison with distilled water (74 mN/m).

Table 8. Effect of different incubation periods on biosurfactant concentration, EI24, and bacterial dry weight.

hours	*P. balearica*		
	EI 24%	Rhamnose con. mg/ml	Dry weight g/50ml
96	0	1.67±0.04	0.13±0.03
168	49±2	6.16±0.13	0.23±0.02
240	56±4	5.42±0.23	0.20±0.03
312	63±2	6.30±0.09	0.23±0.03

Figure 9: Effect of different incubation period on biosurfactant concentration



The isolation of bacterial strains with biosurfactant producing capabilities from sites subject to oil contamination has been undertaken across the globe, including *P. aeruginosa* SG from Xinjiang oil field, China (Zhao et al., 2016), *B. subtilis* MG495086 oil reservoir in Assam, India (Balan et al., 2017) *Ochrobactrum anthropi* HM-1 and *Citrobacter freundii* HM-2 from engine oil contaminated soil in Egypt (Datta et al., 2018) and both *P. aeruginosa* and klebsiella quasivariicola from oil contaminated sea shores in Jeddah, Saudi Arabia (Ibrahim, 2018). This indicates the presence and suitability of distinct biosurfactant producing microorganisms across numerous geographical oil contaminated locations (Motwali et al., 2020).

Assessing oil displacement and droplet collapse allow for rapid screening of a bacteria's efficacy as a biosurfactant producer. The strain presented herein was able to create over 2 cm spread in an oil displacement test (3.0 ± 0.4) and form flat drop in a drop collapse assay. The present value of oil displacement test was higher than that observed by Sharma et al., (2019) who recorded a value of 1.5 ± 0.3 cm by some screened bacterial strains. The present result of dropcollapse assay is in accordance with Vaijayanti (2020) who found that all tested bacterial isolates showed positive results for drop collapse test indicated the occurrence of biosurfactant.

The results also show that the selected bacterium Emb 39 has positive activity on CTAB agar test which considered as evidence of rhamnolipid-production (Verma et al., 2006). The application of quantitative techniques such as surface activity measurement corroborates and adds confidence to the previously offered qualitative assessments. The screened bacterial strain in the current study was able to reduce surface tension to 42±0.3 mN/m, which reveals the ability of this strains to produce surface-active molecules. These results are in accordance with previous reports and Sharma et al., (2015), Eslami et al., (2020) which have suggested a direct relationship between the production of surface-active compounds and a reduction in surface tension. The selected bacterial strain isolated from an oil-contaminated marine environment was identified as *P. balearica*.

Marine bacteria are halo tolerant and can produce novel metabolites, such as biosurfactants to live in such habitats. Strains that belong to genus *Pseudomonas* are the greatest biosurfactant producers (Sharma et al., 2015). The present finding is also in consistent with those of Nejad et al. (2020) who proved that *P. balearica* is a biosurfactant producing bacteria.

According to Noh et al., (2014) the most important factors in increasing biosurfactant yield is the solubility of the carbon source in the culture medium. For instance, palm oil and diesel, as insoluble carbon sources, generally produce more rhamnolipids in comparison with water-soluble carbon sources such as glucose. It has been shown that the highest biosurfactant concentration was produced by *P. balearica* when olive oil was used as the carbon source. Almatawah (2017) and Tan and Li (2018) noted the suitability of olive oil as a substrate for biosurfactant production.

Recently, Sun et al., (2021) have indicated that the highest biosurfactant yields were obtained by *Pseudomonas* sp. using plant oils such as olive oil, soybean oil, and peanut oil as carbon sources. However, the current result disagrees with Wu et al. (2008) who reported a lower biosurfactant yield from *P. aeruginosa* using olive oil than that from glucose and glycerol. Moreover, using olive oil as a carbon source gave the highest emulsion stability. Ezebuiro et al. (2019) concluded that the biosurfactant produced with olive oil showed a high emulsification index. Wasoh et al., (2017) observed emulsification activity against diesel ranging from 55-60 % by *P. aeruginosa* when sunflower oil was used as a carbon source. Additionally, the highest biosurfactant concentration was obtained using urea or NH_4NO_3 as nitrogen sources. Also, Adamczak and Bednarski (2000) found that urea and ammonium salts are optimum nitrogen reservoirs for biosurfactant production. Prieto et al., (2008) reported urea as a best nitrogen source for biosurfactant production by *P. aeruginosa* with emulsification index about 60 while and Saikia et al.,

(2012) Alyousif et al., (2020) reported NaNO3 as the best nitrogen source for biosurfactant production by P. aeruginosa.

The C/N ratio is known to be a vital factor influencing the performance of bacteria in rhamnolipid production (Santos et al., 2002). The highest biosurfactant production and emulsification index for *P. balearica* in this study was obtained at a C/N ratio of 30, while the least yield was recorded at a ratio of 50. Negative correlation (-0.82) was recorded between the C/N ratio and biosurfactant concentration, which agrees with the results of Onwosi and Odibo (2012). Heryani and Putra (2017) reported that the C/N ratio of 12.4 was optimum for the production of a biosurfactant by *Bacillus* sp. and resulted in the highest decrease in surface tension. Khopade et al. (2012) indicated that lower value of C/N ratio (C/N= 20) was improved the emulsification activity by *Nocardiopsis* sp.

The current research found that the best production of biosurfactant and emulsification activity by *P. balearica* was at pH 7, with weak positive (+0.04) correlation detected between pH and biosurfactant concentration. This finding is in agreement with Fouda et al. (2016) who observed that the subsequent increase in pH from 8–10 was followed by a decrease in biosurfactant productivity in *P. aeruginosa* and *B. cereus*. Similarly, maximum biosurfactant from mutated strain of *B. subtilis* and *Pseudomonas* sp. was obtained at pH 7 (Kannahi and Sherley, 2012, Onwosi and Odibo 2012, Alyousif et al., 2020).

However, Elazzazy et al. (2015) reported the maximum biosurfactant production by Virgibacillus salaries was achieved at pH 9. The optimum temperature of operation for *P. balearica* is reported to be 30 °C which was in accordance with Chander et al. (2012). Patil et al. (2014) reported maximum biosurfactant production was at 30 ^{0}C while the best inoculums size was 2% (Roy et al., 2017). Hema et al. (2019) have indicated that the optimum growth and emulsification activity for *Planococcus* sp. was 48 hours of incubation and that emulsification activity decreased by further incubation. After optimisation of the biosurfactant production of *P.balearica*, surface tension was measured to confirm the effect of the selected optimum conditions on biosurfactant activity. The results showed a decrease in surface tension which is in accordance with the data of Asgher et al., (2020).

CONCLUSION

Marine environments polluted with oil contain microorganisms able to produce surface-active agents (biosurfactant). *P. balearica* was the most active isolate and the use of olive oil and urea as carbon and nitrogen sources, pH 7, inoculum size 2% and incubation temperature at 37^{o}C temperature for 312 hr enhanced biosurfactant production.

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Identification and Analysis of Pathogenic NS SNPS in Human Bloom Syndrome Helicase Gene BLM

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ABSTRACT

BLM helicase protein plays important role in DNA replication and maintains the genomic integrity. Variation in BLM helicase gene resulted defect in DNA repair mechanism and are reported to be associated with bloom syndrome (BS) and cancer. Computational analysis of SNPs in BLM helicase gene has been performed to identify, characterize the pathogenic SNPs using bioinformatics approach. SNPs data has been obtained from dbSNP database for human BLM helicase (P54132). There were 1003 SNPs mapped to missense, 19890 SNPs mapped to intron, while 550 SNPs mapped to 5'UTR, 176 SNPs mapped to 3'UTR, 21551 mapped to total SNPs of different variation class and 11 SNPs mapped to pathogenic misense in human BLM helicase gene. 6 nsSNPs of 11 pathogenic missense are found to be deleterious or damaging by all four prediction tools. These 6 nsSNPs rs367543034 of mutation G952V, rs367543023 of mutation H963Y, rs137853153 of mutation C1036F, rs367543029 of mutation C1055Y, rs367543032 of mutation D1064G and rs367543025 of mutation C1066Y can be further investigated along with native protein. These mutations in BLM gene may have potential to be used as an important prognostic marker for detection of cancer, particularly for for surgically-treated lung adenocarcinoma.

KEY WORDS: NSSNP; BLOOM SYNDROME; IN SILICO ANALYSIS; BLM.

INTRODUCTION

BLM gene encodes an important nuclear protein BLM helicase (Eladad et al., 2005), which involved in DNA replication and maintains the genomic integrity (Manthei et al., 2013). BLM is a 3' to 5' DNA helicase that belongs to conserved RecQ helicase family (Imamura et al., 2003). Helicases are very crucial for unwinding duplex DNA to produce the transient single-stranded DNA intermediates necessary for replication, recombination, and repair (Hall et al., 1999, Schmid et al., 1992). In a complex with topoisomerase Topo IIIa and Rmi1/Rmi2, BLM helicase repair DNA double-strand breaks through homologous recombination (HR) pathway (Matson et al., 1994). Consequently, cells lacking functional BLM show ~10-fold raising in chromatid breaks, and mitotic recombination, (Hickson et al., 2003). Bloom syndrome (BS) is a rare autosomal recessive genetic disorder caused by pathogenic variants in the BLM gene. Symptoms of BS include low birth weight, dolichocephaly (long, narrow head), congenital short stature, growth retardation sun-sensitive facial rash, an elevated risk of diabetes mellitus, reduced fertility and immune deficiency (Shastri et al., 2015).

Absence of BLM protein activity causes defect in DNA repair, increased rate of mutations and thus risk of cancer (Arora et al., 2014). BLM gene transcribes a 97.93 kb long precursor-mRNA having 21 exons, which code 1417 amino acid protein. Literature support that a large number of BS patients shows insertion, deletion and missense mutation that change the amino acid or

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nonsense mutations which introduce premature stop in the BLM gene and thus inactivate the BLM helicase (Ellis et al., 1995, Foucault et al., 1997, German et al., 2007 Mclaren et al 2016 Yang et al 2020).

Several articles have stated effectiveness in identifying the deleterious and disease associated mutations, thus predicting the pathogenic SNPs in correlation to their functional and structural damaging properties (Adzhubei et al., 2010 Choi et al 2015). Computational studies have previously provided an efficient platform for evaluation and analysis of genetic mutations for their pathological consequences and in determining their underlying molecular mechanism. Single nucleotide polymorphism (SNPs) is a common genetic variation contributing greatly towards the phenotypic variations (Hecht et al., 2015). SNPs can alter the functional consequences of proteins. In the coding region of gene, SNPs may be synonymous, non-synonymous (nsSNPs) or nonsense.

Synonymous SNPs changes the nucleotide base residue but does not change the amino acid residue in protein sequence due to degeneracy of genetic code. The nsSNPs also called missense variants, alter amino acid residue in protein sequence and thus change the function of protein through altering protein activity, solubility and protein structure. (Calabrese et al., 2009). SNPs have been emerged as the genetic markers for many diseases and there are many SNPs markers available in the public databases. hundreds of new SNPs have been mapped to human BLM genes. However, not all SNPs are functionally important. Despite extensive studies of helicase proteins in human and effect of their polymorphism in cancer (Hecht et al., 2015), no attempt was made to analyze to establish the functional consequences of pathogenic nsSNPs of BLM gene. The aim of this study is to identify the high pathogenic SNPs of BLM gene and determine functional consequences using computational methods.

MATERIAL AND METHODS

SNPs dataset: The SNPs of the BLM helicase gene (Uniport id P54132) were retrieved from the dbSNP database (Sherry et al., 1999). Keyword "Human BLM" used as our search term. Furthermore, it is filtered by selecting variation class as SNV, function class as missense, clinical significance as pathogenic.

Predicting deleterious and damaging nsSNPs: In order to predict the damaging or deleterious nsSNPs, multiple consensus tools were employed by using online tool VEP (http://www.ensembl.org/Tools/VEP). VEP advantages include: it uses latest human genome assembly GRCh38. p10, and can predict thousands of SNPs from multiple tools including SIFT, PROVEAN, Condel, and PolyPhen-2, at a time (McLaren et al., 2016). 11 nsSNP rs-ids were uploaded to VEP tool to get the prediction results.

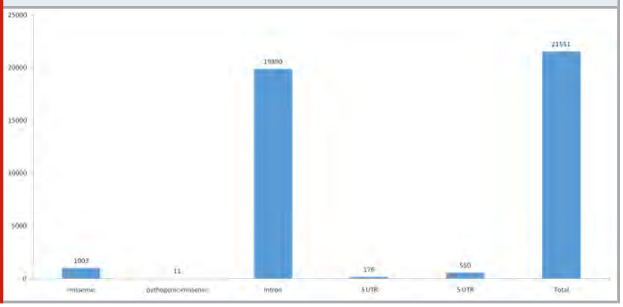
Sift: The algorithm predicted that the tolerant and intolerant coding base substitution based upon properties of amino acids and homology of sequence (Ng PC et al., 2003). The tool considered that vital positions in the protein sequence have been conserved throughout evolution and therefore substitutions at conserved alignment position is expected to be less tolerated and affect protein function than those at diverse positions. SIFT predicted substituted amino acid as damaging at default threshold score <0.05, while score ≥ 0.05 is predicted as tolerated.

Provean: The online tool uses an alignment-based scoring method for predicting the functional consequences of single and multiple amino acid substitutions, and in-frame deletions and insertions (Choi et al., 2012). The tool has a default threshold score, i.e. -2.5, below which a protein variant is predicted as deleterious, and above that threshold, a protein variant is neutral.

Condel (CONsensusDELeteriousness): This tool evaluates the probability of missense single nucleotide variants (SNVs) deleterious. it computes a weighted average of the scores of SIFT, PolyPhen2, MutationAssessor and FatHMM (González-Pérez et al., 2011).

PolyPhen-2: This tool is predicting the structural and functional consequences of a particular amino acid substitution in human protein (Ramensky et al., 2002). Prediction of PolyPhen-2 server [20] is based on a number of features including information of structural and sequence comparison. The PolyPhen-2 score varies between 0.0 (benign) to 10.0 (damaging). The PolyPhen-2 prediction output categorizes the SNPs into three basic categories, benign (score < 0.2), possibly damaging, (score between 0.2 and0.96), or probably damaging (score >0.96).

Figure 1: Number of SNPs in different function class of BLM helicase gene of human from dbSNP database showing missense (1003), pathogenic missense (11), intron (19890), 3UTR (176), 5UTR (550) and Total (21551).



RESULTS AND DISCUSSION

11 rs-ids of pathogenic nsSNPs mapped in human BLM helicase gene was downloaded from dbSNP database of NCBI (Table 1), after filtering variation class SNV, function class missense and clinical significance as pathogenic, there were 1003 SNP mapped to missense, 19890 SNPs mapped to intron, while 550 SNPs mapped to 5'UTR, 176 SNPs mapped to 3'UTR and 21551 mapped to total SNPs of different variation class (Figure 1). Some rsIDs are associated with multiple SNPs and therefore fall in different classes.

Predicting deleterious and damaging pathogenic nsSNPs: In order to predict the damaging or deleterious pathogenic nsSNPs multiple consensus tools were employed. Initially, online tool VEP was used. VEP advantages include: it uses latest human genome assembly GRCh38.p10, and can predict thousands of SNPs from multiple tools including SIFT, Condel, and PolyPhen-2, at a time. 11 nsSNP accession numbers were uploaded to VEP tool and the prediction results were taken on default scores of consensus tools based on sequence and structure homology methods: (a) SIFT (score <-0.5) (b) Polyphen (score >0.96) (c) PROVEAN (score< 2.5) and Condel (score >0.522). In order to get a very high confident nsSNPs impacting structure and function of BLM gene, 6 nsSNPs (Table 1) are found to be deleterious or damaging by all four prediction tools. These 6 nsSNPs rs367543034 of mutation G952V, rs367543023 of mutation H963Y, rs137853153 of mutation C1036F, rs367543029 of mutation C1055Y, rs367543032 of mutation D1064G and rs367543025 of mutation C1066Y.

Table 1. Prediction of 11 pathogenic missense SNPs of BLM helicase gene using prediction tools such as SIFT, Condel, Polyphen and PROVEAN, deleterious predicted by all four tools are shown in bold.

SNP-ids	AA-Change	SIFT	PolyPhen	PROVEAN	Condel
rs746195311	E69K	deleterious	benign	neutral	neutral
rs367543030	S104L	deleterious	Possibly damaging	neutral	neutral
rs1477193473	E488K	tolerated	benign	neutral	neutral
rs200389141	Q548K	deleterious	benign	neutral	neutral
rs367543034	G952V	deleterious	Probably damaging	deleterious	deleterious
rs367543023	H963Y	deleterious	probably damaging	deleterious	deleterious
rs137853153	C1036F	deleterious	Probably damaging	deleterious	deleterious
rs367543029	C1055Y	deleterious	probably damaging	deleterious	deleterious
rs367543032	D1064G	deleterious	probably damaging	deleterious	deleterious
rs367543025	C1066Y	deleterious	probably damaging	deleterious	deleterious
rs367543017	S1093L	deleterious	Possibly damaging	deleterious	Neutral

This analysis shows that six SNPs, G952V, H963Y, C1036F, C1055Y, D1064G and C1066Y have high prevalence for disease association of BLM, the mutation in cysteines (C1036F, C1055Y, C1066Y) and glutamate (D1064V) are in the Zn binding subdomain, which results in the loss of Zn binding upon mutation and alters the function of BLM helicase is reported (Guo et al., 2005). These mutation in RQC domain affect the highly conserved cysteine residues involved in Zn coordination. While mutation in Glycine G952V and mutation in histidine H963Y which alter amino acid residues in the ATPase domain also reported involved in cellular defects (Shastri and Schmidt 2015).

CONCLUSION

This computational analysis of SNPs of the human BLM protein identified 6 highly damaging pathogenic nsSNPs. Prediction analysis shows that SNPs G952V, H963Y, C1036F, C1055Y, D1064G and C1066Y have high prevalence for disease association. Data implies that the reported nsSNPs could potentially alter structure and hence the function of BLM protein resulting in pathogenicity with abnormal symptoms describing the disease states. These nsSNPs associated with significant pathogenicity will offer valuable information in selecting SNPs that are expected to have impending functional influence and contribute in understanding the functional roles of this gene.

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Patient-Attendant Aggression Towards Dental Professionals: A Survey Based Analysis

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ABSTRACT

Violence at work affects the health and safety of dental healthcare workers and studies related to the aggression of patients and their attendants towards dental healthcare staff are scarce. The aim of the present cross sectional study was to assess the frequency and causes of aggressive behavior of patients and their attendants encountered by dental healthcare workers in tertiary care institutes and independent private practices. A self-administered questionnaire consisting of 12 close-ended questions was used to collect data from 286 dentists, postgraduate residents, consultants and assistants. Descriptive statistics were calculated and post-stratification Chi-squared test was used to control effect of gender ($P<0.05$). Majority (45.1%, n=129) of subjects reported encountering violent behavior at least once a week. Statistically significant difference was observed between frequency of violent behavior experienced by females and males, where females reported a higher frequency of such encounters in routine ($P<0.001$) The most commonly encountered aggressive behavior was the use of harsh tone or shouting reported by 73.4% of participants. Most significant reason for aggressive behavior was mishandling at the reception (n=143; 50%) followed by unrealistic expectations of patients (n=106; 37.1%) and culture of dominating healthcare professionals (n=99; 34.6%) as well as socially or professionally influential patients who try to influence the doctor (n=99; 34.6%). Dental healthcare workers frequently encounter aggressive behavior from patients and their attendants. Females are targeted significantly more than males ($P<0.001$).

KEY WORDS: DENTISTS; DENTAL PROFESSIONALS; OCCUPATIONAL HEALTH; PATIENT AGGRESSION; WORKPLACE VIOLENCE.

INTRODUCTION

Violence at work is one of the major deterrents affecting "health and safety" of workers in all sorts of occupations (Pouryaghoub et al., 2017). Aggressive behavior of patients and their attendants towards dental health care providers is an increasingly significant yet an under reported problem (Franz et al., 2010; Duxbury & Whittington., 2005; Cooper & Swanson., 2002). The term 'aggression' is

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used to refer to hostile behavior of varying intensity that leads to harm irrespective of the intent of the aggressor (Edward et al., 2014). Even though the term violence differs in its literal context from aggression, these two terms have been used interchangeably in literature to describe workplace aggression or violence in health care settings. The absence of a universally accepted operational definition of workplace aggression/violence also makes it difficult to draw comparisons between studies from different cultures, regions and specialties (Imran et al., 2013; Hills, 2018, Rhoades et al 2020).

Emergency departments, psychiatric wards and geriatric units apparently have the highest frequency of aggression that have been reported in health care settings. Healthcare settings as mentioned earlier and certain healthcare workers like paramedical staff in emergency department, ambulance staff and nurses are more prone to experience aggression perpetrated by the patients, their attendants or their visitors. However, aggression in healthcare settings is more widespread and needs to be examined within the context of that particular domain of healthcare, cultural/ regional specificity and other associated factors (Aydin et al. 2009 Llor- Ambesh, 2016, Esteban et al 2017 and Lee et al., 2020).

There is a significant gap in literature as far as reporting of aggression of patients and their attendants towards dental health care staff is concerned. There are only a few reports which have studied this phenomenon in dental practice (Pemberton et al., 2000). The aim of this study was to assess workplace aggression against dentists and dental assistants working in tertiary care dental hospitals and private dental practices in the twin cities of Rawalpindi & Islamabad Pakistan. More specifically this study sought to examine the frequency, the factors associated with aggression experienced by dental staff, the implications of such behavior and the suggestions for minimizing such behavior.

MATERIAL AND METHODS

A cross-sectional study was designed and conducted from August 2019 to December 2019. Approval from ethical review committee of Armed Forces Institute of Dentistry, Rawalpindi, Pakistan was sought (Approval # ERC/2019/OA-22). Sample size was calculated using WHO sample size calculator. Keeping confidence level (1-α) at 95%, absolute precision (d) at 0.0539 and anticipated population proportion (P) at 0.319, (Azodo et al., 2011) a sample size of 286 was calculated. General dentists, postgraduate residents in any dental specialty, consultants from all dental specialties and dental surgery assistants who were willing to participate were included in the study. A self-administered questionnaire consisting of 12 close-ended questions was used as the data collection tool.

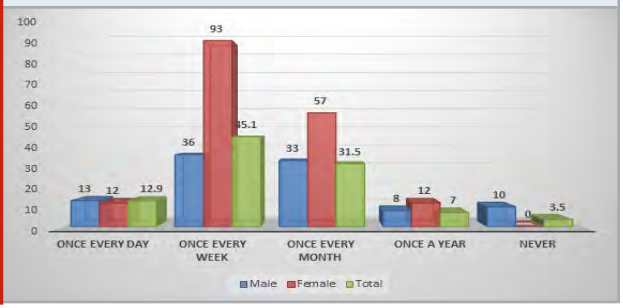
The questionnaire was first pilot – tested to ensure its validity, reliability and relevance. The first part of the questionnaire aimed to collect the demographics including age, gender, practice status and years of experience. In the 2nd part, questions were designed to assess the frequency of aggressive behavior encountered, probable causes of such a behavior and its effect on dental professionals' efficiency. Data was analyzed in SPSS 24. Descriptive statistics were calculated. Post-stratification Chi-squared test was used to control effect of gender. P<0.05 was taken as significant.

RESULTS AND DISCUSSION

The study comprised of 286 study subjects and out of these, 35% (n=100) were male and 65% (n=78) were females. 8.4% subjects (n=24) were consultants in their fields, 36% were residents (n=103), 31.5% (n=90) were interns or house officers, 10.1% were general dentists (n=29), and 14% (n=40) were dental surgery assistants. Majority (46.5%, n=133) of the subjects reported a clinical experience of less than 2 years, while only 7.3% (n=21) had a clinical experience of more than 10 years. Most of the subjects (31.5%, n=90) reported seeing an average of 6-10 patients per day while a smaller percentage (14.7%, n=42) saw more than 20 patients daily. In majority of the cases (69.6%, n=199) dental practices accepted both walk-in patients as well as appointed patients. Only 22% (n=63) practices were solely appointment-based. Study subjects reported varying frequency of aggressive behavior they faced at work, with a good majority (45.1%, n=129) encountering such behavior at least once a week.

A statistically significant difference was observed between frequency of violent behavior experienced by females and males, where females reported a higher frequency of such encounters in routine (P<0.001) (Figure 1). The most commonly encountered aggressive behavior was the use of harsh tone or shouting reported by 73.4% of the subjects followed by verbal abuse, swearing and insult reported by 25.5% while physical assault was reported by 1.09% of the study participants, which is quite alarming.

Figure 1: Frequency of aggressive behavior of patients/ attendants faced by study subjects.



Half of the study subjects (50%, n=143) reported that their manager at work was appropriately trained/capable of managing any patient or attendant with an aggressive attitude. In contrast, 79.4% of the respondents reported that they themselves had not received any formal training in managing aggressively charged or overly emotional patients and 85.7% stated no counselling services were

available to them following any undesirable encounter with a patient, resulting in an emotional setback for the dental staff (Table 1).

Question regarding effect of patient's aggressive behavior on dental staff's efficiency received varying responses (Figure 2). An equal number of respondents (n=99 each, 34.6% each) reported that such a disturbing encounter with patient decreased their efficiency at work and affected the treatment of patients on that particular day while 24.5% (n=70) believed that they were not affected by such encounters.

Table 1. Response to questions about formal training to manage aggressive behaviour.

Question	Response		
	Yes (%)	No (%)	Can't Say (%)
Do you think that your reception or clinic manager is appropriately trained/capable of managing any patient or attendant with an aggressive attitude?	143 (50)	89 (31.1)	54 (18.9)
Do you have any formal training in managing aggressively charged or overly emotional patients?	59 (20.6)	227 (79.4)	-
Are there any counselling services available to you following any untoward happening where the patient or their attendants have behaved aggressively, or their behavior might have resulted in an emotional setback for you?	41 (14.3)	245 (85.7)	-

Figure 2: Effect of patient's aggressive behavior on dentist's efficiency.

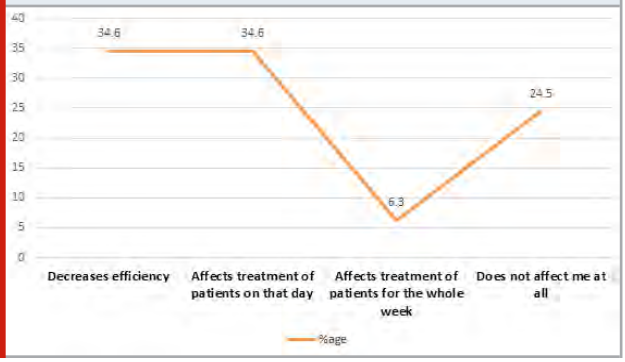
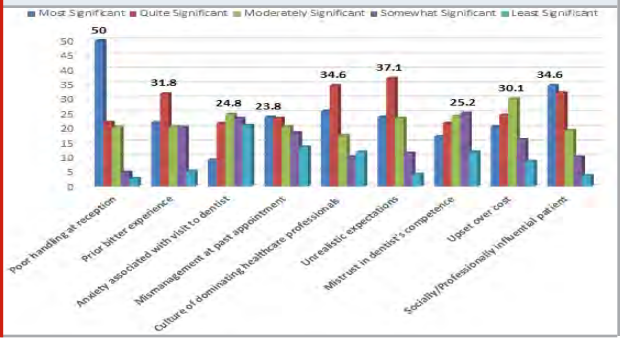


Figure 3: Probable reasons of patients' aggressive behavior towards dentists.



Participating dental health care providers were asked to grade the most probable reasons for patients behaving aggressively or getting overly emotional in the dental office, and the results are highlighted in Figure 3. The factor regarded as most significant reason for aggressive behavior was mishandling at the reception (n=143, 50%) followed by unrealistic expectations of patients (n=, 37.1%) and culture of dominating healthcare professionals (n=99, 34.6%) as well as socially or professionally influential patients who try to influence the doctor (n=99, 34.6%).

Majority (n=103, 36%) of the study subjects believed that both genders were equally liable to show aggressive behavior, and 60.5% (n=173) believed that patients falling in the age group 41 – 60 years were more prone to showing aggressive behavior (Table 2). About 90.2% (n=258) dentists reported that ethnicity of patients did not affect their tendency to show aggressive behavior.

The current study assembled data on aggressive behavior of patients and their attendants towards dental healthcare personnel. The targeted participants were the dental healthcare personal working in different positions and settings of dental clinics/hospitals. The obtained

information can be helpful in designing and planning future strategies regarding management of the aggression of the patients and their attendants in the dental settings. Violent and aggressive behavior of patients or their attendants against dental healthcare professionals poses a serious threat to health and safety at workplace. It not only makes health professionals anxious and tensed, but also diminishes their ability to deliver optimal care to other patients. Very few studies have been published that report the frequency of undesirable encounters of the dental team with patients and their attendants.

Table 2. Age-group more prone to aggressive behaviour among patients.

Age Group	Frequency	Percent
<20 years	6	2.1
21 – 40 years	84	29.4
41 – 60 years	173	60.5
>60 years	23	8

In the present study, majority (45.1%) of the respondents reported facing aggressive behavior at work at least once a week. A study done in UK on postgraduate hospital dentists revealed that 60% dentists had experienced bullying behavior in the last one year (Steadman et al., 2009). This, however, not only included bullying by patients and their attendants but by colleagues, seniors, supervisors and managers. A considerably lower prevalence has been reported by Azodo et al (2011), where majority of the dental health professionals had experienced aggressive behavior only once in the last 12 months. Another study done in Nigeria reported a prevalence of 21.5% violence against dentists and doctors (Abodunrin et al., 2014).

Frequency of violence or aggression directed against females was significantly higher than against male dental healthcare personnel (P<0.001). Although studies have documented female medical healthcare workers as the more common victims of aggressive behavior, (Aydin et al., 2009; Child and Mentes, 2010, Pouryaghoub et al., 2017; Llor-Esteban et al., 2017) no gender predilection in violence against dentists has been reported (Azodo et al., 2011; Steadman et al., 2009; Abodunrin et al., 2014).

In the present study, most of the participants (50%) believed that the most significant reason leading to aggressive behavior of patients was mishandling at the reception/long waiting time followed by patient's unrealistic expectations (37.1%). Interestingly, 50% of the study subjects also believed that their reception manager was not appropriately trained to manage aggressive patients. Similar results have been reported by Azodo et al (2011) and Abodunrin et al (2014), where long waiting time or not being treated on time was cited as the most frequent reason resulting in patient aggression. The aggressive behavior of the patients and their attendants due to long waiting time can further be explained and related to the variations in the complexity and severity of the different treatment modalities in the dental clinics (Tuominen & Eriksson, 2012). These variations make it difficult if not impossible to predict exactly the time duration required for completion of the ongoing dental treatments. Besides there is no universal agreement and no studies reported on the appropriate duration of the various treatments provided in the dental clinics (Jamali et al., 2018), although some researchers suggested different treatment durations for the children of different ages (Aminabadi et al., 2009). However, these suggestions are based on arbitrary assumptions and cannot be generalized and applied for other treatment modalities.

Two interesting factors resulting in aggressive behavior of patients and their attendants were revealed in this study. These included: i. a culture of trying to dominate the healthcare professionals and, ii. patients with social/professional hierarchy who can exercise influence and authority. These factors are more prevalent in the Indian subcontinent i.e. India, Pakistan, Nepal, Bangladesh and Srilanka. Such behavior is perpetuated mainly due to lack of "legal provisions and standards" in these countries that may ensure safety of healthcare workers (Ambesh, 2016; Sharma et al., 2019).

Negative behavior of patients or their attendants, even the use of harsh tone, tends to take a toll on dentist's efficiency. In the present study, 74.5% dental health care providers reported being affected by patient's aggression in one way or another affecting dentists' efficiency and treatment of succeeding patients, while only 24.5% reported not being affected at all. In contrast, 43.2% dental healthcare workers reported feeling "no impact" of patients' behavior in a Nigerian study (Azodo et al., 2011). Results of the present study highlight a need to formulate and implement policy ensuring a health and safety culture for dental healthcare workers. Healthcare workers need to be ensured of safety and should be encouraged while reporting patients' misbehavior, and records must be maintained of such incidents and any ensuing action. Healthcare workers need to be educated how to exercise their rights in order to protect themselves and their practice (Cashmore et al., 2012).

There is also a need to enhance awareness of the general population regarding administration of health facilities especially dental care. Moreover, culture of trying to influence health professionals or the habit to exercise influence owing to one's social status needs to be strongly discouraged. At the moment, there are approximately 25000 registered dental surgeons for a population of 220 million in Pakistan and all those registered are not involved in clinical practice. It is quite evident that practicing dentists are greatly overburdened, are underpaid and in addition, have to face patients' or their attendant's aggressive behavior. Healthcare facilities need to be improved, with delivery of oral healthcare facilities even in rural areas.

The limitation of this study are those inherently associated with survey-based studies. Data derived from these studies may have an element of bias since they are subjective and depend on the truthfulness of the participant. Although the sample size was sufficient, yet the representation of dental surgery staff was inadequate. Also, dental personnel working in government setups where aggression towards healthcare workers and harassment is frequently reported, were not targeted as they were difficult to approach. Future studies should be aimed at including a greater number of healthcare workers from public sector and with an adequate representation of dental surgery assistants.

CONCLUSION

Based on the results of this study, it is concluded that dental healthcare workers in Rawalpindi/Islamabad frequently encounter aggressive behavior from patients and their attendants. Females were targeted significantly more than males (P<0.001). The most common reason of misconduct of patients was mismanagement at reception especially due to long waiting time. Such undesirable encounters with patients decreased efficiency of dentists as well as affected treatment of following patients.

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Evaluation of Some Food Products Produced in Azerbaijan According to the Species Composition and Ecological-Trophic Relations of Fungal Biota

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ABSTRACT

The life activities of living things (plants and animals) used by human beings for food purposes occur in an open system which makes their contact with microorganisms, including fungi, inevitable. As a result, in all products are found either the microorganisms themselves or their metabolites. This leads to a deterioration in the quality and quantity of products. For this reason, to ensure the microbiological safety of products currently used for food purposes is of great importance, in the present work a number of products used for food purposes in Azerbaijan (beef, mutton and chicken, cow's milk, fruits and vegetables) were studied by their species composition and ecological-trophic relationships. It became clear that, studied food products are also one of the habitats of species belonging to different taxonomic groups of fungi. It also became evident that, foodstuffs are one of the habitats of fungi, and in the course of research identified that in the formation of mycobiota of sampled materials involved 63 species of true fungi. Most of the registered fungi (90.5%) belong to sack fungi (Ascomycota), and a small part (9.5%) to zygomycetes (Zygomycota). Among the fungi met both anamorphs (*Aspergillus, Fusarium, Penicillium* and others. species) and telemorphs (G*loeosporium ampelophagum, Monilia fructigena, M.sitophila, Podosphaera leucotricha* and others). Among the registered fungi were identified allergens, toxigens, conventional pathogens, and fungi of whose biotrophy and saprotrophy have not real character. Therefore, in ensuring food safety should be one of today's topical issues inclusion of indicators reflecting both the ecological- trophic relationships of fungi, as well as their ecological- trophic specialization.

KEY WORDS: FOOD MATERIALS, MYCOBIOTA, ECOLOGICAL-TROPHIC RELATIONS, CONVENTIONAL PATHOGEN, ALLERGEN, TOXIGENIC, FOOD SAFETY

INTRODUCTION

As known, the basis of human food consists of products prepared separately from plants, animals, fungi, and bacteria, as well as products made by their participation in various combinations. Although their use changes from time to time (Fernando, 2011), these sources are still on the basis of human nutrition. As the world's population continues to grow, their demand for food is also increasing which creates certain problems in food

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security. It is no coincidence that today, millions of people in many parts of the world are suffering from problems such as food shortages (FAO, 2017). For this reason, the efficient use of existing food sources, as well as the creation of new sources is one of the most important issues of the modern era.

The importance of resolving this issue is related to another point. Thus, in the composition of plant and animal products, which still have a high share in the meeting of people's food needs, also contains substances necessary for the nourishment of microorganisms (Santos Pereira et al., 2019). Almost all plant and animal foods are produced mainly in an open system. For this reason, their contact with microorganisms is inevitable, and in all the produced products are found either the microorganisms or their metabolites (Misihairabgwi et al., 2019).The impact of both microorganisms themselves and their metabolites on human health, as well as on the quality and quantity of plant and animal products, is not always evaluated positively. Therefore, the microbiological safety of raw materials, semi-finished products, as well as finished products intended for food purposes is of great importance, (Makinde et al., 2020).

The primary task in clarifying these issues is considered to be characteristic of the microbiota of food products in terms of number and species composition, as well as the ecological and trophic relationships of the species involved in the formation of this microbiota. Thus, in order to solve any problem, initially, it is necessary to accurately identify its "participants". Extensive research has been conducted to evaluate microbiological, especially bacterial biota of materials intended for food purposes and some related issues have been clarified (Moradali and Rehm, 2020). Stages of production of plant and animal products for food purposes from production to use usually occurs under non-sterile microbiological conditions and therefore from a taxonomic point of view microorganisms, especially fungi, are considered their natural contaminants (De Borba et al., 2020). However, there is not enough research to evaluate the materials intended for food purposes for fungal biota, and there are still many issues that need to be addressed. Therefore, the purpose of the present work was dedicated to the assessment of the species composition of fungi involved in the formation of mycobiota of some plant and animal materials intended for food purposes and to the manifestations of their ecological-trophic specialization.

MATERIAL AND METHODS

Materials for the study were taken from plant (fruits such as apples, pears, grapes, pomegranates, cherries, etc., and vegetables such as tomatoes, cucumbers, cabbage, eggplant, etc.) and animal origin materials (beef, mutton and chicken, cow's milk) intended for food purposes in Azerbaijan. These materials were taken from products sold to people wholesale and retail, and raw materials imported to process. Sampling, certification, and preparation for laboratory analysis were carried out in accordance with the methods and approaches intended for this purpose (Handbook of Mycological Methods, 2006, Neusely da Silva et al., 2018). To separate the fungi from the samples were used from mediums such as Saburo agar, wort -agar, and agarized Czapek. To obtain pure cultures and determined their species composition were used from known determinants (Kirk et al., 2008, Satton et al., 2001).

Table 1. Taxonomic structure of the species involved in the formation of mycobiota of the studied food origin materials

Sample materials	Total number of registered species	Mycota	
		Zygomycota	Ascomycota
Beef	20	2	18
Mutton	17	2	15
chicken meat	23	3	20
Cow's milk (freshly)	12	2	10
Fruit	37	4	33
Vegetables	31	3	28
Total	63	6	57

In the naming of fungi were used from internationally accepted and widely used principles and approaches (Pedro W. Crous et al., 2015). Although the clarification of ecological and trophic specialization of fungi was carried out mainly taking into account the literature, the toxicity of some species has also been identified for both plants (tomatoes, cucumbers, and wheat) and infizor. During carried out this work were used from the methods and approaches used in our previous work (Bakshaliyeva et al., 2020, Yusifova et al., 2020).

RESULTS AND DISCUSSION

During the analysis of fungal biota of plant and animal origin products intended for food purposes determined that in the formation of their mycobiota mainly participants real fungi (Mycota) that the information on their taxonomic structure summarized in the Table 1. As seen, 63 species (*Alternaria alternat, A.mali, A.solani, A.tenuissima, Aspergillus flavus, A.fumigatus, A.niger, A.ochraeus, A.repens, A. terreus,*

A.versicolor, Botrytis cinerea, Candida alpicans, Chaetomium cellulolyticum, Cladosporium cladosporides, C.herbarum, Coniothyrium diplodiella, Debaryomyces hansenii, Endomuces vernalis, Fuzarium moniliforma, F.oxysporum, F.semitechium, F.solani, Geotrichum candidum, Gloeosporium ampelophagum, Gloeosporium fructigenum, Guignardia bidwellii, Monilia fructigena, M.sitophila, Mucor hiemalis, M.mucedo, M.rasemous, Paecilomyces variottii, Penicillium camemberti, P.citrinum, P.chrysogenum, P.cuclopium, P.decumbens, Penicillium digitatum, P.funiculosum, P.expancum, Penicillium glaucum, P.purpurogenium, Phoma rostrupii, Ph.uvicola, Phyllosticta mali, Podosphaera leucotricha, Rhizobus nigricans, Rh.stolonifer, Saccharomyces cerevisia, Saccharomyces vini, Sclerotinia fructigenum, Sclerotinia libertiana, Sporotrichum camis, Stachybotrys chartarum, Thamnidium elegans, Trichoderma lignorum, T.viride, Trichothecium roseum, Torulopsis candida, Venturia inaequalis and *Verticillium dahliae* and *Yarrowia lipolytica*) of fungi takes part in the sample food materials, most of which (90.5%) belongs to the sac fungi(Ascomycota) and a small proportion (9.5%) to the zygomycetes (Zygomycota). Among the fungi met both anamorphs(*Aspergillus, Fusarium, Penicillium* and others. species) and telemorphs (*Gloeosporium ampelophagum, Monilia fructigena, M.sitophila Podosphaera leucotricha*, and others).

It is known that between fungi and other living things, including plants and animals have different relationships. Sometimes, depending on the form of this relationship, the nature of the functions performed by fungi also changes. For this reason, the characterization of fungi from this aspect was of interest both from a scientific and practical point of view. When characterizing the recorded fungi from this aspect, became clear that among the recorded fungi, true biotrophs were not found, saprotrophs made up only 11.1% of the total fungi. The reason why not found real biotrophs was that they are not biologically alive, although all of the sampled materials belonged to living things, (Naranjo-Ortiz and Gabaldon, 2019).

Table 2. Characteristics of fungi species by the manifestations of ecological-trophic specialization recorded in the analyzed materials.

Analyzed products	Total	Number of fungi species, including			
		Conditional pathogens (%)	Allergens (%)	Toxigens (%)	Those whose status is unknown (%)
Beef	20*	25,0	30,0	55,0	20,0
Mutton	17	23,5	29,4	52,9	29,4
chicken meat	23	26,1	30,4	56,5	26,1
Cow's milk (freshly milked)	12	16,7	25,0	41,7	58,3
Fruit	37	16,2	27,0	51,3	18,9
Vegetables	31	25,8	32,3	48,4	22,6

Note: * - some fungi have a dual, some a triple (allergen, toxigen, and conventional pathogen) feature, for this reason, the sum of the data in % in the table is more than 100

Fungi also differ by ecological-trophic specialization - toxigenic, allergenic, conditionally pathogenic. Characterization of fungi from this aspect is also important in terms of biosafety and hygienic requirements for the nutritional value of food materials of both plant and animal origin. When characterizing the registered fungi from this aspect, became clear that among the registered fungi there were species that have been confirmed to be toxigenic, allergenic, and conditionally pathogenic, and their specific gravity was significant (Table 2). As seen, the materials differed from each other in these respects. Thus, the specific gravity of conventional pathogens was found in vegetables, the specific gravity of allergens in chicken meat, and the specific gravity of toxigens in fruits.

As noted, plant and animal foods play an important role in the human diet, and today there is no alternative source that can replace them. Production of almost all food products, transportation and storage of finished products, and other processes carried out under conditions not fully compliant with microbiological sterility (Muradov et al., 2011). The composition of various nutrients rich in various organic and inorganic substances. These nutrients suitable not only for humans, also for other living things. Therefore to develop food safety principles one of the very important issues. From the obtained results became clear that materials that have been researched and widely used for food purposes in the world, including Azerbaijan, are no exception in this regard. All of them characterized by one of the places where fungi were found. On the other side, from the obtained results became clear that animal products characterized by a lack of fungal biota compared to plant-based foods (Table 1).

Thus, the number of species of fungi involved in the formation of mycobiota in beef was 1.85 times less than in fruits, and 1.55 times less than in vegetables. Similar comparisons with other products are always

in favor of plants. This is due to the predominance of polysaccharides among the components of plants and the fact that it is a more suitable food source for fungi. Among the reasons for the widespread spreads of fungi in plant materials the acidity of the environment also plays a role. Thus, the acidity of meat is neutral and high (towards alkalinity), while that of fruits and vegetables is generally neutral and low (towards acidity). An acidic environment is more conducive to the growth of fungi, which was confirmed in our previous studies (Bakshaliyeva et al, 2020).

Based on the interaction of fungi with other living things formed over many years stands their attitude to food, and ecological-trophic features (Naranjo-Ortiz and Gabaldon, 2019). Many studies have confirmed the importance of this approach, both in terms of the functions performed by fungi in their habitat, as well as in terms of assessing the nutritional value of the substrates with which they come into contact (Snyder et al. 2019). From the results carried out of our research became clear that although a wide range of fungi does not participate in the formation of mycobiotas of plant and animal origin food materials, the predominance of polytrophs among them can be assessed as a negative case. Thus, the adaptability of polytrophs especially in terms of meeting their food needs higher than other ecological groups (true saprotrophs and biotrophs), which allows them to more widespread.

The specialization of fungi from the point of view of ecological-trophic relations also different (Richards et al., 2017) and this does not manifest itself in all fungi. Thus, the form of expression of the ecological-trophic specialization of fungi manifests itself in the forms of conditional pathogenicity, allergenity, and toxigenicity. Fungi complying with this characteristic participates in the mycobiota of studied animal and plant food materials and their specific gravity sometimes more than 50% (Bakshaliyeva, 2017). There are enough research materials about the negative impact of fungi that meet this characteristic on the health of other living things, especially humans. However, the sanitary-epidemiological rules and regulations adopted in many countries, including the Republic of Azerbaijan, do not contain indicators regulating the activity of these fungi.

Therefore, the inclusion of indicators aimed at ensuring food safety should be one of today's topical issues. It would be useful to explain our opinion with the information obtained about toxigenic fungi. So that, the number of fungi species that synthesize toxic substances is more than 300, and the number of mycotoxins they produce is more than 500 (Cinar and Onbashı, 2019). Mycotoxins are toxic secondary metabolites produced by various filamentous fungi, of which Fusarium, Aspergillus and Penicillium are the three main genera (Greeff-Laubscher et al., 2020).

With the development of science and technology, the probability that this number will increase is real, and among the mycotoxins synthesized by toxigenic fungi known to science today are those that adversely affect human health in any concentration (Ogunade, 2018). Therefore, the permissible number of fungi in food substances should be specified on the basis of specific groups, but not in generally. Since in similar documents in many countries this does not exist. In this regard, it is necessary to pay special attention to the fact that some fungi carry all of the mentioned features.

For example, in research has been confirmed that A. niger has all the mentioned features.It is impossible to give a definitive opinion about some of the fungi isolated in studies because literature data was not found about on their characterization according to their ecological-trophic specialization in this or that research. On the other side, although some of them have phytotoxic activity against plants, but they do not show such a feature against to infizors. For this reason, it is not possible to give an unequivocal opinion about their status, and in the study was considered expedient recorded their status as groups of unknowns (Table 2) and to clarify this in future studies.

CONCLUSION

Thus, various meats, fruits, and vegetables intended for food purposes in the Republic of Azerbaijan, have been characterized as one of the places for feeding and habitats of fungi. It was determined that in the formation of mycobiota of sampled food materials involves the species of fungi characterized by diversity both in terms of ecological-trophic relations and forms of its specialization. The presence of toxigens, allergens, as well as opportunistic pathogens among the registered fungi, allowed to emphasize the need specification of indicators regulating the activity of this type of fungi for the adoption of the sanitary-epidemiological rules and regulations related to food products.

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Reproductive Toxicity of Gemcitabine on Breeding and Fertility in Male Albino Rats

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ABSTRACT

Although chemotherapy for malignancies is highly effective, their related gonadotoxic side effects may severely impair fertility and cause gonadal toxicity in male patients.The aim of the present work was to investigate influence of Gemcitabine toxicity on reproductive system of albino male rats (breeding and fertility tests). Animal experimental study conducted in zoology department, College of science, King Saud University during period from June to October 2014 using albino rats (Rattusnorvegicus) (Wistar strain). Males were divided into four different groups (control" 0 mg/kg",7 mg/kg,14 mg/kg, and 21 mg/kg).Male fertility index, female fertility index, pregnancy index, number of pregnancies per pregnancy and total number of births decreased in all groups treated. Mean number of implantation sites per female and the implantation index also decreased significantly ($p \leq 0.05$) and the rate of paradise loss before implantation increased at both doses 14 and 21 mg / kg, while the rate of loss of fetuses after implantation also increased. The histological examination in both the testis and the epididymis showed that they were significantly affected at the level of the three doses, and the effects ranged between moderate and severe. Histological examination of testis segments at a dose of 7 mg / kg showed atrophy of spermatogenic epithelial degeneration in many seminal tubules in most animals. Fluid accumulation was observed in some cases and sperm retention. It is concluded that the drug can be considered highly toxic to the male reproductive system, and despite the severity of the observed effects, it has been recovered to a large extent.

KEY WORDS: FERTILITY-GEMCITABINE-RATS-SPERMS-TESTIS-TOXICITY.

INTRODUCTION

The incidence of cancers commonly diagnosed in the adolescent and young adult population, including Hodgkin and non-Hodgkin lymphoma, acute lymphocytic leukemia and testis cancers, is on the rise worldwide (Okada & Fujisawa, 2019, Chan, 2013), Siegel et al., 2016). Simultaneously, the latest combination chemotherapy treatments provide the safety and efficacy and have improved the survival rates of these patients to more than 75%–90%, making them more able to be fathers and form family. Even that chemotherapy and radiation therapy for malignancies are highly effective, their related gonadotoxic side effects possibly will severely impair fertility in agent- and dose-dependent manners and may cause impermanent or permanent gonadal toxicity in male patients (Wong et al., 2009). Where 24% of the cases suffered from persistent azoospermia or severe oligozoospermia. On the other hand, a n important question was presented about the efficacy of post-therapy spermatozoa for conception, either naturally or through assisted reproductive technologies, where, many of the survivors have a complete return of sperm production.

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Gemcitabine (GCB) is a pyrimidine antimetabolite exceedingly used in various solid tumors as a single treatment or as a component of multidrug plans (Okada & Fujisawa, 2019).

Most patients have a good tolerance for GCB, however, sometimes life-threatening complications occur. where, the main side effects were laboratory variations (mild proteinuria transaminase elevation, myelosuppression, and hematuria) (Chan, 2009, Siegel et al., 2016). The main factors raising GCB toxicity were; alcohol abuse, combination with platinum derivatives or taxanes, and liver and kidney diseases (Trost & Brannigan, 2012, Hryciuk et al., 2018). There is no obvious evidence about the dose amendment of GCB, and clinical decisions are mainly made depend on experimental bases. The aim of the present study was to investigate the influence of Gemcitabine toxicity on reproductive system of albino male rats (breeding and fertility tests).

MATERIAL AND METHODS

This animal experimental study was conducted in zoology department, College of science, King Saud University, Saudi Arabia. The study was conducted during the period from the beginning of June to the end of October 2014.

Animals: Sexually mature male and female albino rats (Rattus norvegicus) of the Wistar strain, with their ages ranged between 8-10 weeks and weights of 220-250 gm, were obtained from animal house, College of Pharmacy, King Saud University.

Drugs: Gemcitabine is available from the manufacturer in packages containing either 200 mg or 1 gram of salicetabine hydrochloride prepared with mannitol (200 mg and 1 g respectively) and sodium acetate (12.5 mg and 62.5 mg respectively) (Casciato, 2004, Eli Lilly and Company, 2007). It was dissolved in a 0.9% solution of sodium chloride and remained stable for 24 hours at room temperature. The drug was not cooled in the refrigerator after it was thawed, to avoid any crystallization, (Eli Lilly and Company, 2006, Mini et al., 2006).

Experimental Design: Animals were dealt with and the various experiments and tests were designed in general according to the guidelines and standards used in estimating the toxicity of chemical compounds on the reproductive system (Clegg et al., 2008). Males were divided into four different groups (control" 0 mg/kg",7 mg/kg,14 mg/kg, and 21 mg/kg). Each group of the four groups included 20 rats, which were divided into two subgroups, each of which included 10 rats, so that the different tests were applied to the first subgroup immediately after the end of the treatment period (9 weeks) in order to find out the harm caused by the drug, while different tests were applied to the group. The second subset after one of the spermatogenesis cycles has passed, with the aim of knowing whether or not the reproductive system functions recover and return to a normal state.

The animals of each group were injected into the peritoneal cavity "IP" once a week for a period of 9 weeks. The control group was injected with a physiological solution, while the three treated groups were injected with a physiological solution in which the drug was dissolved according to the specified dose. During the injection period, the animals were monitored daily in order to follow up the appearance of disease signs resulting from drug toxicity or mortality, and their weights were recorded at the beginning of the experiment and then weekly. Immediately after the end of the injection period (10 males from each group) or after a complete sperm cycle equivalent to nine weeks (10 males - recovery group), then the males were mated with healthy females at a rate of (1: 2).

The males were sedated after the end of the treatment period and mating with ether, and then killed, and blood was collected from the arterial stem in the neck and left to coagulate at room temperature for an hour, then the serum was collected after centrifugation at 3000 rpm for 20 minutes and kept at -80 ° C. To measure testosterone concentration at a later date (Foster & Harris, 2005). After killing males and collecting blood, the abdomen was incised and the internal organs were fully exposed and examined, and any anatomical or pathological changes were recorded. The organs of the reproductive system consisting of the testes, epididymis, prostate gland, seminal vesicles, and associated clotting glands were removed, and the adipose tissue attached to them was trimmed and weighed all. The weight of the different organs was expressed as absolute weight and as relative weight to body weight, which was calculated according to the formula (member weight / body weight x 100) (Andrade et al., 2002, Yu et al., 2009). The left testis and left epididymis were used to assess the toxic effect of the drug on histopathology.

In order to determine the effect of treating male rats with the drug gemcitabine on their fertility and on the resulting offspring, they were mated immediately after the end of the treatment period with untreated females, at the rate of 2 females for each male. After that, half of them were killed on day 20 of pregnancy and the other half were left to complete the normal pregnancy period and give birth to their young, which were followed up until the end of the breastfeeding period, and the various indicators of male fertility, rates of fetal loss, birth survival, and the incidence of abnormalities, which in general give a complete picture of Condition and efficiency of the reproductive system after treatment with the drug.

Statistical Analysis: The obtained data were represented as mean ± standard error of mean ± SE and a significant level of P 0.05 and P 0.01 was adopted. For the statistical analysis of the data, both SPSS version 16 and SigmaStat version 3.5 were used. Data for the mating index, fertility

index for males and females, and pregnancy index were analyzed using chi-square, and in case of differences between groups.

RESULTS AND DISCUSSION

For groups treated with Gemcitabine and identified to study recovery from the toxic effects of the drug, the various external observations observed during the drug treatment period disappeared and the animals returned to normal. After the autopsy and examination of the internal organs, all were healthy except for atrophy and redness of the lung in one of the subjects of the higher dose and the fibrosis of some lung lobes in one of the members of the medium dose and one of the members of the lower dose. It was also noted that the testicles atrophied in one of the subjects in the third dose and their stature was soft compared to the normal state.

Table 1 shows the different effects of the drug gemcitabine on the different indicators of mating and fertility and on the offspring resulting from mating the drug-treated males with untreated females. It is evident from the data of this table that the mating index was not affected by the drug-treated males, as it recorded values equal to that of the control group. As for the male fertility index, it decreased in all groups treated with gemcitabine, and in the 7, 14 and 21 mg / kg doses treated groups, 70%, 70% and 66.67% were recorded, respectively, compared to 90% in the control group. It is evident from these data, as well, that there is a significant decrease in the female fertility index and the pregnancy index. Where the female fertility index decreased, and at doses 7, 14 and 21 mg / kg, the percentage was 55%, 50% and 50%, respectively, compared to 90% in the control group, and the pregnancy index at the same doses scored 68,75% and 55,55. % And 56,25%, respectively, compared to 90% in the control group. The number of pregnancies per pregnancy also decreased at all doses, but this decrease was not significant only in the 14 mg / kg group (p 0.05).

Table 1. The effect of gemcitabine on different mating indicators, autopsy results of pregnant females, and birth data immediately after the end of treatment (Mean ± SE).

Parameters	Dose			
	Control	7 mg	14 mg	21 mg
Male mating index	100	100	100	100
Male fertility index	90	70	70	66.7
Female fertility index	90	55*	50**	50**
Pregnancy index	90	68.75	55.55*	56.25*
Caesarean section data				
Litter size/dam	11.1 ± 1.34	7.71 ± 2.31	5.71 ± 1.06*	8.67 ± 2.17
Implantation sites/dam	13.3 ± 0.52	10.29 ± 1.79	8.86 ± 1.86*	8.83 ± 2.19*
Dead fetuses/litter	0	0	0.14 ± 0.14	0
Resorptions/litter	2.2 ± 1.33	2.57 ± 1.41	3.14 ± 1.39	0.17 ± 0.17
Corpora lutea/dam	14.5 ± 0.7	15.43 ± 0.87	13.71 ± 0.81	15.83 ± 1.01
Implantation index	92.52 ± 2.76	70.33 ± 13.36	62.46 ± 10.74*	58.82 ± 15.41*
% Preimplantation loss/litter	7.48 ± 2.76	29.67 ± 13.36	37.54 ± 10.74*	41.18 ± 15.41*
% Postimplantaion loss/litter	16.05 ± 9.48	26.21 ± 14.92	36.5 ± 13.64	1.52 ± 1.52
Fetal body weights (g)	4.00 ± 0.17	4.33 ± 0.3	4.33 ± 0.25	4.46 ± 0.2
natural delivery data				
Total no. of delivered pups	95	42	38	29
Live pups delivered/litter	10.56 ± 0.96	8.4 ± 2.16	9.5 ± 2.33	9.67 ± 1.33
Live birth index	92.9 ± 4.14	100	96.43 ± 3.57	100
pups body weights at PND 0(g)	5.72 ± 0.12	6.61 ± 0.44	5.89 ± 0.25	6.50 ± 1.04
4-days survival index	95.9 ± 2.18	75.00 ± 25.0	80.72 ± 11.04	100
pups body weights at PND 4(g)	9.11 ± 0.41	9.24 ± 0.39	9.40 ± 1.64	9.82 ± 0.78
weaning index	90.87 ± 6.27	92.86 ± 7.14	100	100
pups body weights at PND 21(g)	38.44 ± 1.43	41.95 ± 2.54	44.08 ± 7.13	41.14 ± 3.03
Sex ratio (% males/litter)	52.7 ± 4.3	58.5 ± 2.3	43.85 ± 2.7	49.0 ± 3.9
Externally malformed fetuses/litter	0	0	0	0

* Significantly different from control (p ≤ 0.05).PND (Postnatal Day).

The mean number of implantation sites per female also decreased significantly (p ≤ 0.05) at both doses 14 and 21 mg / kg. There was no significant change in the number of dead or absorbed embryos per pregnancy. Regarding the implantation index, it was significantly decreased (p 0.05) at each of the 14 and 21 mg / kg doses, and the

rate of paradise loss before implantation for these two groups also increased significantly (p 0.05), while the rate of loss of fetuses after implantation also increased. No significant increase was recorded. There was no effect on the different birth indicators nor on the proportion of males in each pregnancy, and no external abnormalities were recorded in any of the pregnancies or births, but the total number of births was significantly reduced in the drug-treated groups compared to the control group. (Table 1)

Regarding the drug recovery group, it is noticed from Table 2 that all indicators affected by the drug return to levels equal to or close to the control group levels, and no significant differences were observed on any of these indicators or measurements except the number of live births at the dose of 14 mg / kg. (Table 2)

Table 2. The effect of gemcitabine on different mating indicators and birth data in the recovery group after a complete spermatogenic cycle after cessation of treatment with the drug (Mean ± SE).

Parameters	Dose			
	Control	7 mg	14 mg	21 mg
Male mating index	100	100	100	100
Male fertility index	80	80	75	80
Female fertility index	80	80	75	80
Pregnancy index natural delivery data	80	80	75	80
Total no. of delivered pups	106	89	59	98
Live pups delivered/litter	13.25 ± 0.53	11.13 ± 0.72	9.83 ± 1.4*	12.25 ± 0.59
Live birth index	100	100	100	100
pups body weights at PND 0(g)	5.63 ± 0.11	6.15 ± 0.21	6.16 ± 0.35	5.42 ± 0.13
4-days survival index	98.33 ± 1.09	98.96 ± 1.04	100	100
pups body weights at PND 4(g)	8.28 ± 0.27	8.82 ± 0.35	9.44 ± 0.81	8.15 ± 0.48
weaning index	73.44 ± 4.38	65.40 ± 9.60	72.32 ± 9.90	55.36 ± 5.36
pups body weights at PND 21(g)	41.67 ± 1.18	41.86 ± 2.08	45.82 ± 2.79	41.01 ± 5.60
Sex ratio (% males/litter)	48.39 ± 5.44	46.89 ± 7.84	54.07 ± 4.78	56.59 ± 6.99
Externally malformed fetuses/litter	0	0	0	0

*Significantly different from control ($p \leq 0.01$).
PND (Postnatal Day)

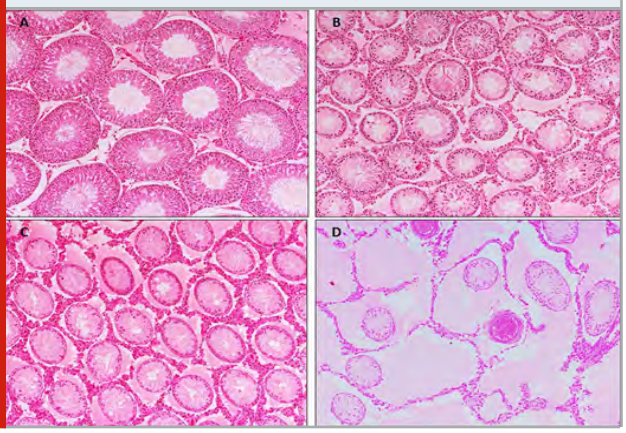
The histological examination of the testis and epididymis segments in the control group showed in the dissected animals immediately after the end of the treatment, as well as that of the morgue after the passage of time after the cessation of the treatment of normal tissue composition. All types of germ cells appeared naturally organized inside the seminiferous tubules, and Leydig cells appeared distributed in the tissue areas between the seminiferous tubules with the blood vessels. In the epididymis, the tubules appeared naturally in terms of cellular composition of tubule walls, interstitial tissues, and sperm content of the tube lumen. As for the animals treated with the drug, the histological examination of the histological sectors in both the testis and the epididymis showed that they were significantly affected by the treatment with the drug at the level of the three doses, and the effects ranged between moderate and severe between the members of the single treatment group and between the three doses. Also, these effects continued, to varying degrees, even after some time had passed since treatment with the drug was discontinued in animals of the recovery group from the effect of the drug.

Histological examination of testis segments at a dose of 7 mg / kg showed atrophy of spermatogenic epithelial degeneration in a large number of seminal tubules in most animals. Most of the histological changes observed in this group can be summarized as follows: Some seminiferous tubules appeared irregular epithelia, missing some types of germ cells. Presence of seminal tubules devoid of all cells except for Sertoli cells and very few spermatogonia. The lumen of some seminiferous tubules contained cellular necrotic materials, and residues of a darker pigment, Eosinophilic debris. Some seminiferous tubules contained cells with pyknotic nuclei and evidence of these cells' degradation. Separation of spermatocytes and spermatids from the epithelialization of the seminiferous tubules as a sign of the onset of the process of germ cell sloughing or exfoliation and the formation of cell masses within the lumen of the tube. Vacuoles form in Vacuolation of sertoli cells. Retention of elongated spermatids (Step 19 Step 19) occurs at the lumen of the tubules and near the basement membrane of the seminiferous tubules in stages IX - XII. The presence of elongated spermatids in an irregular position and scattered unlike the normal state.

At a dose of 14 and 21 mg / kg, the atrophy of a very large number of the seminiferous tubules was observed, and in some cases the atrophy of all the seminiferous tubules

in the transverse sections examined. In most cases, these tubes contained only Sertoli cells or a few spermatogonia, and in some cases there were some sperm cells. Among the most prominent histological changes observed, in addition to the above, are the following: They form Giant multinucleated cells. In some cases, hyperplasia was observed in Leydig cells, where their number was observed to increase at a rate more than normal. Dilated interstitial tissue spaces due to the accumulation of an amount of fluid in some cases. Edema. Too large shrinkage of some seminiferous tubules. The basement membrane of the seminiferous tubules is thickened. In some cases, at a dose of 14 mg / kg, hematopoietic infiltration was observed in the interstitial tissue, where red blood cells were observed outside the vessels, and in one of the members of this group heavy bleeding occurred, and the interstitial tissue was observed which was filled with red blood cells. Regarding the treatment group, the histopathology was observed in most of the cases examined in the three doses to a position very close to the normal structure, with a small number of seminal tubes containing only Sertoli cells and a few spermatogonia. However, there remained a small number at different doses in which the seminiferous tubules did not recover, as they were seen in a state of semi- or partial atrophy. Fluid accumulation was observed in some cases and sperm retention (Figure 1).

Figure 1: Sections of testis of a rats from the four groups.
A: Section of testis of a rat from the control group shows a group of seminiferous tubules at different stages of the seminiferous epithelial cycle (H&E, x 100).
B: Section of testis of a rat at dose of 7 mg shows most of the seminal tubules atrophied (H&E, x 100).
C: Section of testis of a rat at dose of 14 mg shows atrophy included all the seminiferous tubules, with only the spermatogenic cells remaining in most of them. (H&E, x 100).
D: Section of testis of a rat at dose of 21 mg shows a significant expansion of the interfacial area occurred as a result of fluid accumulation(edema). All the seminiferous tubules were atrophied and their cavities blocked due to the extension of the Sertoli cell growths. (H&E, x 100).



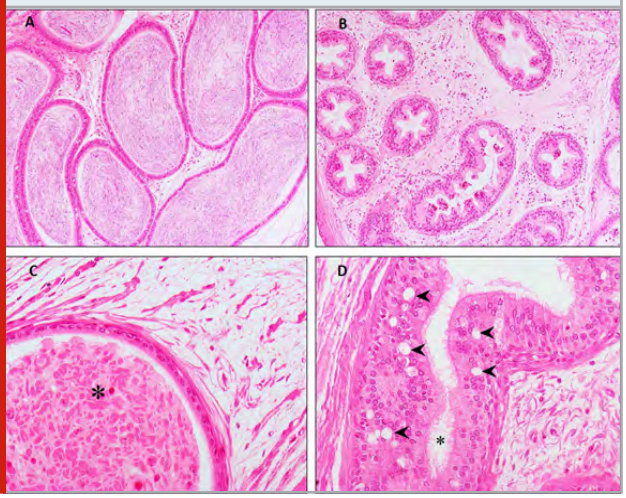
Histopathological examination of epididymal segments showed the effect of treatment with the drug gemcitabine, which caused significant damage to the epididymal tubes, especially in the lining epithelial cells. The tail of the epididymis was the most affected part, while the head of the epididymis was not significantly affected, and its epithelial cells appeared almost similar to their counterparts in the control group except that they contained few or no sperms and an increased number of damaged cellular remnants within them. The tissue damage seen in the tail of the epididymis can be summarized as follows: Thickened tubular epithelium, which is composed of several layers of Stratified epithelium, and sometimes the cells appear to be very high. Absent or low sperm count inside the epididymal tubes.

The presence of remnants of immature germ cells, abnormal germ cells, and largely necrotic cells in the tube lumen was observed. Significantly reduced diameter of the epididymal tubes. The presence of a relatively large amount of connective tissue that surrounds the epididymal tubes, especially fibroblasts, and the space between the tubes seemed to be wider than in the normal case. Significant damage to the epithelium and the emergence of gaps sometimes. Cribriform changes. Abnormal material in the lumens of the epididymal tubes. White blood cells appear near some tubes as an indicator of Chronic inflammation.

In the recovery group, the histological structure of the epididymis returned to its normal position in most animals, especially the epithelialization of the epididymal tubes, and only an increase in the percentage of cellular remnants was observed in some cases, as well as the absence of spermatozoa in animals in which the testicles had not regained their normal structure. In some cases, these tubes appeared to be heavily filled with material stained by the PAS stain, and sometimes the epithelium was seen to be folded and with many vacuoles (Figure 2).

The current study aimed to investigate the toxic side effects of one of the most prominent relatively new anti-cancer drugs on the reproductive system of male rats, as an animal model through which similar effects can be predicted on humans in the absence of any information or studies dealing with that. It is known that the process of spermatogenesis is similar to a large extent in many of its characteristics between humans and experimental animals, especially mice and rats (Meistrich, 2013).The mating index is a reliable measure of normal sexual behavior and the presence of sexual desire, and it also provides indirect information related to the function and state of the hypothalamic-pituitary-epiphysis axis (Holson et al., 2006). This indicator can be affected by many factors, including physical damage, acute intoxication, or changes in the neuroendocrine-gonadal axis that affect sexual desire or hormonal balance (Parker, 2006).

Figure 2: Sections of epididymis of rats from the four groups
A: Section of a rat epididymis of the control group. Note the normal structure of the epithelial layer of the epididymal tubes, the filling of the epididymal tubes with sperm and the normal volume of the interstitial tissue. (H&E, x 100).
B: Section of a rat epididymis at dose of 7 mg. Note the significant contraction of the epididymal tubes, the absence of sperm, the fold of the epithelium, and the expansion of the interstitial space. (H&E, x 100).
C: Section of a rat epididymis at dose of 14 mg. Note that the epididymal tube lumen is filled with abnormal contents and dissolved eosin-stained cellular residues (the star). (H&E, x 400).
D: Section of a rat epididymis at dose of 21 mg. Note the formation of vacuoles in the epithelium of the epididymal tubes (arrowheads), their detachment and pushing towards the tube lumen (the star) causing near closure. Note also that the tube is empty of sperm. (H&E, x 400).



The results of the current study indicated that there was no change in the mating index due to the treatment with the drug gemcitabine, and this indicates that the drug did not inflict any harm on sexual behavior as a result of not affecting the hormonal neurogenic gynecological axis. The Male Fertility Index measures their ability to produce sperm that can induce pregnancy in females. Because the occurrence of mating does not necessarily imply the occurrence of pregnancy, this indicator provides additional and valuable information. Fertility index can be affected by many factors like those that affect the mating index, in addition to several other factors, including the process of sperm maturation, transfer through the reproductive tracts, and their ability to fertilize eggs. The pregnancy index measures the ability of females to reach pregnancy (Parker, 2006).

Pregnancy success is considered the most effective measure of an animal's ability to produce gametes, mate and produce live offspring (Dixon, 1986). This indicator is affected by the same factors that affect the fertility index in males (Parker, 2006). The results of the current study showed that the treatment of males with the drug Gemcitabine caused a significant reduction in both indicators of fertility and pregnancy compared to the control group.

A 90% reduction in the sperm available for ejaculation in rats and other laboratory animals by surgery does not affect fertility (Amann, 1986), and if chemical treatment leads to the same decrease in the number of sperm ejaculated during mating, the changes in reproductive function will not be Sufficient to cause a significant decrease in fertility or pregnancy size ((MEISTRICH, 1982); (Keller, 2006)). However, despite the observed decrease in the fertility index at all doses used in the current study, the number of sperms in the epididymis did not decrease in the drug-treated groups below the limit affecting fertility except in the group treated with the large dose, which confirms that this decrease in fertility is a result of Basically, the quality of the sperms available for ejaculation is low, as indicated by the results of estimating their motility and the percentage of their abnormalities. This is supported by the results of the study conducted on 5-azcytidine.

In rats treated at a dose of 4 mg / kg for 11 weeks, the drug reduced the number of sperms in the epididymis to more than 90%. However, these animals were still able to mate and fertilize eggs. Most of the resulting embryos were abnormal as was proven by examination on the second day of the occurrence of pregnancy, and they also died before implantation (Doerksen & Trasler, 1996). Loss before implantation represents the number of eggs that were not fertilized or that was fertilized but was lost before implantation. It was evident from the results of the current study that the treatment of male rats caused an increase in the pre-implantation loss rate at the level of all the doses used, although the statistical analysis did not indicate its significance except at the medium and large doses. Any defect in the genome coming from the father as a result of exposure to chemicals may have severe consequences on the vitality and growth of the embryos (Kelly et al., 2003).

Interpretation of the loss prior to implantation may require information on the extent to which the agent induces mutations (Zenick, H. and Clegg, 1989). In order to determine the cause of the increased loss before implantation, additional studies must be conducted, including direct examination of fertilized eggs and early embryos. It must also be realized that the loss of embryos before and after implantation occurs naturally in untreated rodents as is the case with treatment, which contributes to the natural variation between the number of births per pregnancy (Parker, 2006).The number of embryos per pregnancy and the number of live births are influenced by the number of eggs available for fertilization, the fertilization rate, the implantation rate, the percentage of implanted embryos that survive the due date, and sperm measurements such as movement and number (Holson et al., 2006). The weight of the newborns after birth and throughout the growth period, as well as their survival rates, depend on their weight at birth, on

gender, on the natural formation of the individual, on the number of births per pregnancy, and on the ability of the newborn to breastfeed. Any defect in these indicators may indicate the effect of the toxic agent on one of these factors (Parker, 2006).

Determination of sex in mammals depends on the male through fertilization of the egg with a sperm that carries either of the Y or X chromosomes. Therefore, an effect on the production of a specific type of them or in its transmission through the reproductive tracts or in its ability to fertilize may result in a change in the sex ratio. There are also influences that may cause selective loss of one of the sexes, or they may have an effect on the external appearance by interfering with the process of growth of the reproductive system and thus lead to a change in the sex ratio in the births or the production of births bearing the characteristics of both sexes (Parker, 2006).

The tissue structure of the testis is the most sensitive indicator for detecting reproductive toxicity (Parker, 2006). It was observed in this study the occurrence of significant tissue damage in both organs as a result of treatment with the drug gemcitabine. Histological manifestations of the damage caused by anti-cancer drugs are characterized by depletion of germ cells, and most tissue sections show complete loss of them, as the seminiferous tubules appear to be devoid of all germ cells except for Sertoli cells, and sometime there can be a few sporadic spermatogonia, sperm cells and spermatogonia. The seminiferous tubules appear atrophied while the Leydig cells remain normal in appearance (Schilsky et al., 1980).

In addition to the manifestations of previous tissue damage, the histological observations recorded in this study also included a failure in sperm release and retention in the later stages of the spermatic epithelial cycle, the appearance of vacuoles in Sertoli cells, the formation of multinucleated giant cells, and the occurrence of hemorrhage in the interstitial tissues. The accumulation of fluid in the interfacial tissue. The histological composition of the epididymis was also affected, and the epithelium lining its tubes was bent and thicker than normal. Sometimes these cells separated and headed into the tube lumen. The accumulation of foreign substances positive for the periodic acid-Schiff stain was observed that filled the cavities of the epididymal tubes.These histopathological observations are consistent with the tissue damage caused by anticancer drugs that has been reported by several studies (Kelly et al., 2003, Oakes et al., 2007).

Regardless of the initial site of damage, most testotoxins will cause germ cell lysis and a decrease in their number. If the effect is severe or lasts for a long time, the end result will be a seminiferous tubule containing only Sertoli cells. Although Sertoli cells are very sensitive to dysfunction, they are exceptionally resistant to cell death (Creasy, 2001). The formation of vacuoles in Sertoli cells is one of the most prominent morphological responses to damage, and optical microscopy does not provide an opportunity to determine whether these vacuoles originate within them or between adjacent cells (Russell et al., 1991). The vacuole formation is followed by germ cell lysis and its irregularity or detachment, and the normal separation of the cells indicates the primary effect on the intercellular connections between the germ cells and Sertoli cells. Despite the severe effect, Sertoli cells remain intact and line the partially or completely empty tubes (Creasy, 2001).

The formation of vacuoles in Sertoli cells and failure to release sperm are all indications that Sertoli cells have been malfunctioning as a result of treatment with the drug gemcitabine. It is not possible, from the results of the current study, to know whether this defect was due to the direct effect of the drug or if it was the result of germ cell degeneration, which in turn caused a dysfunction in Sertoli cells. In general, Sertoli cells in adult animals are not affected by most anticancer drugs because they do not divide (Trottmann et al., 2007), and this may suggest the second possibility as a cause of impaired function.The most important characteristic of cytotoxicity specialized in a type of germ cell is the rapid programmed cell death of this type of cell and the infected cells are ingested by Sertoli cells, leaving the tubes free of it.

This early event is followed by a rapid inhibition in the growth of the generations following the affected generation during the rest of the spermatogenesis process. The death of a specific type of germ cell will eventually leave the seminal tube empty of cells except for Sertoli cells and germ cells that precede the target cells with the toxic effect, and this gives the impression that the process of sperm formation has stopped, and in fact the unaffected cells continue to grow but It is killed as soon as it reaches the target stage with the toxin (Creasy, 2001). This may explain the emergence of some seminal tubules in rats treated with gemcitabine devoid of certain types of germ cells, especially spermatocytes and round and elongated spermatids.

The volume of interstitial fluid increases in many cases, such as obstruction of lymphatic drainage, damage to the epithelial lining of blood vessels as in the case of exposure to cadmium, or as a secondary result of decreased spermatogenesis and shrinkage of the seminal tubules, and this damage is usually associated with an increase in testicular weight (Creasy, 2001). This is consistent with the observations observed in the current study, as the tissue samples in which an accumulation of fluid was recorded in the interstitial tissues was observed to have a high weight compared to the rest of the members of the group to which it belongs. It led to this kind of tissue damage. Histological examination did not indicate that the Leydig cells were affected by the drug, as no change in their phenotype was observed from the control group (Lanning et al., 2002). The results of the current study, regarding the unaffectedness of Ledge cells, agree with what is known about the

latter in terms of their resistance to anti-cancer drugs (Fosså & Magelssen, 2004) and the reason for this is due to the rate of its slow division (Puscheck et al., 2004).

Histological examination of testicular tissue in medium dose animals showed severe hemorrhage in one of their subjects, which led to widespread proliferation of blood cells in the obvious tissues outside the blood vessels (Eli Lilly and Company, 2007), and there are several reports indicating the occurrence of vascular toxicity associated with the drug in a number of cases (Muñoz et al., 2002). The recording of this drug's toxic effects on the blood vessels may explain the hemorrhage that was recorded in this study, as it is believed that it may cause damage to the epithelial cells lining the small blood vessels spreading in the interstitial tissue, which led to the influx of blood cells to this tissue and their spread around the seminiferous tubules.

CONCLUSION

The results of the current study showed the possibility that there was no effect of the drug gemcitabine on the hormonal axon of the reproductive system, as it was evident from the fact that none of the testosterone concentration in the blood, the mating behavior, or the main testosterone producing Leydig cells were affected in the drug-treated groups compared to the control group. From this, it can be concluded that the significant damage caused by the drug to the germ cells, the histological composition of the epididymis, the quantitative and qualitative sperm measurements, and the fertility indicators are mainly due to the direct effect of the drug on the germ cells themselves.Although the largest dose used in the current study represents only one-tenth of the corresponding therapeutic dose in humans (1200 mg / m2), it caused significant damage to the tissue structure of the testicle, and it also caused the quantitative and qualitative measurements of sperm to be greatly reduced. Which ultimately reduced fertility in males treated with the drug. Accordingly, the drug can be considered highly toxic to the male reproductive system, and despite the severity of the observed effects, it has been recovered to a large extent.

Conflict of Interest: Authors declares no conflicts of interests to disclose.

Ethical Clearance Statement: The Current Research Work Was Ethically Approved by the Institutional Review Board (IRB) of Taibah University, Madinah, Saudi Arabia.

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Changes in Levels of Formed Elements in Pig Blood with Reference to Activity in Conditions of their Eleovite Use

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ABSTRACT

In the presented work, it was possible to show the connection between the type of higher nervous activity of pigs and the dynamics of the content in their blood of the main varieties of corpuscular elements under the influence of the biological stimulator eleovite. The highest level of erythrocytes, leukocytes and platelets in the outcome was found in pigs with a strong balanced mobile type of higher nervous activity. The smallest number of them was noted in animals with a weak type of higher nervous activity. This state persisted even after the use of a biological stimulant - the most pronounced changes in the levels of blood corpuscles occurred during the study in pigs with a strong balanced type of higher nervous activity. The lowest level of erythrocytes, leukocytes and platelets in comparison with the rest of the pigs during the entire study was characteristic of animals with a weak type of nervous processes. As a result of the study, it became clear that the introduction of a biological stimulant has an effect on the content of erythrocytes, leukocytes and platelets in the blood of animals, largely mediated by regular influences of the cerebral cortex. At the same time, the dynamics of the level of blood corpuscles with the use of eleovite largely depends on the strength of the excitation processes during the implementation of higher nervous activity in animals.

KEY WORDS: PIGS, TYPES OF HIGHER NERVOUS ACTIVITY, ELEOVITIS, ERYTHROCYTES, LEUKOCYTES, PLATELETS.

INTRODUCTION

The continuous increase in the world's population creates a great need to increase the volume of food production (Zavalishina, 2020b), especially of meat origin (Zavalishina, 2020c). Currently, there is a continuous improvement of technologies for raising farm animals and especially pigs as very fast-growing and highly productive (Sharnin, 2006). Of great importance in the formation of individual biological and productive characteristics of pigs is the level of functioning of their brain, and especially in terms of higher nervous activity. For this reason, there is an active improvement of tests to determine the type of higher nervous activity in pigs (Zotko, 2011).Earlier observations of the behavior of young pigs of different ages revealed that already soon after birth, they actively form reflexes to a specific nipple of the sow (Zavalishina, 2020a). Soon after birth, piglets clearly understand and identify the sounds associated with the feeding process (Kabanov, 2002). Moreover, the formation of these conditioned reflexes in piglets can be inhibited by the peculiarities of the functioning of their central nervous system (Smirnov, 2011). In this regard,

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there is great interest in the study of the dynamics of individual indicators of pigs, which occurs under the influence of environmental factors (Tkacheva and Zavalishina, 2018).

Currently, there is a methodology available in application for assessing conditioned reflex processes in pigs (Trokoz, 2012). It was possible to establish that animals with different types of higher nervous activity differ in hematological parameters, in the level of meat and milk productivity, the rate of maturation, the level of fertility, and do not equally respond to a decrease in stress. This was largely due to the presence of stress resistance in the first category of animals, and explicit stress sensitivity in the second (Kokorina, 1986).Also, a connection was found between the features of processes in the nervous system and the course of functions in the body of animals, which helped to make a proposal about the greatest resistance of animals to abrupt changes in environmental conditions in the presence of a strong balanced type of higher nervous activity and the least resistance of animals in the presence of a weak type of higher nervous activity (Zavalishina, 2018a; Zavalishina, 2018b).

Modern animal husbandry technologies imply changes in the previous stereotype of keeping animals. The need for quick and successful adaptation of animals without stressing all body systems becomes clear. This is due to the fact that in the case of insufficient adaptation-compensatory processes, they cannot sufficiently neutralize the negative effects of the environment on the body, in addition, they will quickly deplete, worsening the general condition of animals and lowering their level of productivity (Shcherbinin, 2011). It is recognized that all parts of the nervous system participate in the formation of the reactivity of the organism, the functional readiness of which determines any reactions of the organism to the effects of the environment. It becomes clear that the peculiarities of the general reactivity of an animal's organism can be understood only by taking into account the indicators of its central nervous system (Yurchenko, 2009).

Table 1. The number of erythrocytes in the blood of pigs with different types of higher nervous activity against the background of the use of a biological stimulator

The number of erythrocytes in the blood of pigs with different types of higher nervous activity					
Terms of research		strong balanced agile, n=28	strong balanced inert, n=31	strong unbalanced, n=26	weak, n=25
The initial state		6.7±0.44	6.4±0.65	6.0±0.45*	5.5±0.57**
After the first introduction eleovita, day	3	7.3±0.52	6.7±0.38	6.3±0.51*	5.8±0.47**
	7	7.7±0.61 P<0.05	7.3±0.57 P<0.05	6.8±0.48* P<0.05	6.1±0.43** P<0.05
	12	7.5±0.39 P<0.05	6.9±0.48 P<0.05	6.7±0.37* P<0.05	6.0±0.46** P<0.05
	16	7.2±0.45	6.7±0.63	6.3±0.42*	5.7±0.38**
	21	6.8±0.60	6.4±0.73	6.1±0.76*	5.4±0.54**
After repeated introduction of eleovite, da	3	7.4±0.43 P<0.05	6.8±0.35	6.3±0.51*	5.7±0.44**
	7	7.8±0.52 P<0.05	7.2±0.47 P<0.05	6.9±0.42* P<0.05	6.2±0.50** P<0.05
	12	7.5±0.33 P<0.05	7.0±0.49 P<0.05	6.7±0.39* P<0.05	6.0±0.43** P<0.05
	16	7.3±0.42	6.7±0.44	6.2±0.40*	5.5±0.47**
	21	6.8±0.46	6.3±0.39	6.0±0.45*	5.3±0.48**

Note. Significance of differences in indicators in comparison with animals of a strong balanced mobile type of higher nervous activity *–p<0.05; ** - p<0.01, p - reliability of the dynamics of indicators in animals of each type of higher nervous activity in comparison with the initial state.

In this case, special importance should be attached to the type of higher nervous activity as a factor significant for the reactivity of the organism as a whole (Pavlov, 1951). At the same time, it has not yet been possible to connect the individual characteristics of the hematological parameters of pigs and the type of their higher nervous activity. In this regard, it is of great interest to elucidate the relationship between the influence of the type of higher nervous activity on the level of the amount of basic formed elements in their blood that is significant for the growth and development of animals. This is especially important in conditions of use of a biological stimulator in pigs with different types of higher nervous activity.The aim of this work is to assess the influence of

the activity of the nervous system on the dynamics of the amount of the main blood corpuscles in pigs under the conditions of using a biostimulator.

MATERIAL AND METHODS

The work was carried out in full compliance with the ethical standards defined by the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (adopted in Strasbourg on March 18, 1986 and was fully approved in Strasbourg on June 15, 2006) and supported by the local ethics committee of the Moscow State University of Food Production (Protocol No11 of January 17, 2018). The study was carried out on six-month-old large white pigs taken to the tribe, a total of 110 heads. When taken under observation, all the pigs were examined for their type of higher nervous activity. The determination of this characteristic was based on an assessment of the characteristics of the animal's behavior, on the elucidation of the characteristics of its reaction to the researcher, to giving him food, to sharp and unexpected stimuli of a sound and light nature. The conclusion about the type of higher nervous activity was carried out in gilts on the basis of the results of tests that assess the strength, mobility and balance of nervous processes: "Formation and extinction of a conditioned reflex", "Feed to a hungry animal", "Test for an unexpected sound stimulus" (Trokoz, 2012).

All pigs, regardless of the type of higher nervous activity, underwent biological stimulation of their bodies twice by injecting a multivitamin preparation eleovita (manufactured by the company "Askont+", Russia), 2.0 ml intramuscularly in the thigh area. The drug was administered twice with an interval of 21 days. After the first biological stimulation, all animals were examined on days 3, 7, 12, 16 and 21. Re-introduction of eleovite was carried out on the 22nd day after its first injection with the examination of the gilts after the second injection of the preparation also on the 3, 7, 12, 16 and 21 days after the second injection.In all pigs, the number of erythrocytes, the total number of leukocytes and the concentration of platelets in the blood was determined by conventional methods. Statistical processing of the obtained digital material was carried out using Microsoft Excel using the Student's test, correlation and analysis of variance.

Table 2. Correlation relationships between the number of erythrocytes in the peripheral blood and the properties of higher nervous activity in pigs under conditions of using eleovite

Terms of research		The number of erythrocytes in the blood of pigs with different types of higher nervous activity		
		property strength	property poise	property mobility
The initial state		0.58**	0.55**	0.52**
After the first introduction eleovita, day	3	0.52**	0.48*	0.45*
	7	0.42	0.40	0.39
	12	0.40	0.38	0.32
	16	0.52**	0.47*	0.47**
	21	0.57**	0.54**	0.51**
	3	0.51**	0.46*	0.46*
After repeated introduction eleovita, day	7	0.42	0.41*	0.38
	12	0.40	0.39	0.31
	16	0.51**	0.47*	0.46*
	21	0.59**	0.56**	0.53**

Note: the reliability of the correlation coefficients: * p <0.05; ** p <0.01.

RESULTS AND DISCUSSION

The initial number of erythrocytes in the blood of animals showed its dependence on the type of higher nervous activity available to them (Table 1).Before the use of eleovite, the content of erythrocytes in the blood of the observed pigs remained within the normal range. At the same time, in animals with a strong balanced mobile type of higher nervous activity, this indicator exceeded those in individuals with a strong balanced inert type, with a strong unbalanced type and with a weak type of higher nervous activity by 4.7%, by 11.7% (p <0.05) and by 21.8% (p <0.01), respectively.

The introduction to pigs of all types of higher nervous activity of eleovite caused them to change the quantitative content of erythrocytes in their blood. The observed dynamics of their level consisted in all cases in an increase in the number of these cells. The reliability of changes in all types of higher nervous activity observed in the work of gilts was noted on the 7th day after administration of the drug and was maximum during

these periods. Subsequently, the level of erythrocytes began to decrease. By 12 days after injection, it remained significantly higher than the initial level, and by 16 and 21 days after the first injection, it did not differ significantly from the outcome level.

At the same time, the degree of increase in the number of erythrocytes in the blood of the observed animals differed depending on the type of higher nervous activity they had. So, in animals on the 7th day after administration of the drug in the presence of a strong balanced mobile type of higher nervous activity, this indicator increased by 14.9%, in those with a strong balanced inert type by 14.0%, in the presence of a strong unbalanced type by 13.3%, in those who had a weak type of higher nervous activity by 10.9%.After the second injection of eleovite in the blood of pigs, a similar increase in the number of erythrocytes was repeatedly observed with its maximum level on the 7th day after administration of the drug and a subsequent decrease in this indicator, as after the first use of the drug.

Thus, the level of erythrocytes in the blood of pigs was closely related to the activity of their cortical processes. With their low amount in the blood, one can suspect the presence of weakness of these processes, and a consistently high number of erythrocytes gives reason to talk about the presence of strong and balanced processes in the animal's brain during the implementation of higher nervous activity.Considering that the highest levels of erythrocytes in the blood are characteristic of pigs with a strong balanced mobile type of higher nervous activity, it was of great interest to find out the severity of the relationship of each of these properties with the level of erythrocytes in the blood of animals, and, consequently, with the activity of erythropoiesis.

Using correlation analysis, the authors were able to establish the following. The highest values of the correlation coefficients of the level of erythrocytes in the blood of animals with a separate property of their nervous processes were found in the outcome with strength ($r = 0.58$; $p < 0.01$), with equilibrium ($r = 0.55$; $p < 0.01$), with mobility ($r = 0.52$; $p < 0.01$). The values of the correlation coefficients given in Table 2 between the properties of the nervous processes of the observed pigs and the level of erythrocytes in their blood proves that they have a clear control on the part of the central nervous system over the production of erythrocytes in the bone marrow. In this case, the most significant for the course of erythropoiesis were two properties of the processes of the central nervous system - strength and balance. The property of mobility of processes in the cerebral cortex of pigs was to a somewhat lesser extent associated with erythropoiesis in the bone marrow, but not so much that it could be neglected when considering this issue.

Table 3. The strength of the influence of the main properties of nervous processes on the level of erythrocytes in the blood of pigs, $\eta^2 x$

Terms of research		Properties of nervous processes in the examined gilts		
		property strength	property poise	property mobility
The initial state		0.16*	0.19*	0.14*
After the first introduction eleovita, day	3	0.15*	0.18*	0.10
	7	0.05	0.04	0.04
	12	0.09	0.06	0.06
	16	0.11	0.12	0.07
	21	0.16*	0.19*	0.13*
After repeated introduction eleovita, day	3	0.14*	0.17*	0.09
	7	0.06	0.05	0.04
	12	0.10	0.09	0.06
	16	0.10	0.11	0.08
	21	0.17*	0.19*	0.15*

Note: reliability of indicators - * $p < 0.05$; ** $p < 0.01$.

After the first and second injection of eleovite, the strength of the correlations of all the properties of the nervous processes taken into account weakened by the 3rd day, and by the 7th day it lost its reliability. After the first and after the second administration of the biostimulant on the 12th day, the correlation coefficients decreased further, without changing the reliability. On the 16th and 21st days of observation, after both injections, an increase in the correction coefficients was noted with the achievement of the reliability level. These changes in the correlation coefficients took place after both injections of eleovite, ensuring the achievement on the

21st day in both cases of the values of the correlation coefficients characteristic of the levels of the initial values.

The found changes in the values of the correlation coefficients in gilts with different types of higher nervous activity after the initial and after repeated use of the biostimulator indicate a temporary weakening of the control from the cerebral hemispheres of the brain over the red sprout of the bone marrow under conditions of exposure to the body that can intensify hematopoietic processes.This opinion was confirmed by the data of the analysis of variance carried out, the results of which are given below (Table 3).

All considered properties of nervous processes influenced the level of erythrocytes in the blood of pigs. The greatest influence on their amount in the blood in the initial state was demonstrated by the strength and balance of cortical processes. At the same time, the influence of mobility was more modest, but it was at the level of reliability. When using a biological stimulator, the effect of the considered properties of cortical processes on the number of erythrocytes in the blood of animals decreased up to 12 days of observation, and then began to increase. It becomes clear that under the influence of eleovite on the body of the animal, nervous processes control erythropoiesis weaker. Moreover, the property of strength and the property of balance of nervous processes lost the reliability of the strength of their influence on erythropoiesis between 7 and 16 days after the first and second administration of the drug. At the same time, after the injection of a biological stimulant, the property of mobility of nervous processes in the central nervous system quickly lost its effect on erythropoiesis in the observed gilts and restored it only on day 21 after the first and second use of the drug.

Thus, the properties of nervous processes in the central nervous system, and, therefore, the type of higher nervous activity largely determines the level of erythrocytes in the blood of pigs. It is clear that against the background of a temporary loss of strict control on the part of the central nervous system over the content of erythrocytes in the blood, under the action of a biostimulator, their number can actively and physiologically beneficially increase in the blood of pigs with strong types of higher nervous activity. The most significant in the process of increasing the content of erythrocytes under conditions of biostimulation are the properties of higher nervous activity - strength and balance. In this regard, the most pronounced increase in erythrocytes occurs in the blood of animals with a strong balanced mobile type.

Table 4. The total number of leukocytes in the blood of pigs with different types of higher nervous activity against the background of the use of a biological stimulator

The total number of leukocytes in the blood of pigs with different types of higher nervous activity					
Terms of the study		strong balanced agile, n=28	strong balanced inert, n=31	strong unbalanced, n=26	weak, n=25
The initial state		15.2±0.92	14.2±0.86	12.9±0.47*	11.5±0.56**
After the first introduction eleovita, day	3	17.2±0.45 P<0.05	14.2±0.71* P<0.05	15.9±0.66 P<0.05	12.5±0.49** P<0.05
	7	18.9±0.64 P<0.01	17.2±0.72 P<0.01	15.5±0.52* P<0.01	13.1±0.63** P<0.05
	12	17.2±0.58 P<0.05	15.8±0.74 P<0.05	14.9±0.81* P<0.05	12.7±0.58** P<0.05
	16	16.3±0.60	15.0±0.54	13.8±0.49	12.2±0.45**
After repeated introduction of eleovite, day	21	15.5±0.55	14.3±0.63	13.2±0.65*	11.7±0.57**
	3	16.8±0.63 P<0.05	16.0±0.71 P<0.05	14.4±0.60* P<0.05	12.7±0.54** P<0.05
	7	18.6±0.59 P<0.01	17.0±0.48 P<0.01	16.2±0.37* P<0.01	13.2±0.52** P<0.05
	12	17.5±0.44 P<0.05	15.9±0.49 P<0.05	15.0±0.62* P<0.05	12.8±0.57** P<0.05
	16	16.6±0.75	15.4±0.62	14.4±0.57*	12.5±0.60**
	21	15.7±0.48	14.7±0.54	13.3±0.50*	11.8±0.63**

Note. Significance of differences in indicators in comparison with animals of a strong balanced mobile type of higher nervous activity * - p <0.05; ** - p <0.01; p - reliability of the dynamics of indicators in animals of each type of higher nervous activity in comparison with the initial state.

The strong balanced inert type is somewhat inferior to him, the strong unbalanced type and the very significantly weak type of higher nervous activity are even more inferior. This can be explained by the fact that in the presence of a strong type of higher nervous activity, the properties of strength and balance

characteristic of the processes of the central nervous system, to a large extent, activate metabolic processes throughout the body. This is able to lay the foundation for maintaining the level of red blood cells at a higher level in the event of any external influences on the body, including non-immune ones.

Before the use of the biological stimulant, the number of leukocytes in the blood of the examined pigs corresponded to the normative values and was associated with the type of higher nervous activity they had (Table 4).Before the first injection of eleovite, the greatest number of leukocytes was characteristic of gilts with a strong balanced mobile type of higher nervous activity. This indicator in these animals exceeded those in individuals with other types of higher nervous activity - strong balanced inert type, strong unbalanced type and weak type, respectively by 7.0%, by 23.6% ($p<0.05$) and 32.2% ($p<0.01$).

The use of eleovite was accompanied in all animals by the dynamics of the level of leukocytes in the blood. Moreover, in pigs with different types of higher nervous activity, different severity of this dynamics was noted. In all four groups collected, taking into account the existing type of higher nervous activity, compared with the initial values, there was a significant increase in the outcome level after the first and after the second injection of eleovite on days 3.7 and 12, followed by a return of the indicator to the outcome level.

The most pronounced increase in their level was noted in gilts with a strong balanced mobile type of higher nervous activity. On the seventh day after the first injection of the drug, they showed the greatest increase in the level of blood leukocytes compared to the initial state (by 24.3% at $p<0.01$). The least pronounced increase (by 13.9%) was noted in gilts with a weak type of higher nervous activity. At the same time, the pigs with a strong balanced inert (by 21.1%) and strong unbalanced (by 20.1%) had an intermediate degree of growth in the number of leukocytes in their blood and did not differ among themselves. In the subsequent periods of observation, they were found to decrease by 21 days and increase to a comparable degree after repeated administration of eleovite up to 12 days, followed by a decrease to the initial level by the end of observation (21 days after repeated administration of the multivitamin).

Table 5. Correlation relationships between the level of the total number of leukocytes in the peripheral blood and the properties of the higher nervous activity of pigs under the conditions of using eleovite

		Properties of nervous processes in the examined gilts		
Terms of the study		property strength	property poise	property mobility
The initial state		0.57**	0.53**	0.46*
After the first introduction eleovita, day	3	0.48*	0.43*	0.40*
	7	0.39	0.37	0.31
	12	0.41	0.40	0.36
	16	0.43*	0.46*	0.41*
	21	0.55**	0.56**	0.50**
After repeated introduction eleovita, day	3	0.47*	0.46*	0.41*
	7	0.38	0.39	0.30
	12	0.40	0.40	0.32
	16	0.43*	0.43*	0.36*
	21	0.55**	0.53**	0.47**

Note: the reliability of the correlation coefficients - * $p<0.05$; ** $p<0.01$.

Thus, it can be assumed that the level of leukocytes in the blood of pigs is closely related to the activity of their cortical processes. In this regard, a low number of leukocytes in the blood can indicate their weakness, and a consistently high content of leukocytes in the blood can be considered a marker of the presence of strong, balanced and mobile processes of excitation and inhibition in the animal's brain. Taking into account the revealed connection between the levels of leukocytes in the blood of pigs and the type of higher nervous activity they have, it was of great interest to find out the severity of this connection for each of the properties of a strong balanced mobile type of higher nervous activity. Applying correlation analysis, the work was able to establish the following (Table 5).

In the outcome, the highest values of the correlation coefficients of the level of leukocytes in the blood of animals were found with strength ($r = 0.57$; $p<0.01$) and with equilibrium ($r = 0.53$; $p<0.01$) of nervous processes. The values of the correlation coefficients given in table 5 between the properties of the nervous processes of pigs

and the level of leukocytes in their blood confirms the presence of a clear control by the central nervous system over the production of leukocytes by the bone marrow. At the same time, the most significant for leukopoiesis throughout the entire observation were two properties of processes in the central nervous system: strength and balance.

It turned out that the property of mobility of processes in the cerebral cortex is to a lesser extent related to the production of leukocytes in the bone marrow, but not so much that it could be neglected when considering this issue. After the first and second injection of eleovite, the strength of the correlations of all the properties of the nervous processes taken into account weakened by the 3rd day, and by the 7th day it lost its reliability. And after the first and after the second administration of the biostimulant on the 12th day, the correlation coefficients experienced a tendency to increase. After both injections of eleovite, by 16 days the values of the correlation coefficients increased to the level of reliability, and by 21 days in both cases the values of the correlation coefficients reached the initial level.

The observed changes in the values of the correlation coefficients in gilts of different types of higher nervous activity after the primary and after repeated use of the biostimulator indicated the onset of a temporary weakening in animals of the regulatory function of the cerebral hemispheres under conditions of stimulation of the body's metabolism from the outside.This point of view was confirmed by the results of the analysis of variance (Table 6).

Table 6. The strength of the influence of the properties of nervous processes on the total level of leukocytes in the blood of pigs, η^2_x

Terms of the study		The property of nervous processes in the examined gilts		
		property strength	property poise	property mobility
The initial state		0,24**	0,22**	0,17*
After the first introduction eleovita, day	3	0,16*	0,15*	0,12
	7	0,08	0,06	0,05
	12	0,10	0,08	0,07
	16	0,15*	0,14*	0,11
	21	0,23**	0,21**	0,17*
After repeated introduction eleovita, day	3	0,09	0,05	0,04
	7	0.38	0.39	0.30
	12	0,10	0,08	0,07
	16	0,16*	0,15	0,11
	21	0,24**	0,22**	0,17*

Note: reliability of indicators - * p <0.05; ** p <0.01.

All the considered properties of nervous processes influenced the level of leukocytes in the blood of pigs. The greatest influence on the number of leukocytes in the blood was manifested by the strength and balance of cortical processes, while the influence of mobility was somewhat more modest. At the first application of a biological stimulator, the effect of the properties of cortical processes on the leukocyte content in the blood of pigs decreased until 7 days of observation, and then began to grow.In the process of repeated influence of eleovite on the body of the animal, the nervous processes also affected leukopoiesis weaker, and the property of strength and the property of balance in nervous processes also lost the reliability of their influence on leukopoiesis from 7 to 12 days. At the same time, after both injections of the biological stimulant, the property of mobility of nervous processes in the central nervous system lost its power of influence on leukopoiesis in the observed gilts from 3 to 16 days.

Thus, the properties of nervous processes in the central nervous system, and, therefore, the type of higher nervous activity largely determines the level of leukocytes in the blood of healthy pigs. As a result of the study, it became clear that animals with strong types of higher nervous activity against the background of a temporary loss of strict control from the central nervous system over leukopoiesis, under the action of a biostimulator, are able to very actively increase the number of leukocytes in their blood. At the same time, the properties of strength and balance were the most significant for the development of leukocytosis in pigs under conditions of biostimulation. In this regard, the most pronounced leukocytosis is noted in animals that received eleovitis, with a strong balanced mobile type, a strong balanced inert type is somewhat inferior to it, to which a strong unbalanced type is inferior. An increase in the number of leukocytes in the blood of pigs with a weak type of higher nervous activity is even less recorded. This pattern can

be explained by the fact that the properties of strength and balance during the implementation of processes in the central nervous system contribute significantly to the activation of metabolic processes throughout the body. This lays the foundations in these animals for a pronounced reaction of the level of leukocytes to any external influences, including biostimulation of a non-immune nature.

Before the start of the use of the tested biological stimulant, the number of platelets in the blood of pigs had a clear connection with their type of higher nervous activity (Table 7) and were within the normal range.Before the first injection of eleovite, the greatest number of platelets was found in the blood of pigs with a strong balanced mobile type of higher nervous activity. This indicator in these animals was higher than that in individuals that had other types of higher nervous activity - strong balanced inert, strong unbalanced and weak types of higher nervous activity, respectively by 6.5%, by 13.8% ($p<0.05$) and by 26.1% ($p<0.01$).

The use of eleovite was accompanied in pigs by the dynamics of the level of platelets in their peripheral blood. For animals of each type of higher nervous activity, the characteristic dynamics of their level was revealed. It was associated with a significant increase in their number already on the 7th day after the first injection of the drug (strong balanced mobile by 15.8%, strong balanced inert by 13.4%, strong unbalanced by 11.8%, weak by 10.1%). In all four observation groups, formed taking into account the type of higher nervous activity in animals, there was a significant increase in the number of platelets after the first injection of eleovite on the 12th day of observation. The greatest increase in their level during these periods was noted in gilts that had a strong balanced mobile type of higher nervous activity.

Table 7. The number of platelets in the blood of pigs of various types of higher nervous activity against the background of the use of a biological stimulator

The number of platelets in the blood of pigs with different types of higher nervous activity					
Terms of the study		strong balanced agile, n=28	strong balanced inert, n=31	strong unbalanced, n=26	weak, n=25
The initial state		362.3±1.25	340.2±0.98	318.4±0.72*	295.2±0.83**
After the first introduction eleovita, day	3	384.4±0.65	361.8±0.71	337.5±0.84*	295.6±0.64**
	7	419.6±0.72 p<0.05	385.7±0.78 p<0.05	355.9±1.07* p<0.05	325.1±0.99** p<0.05
	12	402.6±1.15 p<0.05	376.5±0.93 p<0.05	352.6±0.90* p<0.05	319.7±0.78** p<0.05
	16	386.3±1.00	357.6±0.75	336.2±0.68*	299.4±0.57**
	21	360.4±0.83	343.7±0.72	320.1±0.76*	290.6±0.81**
After repeated introduction eleovita, day	3	386.3±0.72	360.7±1.05	339.2±0.87*	299.5±0.84**
	7	420.6±0.52 p<0.05	387.0±0.74 p<0.05	362.7±1.03* p<0.05	322.6±0.67** p<0.05
	12	404.5±0.67 p<0.05	378.4±0.52 P<0.05	350.4±0.64* p<0.05	316.3±0.77** p<0.05
	16	386.3±0.92	359.1±0.73	339.9±0.84	300.1±0.79**
	21	365.2±0.80	346.0±0.86	320.6±0.72*	292.6±0.42**

Note. Significance of differences in indicators in comparison with animals of a strong balanced mobile type of higher nervous activity * - $p<0.05$; ** - $p<0.01$; p - reliability of the dynamics of indicators in animals of each type of higher nervous activity in comparison with the initial state

So, on the 12th day after the first injection of the drug in animals, the following degree of excess of the initial level of platelets in the blood was noted: in pigs with a strong balanced mobile type by 11.3% ($p<0.05$), in a pig with a strong balanced inert by 10.7%, with a strong unbalanced by 10.7%, in gilts with a weak type of higher nervous activity by 8.3%. After the repeated injection of eleovite, the dynamics of the platelet level in animals was similar to that after its first injection. At the same time, in all cases, the number of platelets in the blood of animals was restored at the level of outcome by 21 days after the first and second injection of the tested multivitamin.

Thus, the level of platelets in the blood of healthy pigs is closely related to the activity of their cortical processes. In this regard, with their low amount in the blood, it can be assumed that animals have weakness in the processes of higher nervous activity. At the same time, their high number gives reason to assume that the animal has strong, balanced and mobile processes of excitation and inhibition in the brain.Considering

that the highest levels of platelets in the blood are characteristic of gilts with a strong balanced mobile type of higher nervous activity, it was of great interest to clarify the severity of the relationship between each of these properties with the concentration of platelets in their blood, and, consequently, with the intensity of thrombocytopoiesis.

Applying the correlation analysis, the following was established in the work. In the outcome, the highest values of the correlation coefficients of the level of platelets in the blood of animals were found with strength ($r = 0.55$; $p < 0.01$) and with equilibrium ($r = 0.53$; $p < 0.01$).The values of the correlation coefficients given in table 8 between the properties of the nervous processes of pigs and the level of platelets in their blood confirm the presence of a clear control by the central nervous system over the production of platelets in the bone marrow. The most significant for thrombocytopoiesis were two properties of the processes of the central nervous system: strength and balance. The property of mobility of processes in the cerebral cortex was to a lesser extent associated with the production of platelets in the bone marrow, but not so much that it could be neglected when considering this issue.

After the first and after the second injection of eleovite, the strength of the correlations of all the properties of the nervous processes taken into account by the 3rd day somewhat weakened, and between the 7th and 12th days of observation it lost its sufficiency. After the first and after the second administration of the biostimulant on the 16th day, an increase in the correlation coefficients was noted with the achievement of the level of reliability. In the subsequent periods of observation, an increase in the correlation coefficients was recorded in all animals, reaching the initial values on day 21 after both injections of the drug.

Table 8. Correlation relationships between the level of platelets in peripheral blood and the properties of higher nervous activity in pigs under conditions of using eleovite

		Properties of nervous processes in the examined gilts		
Terms of the study		property strength	property poise	property mobility
The initial state		0.55**	0.53**	0.50**
After the first introduction eleovita, day	3	0.45*	0.46*	0.44*
	7	0.39	0.40	0.35
	12	0.35	0.34	0.32
	16	0.46	0.41*	0.42*
	21	0.55**	0.54**	0.49**
After repeated introduction eleovita, day	3	0.44*	0.43*	0.45*
	7	0.38	0.41	0.38
	12	0.34	0.33	0.32
	16	0.45*	0.42*	0.41*
	21	0.54**	0.53**	0.49*

Note: the reliability of the correlation coefficients - * $p<0.05$; ** $p<0.01$.

The found changes in the values of the correlation coefficients in pigs with different types of higher nervous activity after the first and after repeated application of eleovite indicate the development of a weakening of the regulatory function of the cerebral hemispheres of the brain under conditions of biostimulation of the organism from the outside. These data were confirmed by the results of the analysis of variance carried out in the study (Table 9).

All the considered properties of nervous processes influenced the level of platelets in the blood of pigs. The most reliable effect on their number in animals under standard conditions was shown by the strength and balance of cortical processes. The effect of mobility on platelet count was more modest and did not reach the level of reliability. When using a biological stimulant, the strength of the influence of the properties of cortical processes on the number of platelets in the blood of pigs decreased, losing the reliability of strength and balance on days 7 and 12 after the first and second administration of the drug.The data obtained allow us to believe that the properties of the nervous processes occurring in the central nervous system of pigs, and, consequently, the type of their higher nervous activity, determine the level of platelets in the blood of these animals.

This pattern is only temporarily violated under the conditions of the use of a biostimulator, followed by a rapid restoration of control of nervous processes over thrombocytopoiesis and the level of thrombocythemia. At the same time, in pigs, even against the background

of biostimulation, the properties of strength and balance of nervous processes are most significant for the level of platelets in the blood. This can be explained by the fact that in animals with a strong type of higher nervous activity, the properties of strength and balance of the central nervous system contribute to the activation of metabolic processes in all tissues, which lays the foundation for a pronounced reaction of the platelet level to any external influences on the body, including those of a non-immune nature.

Table 9. The strength of the influence of the properties of nervous processes on the number of platelets in the blood of pigs, η^2x

		The property of nervous processes in the examined gilts		
Terms of the study		**property strength**	**property poise**	**property mobility**
The initial state		0.15*	0.19*	0.13
After the first introduction eleovita, day	3	0.14*	0.17*	0.12
	7	0.11	0.12	0.10
	12	0.09	0.11	0.08
	16	0.13*	0.18*	0.12
	21	0.15*	0.19*	0.13
After repeated introduction eleovita, day	3	0.15*	0.18*	0.12
	7	0.11	0.12	0.09
	12	0.09	0.10	0.07
	16	0.15*	0.17*	0.12
	21	0.16*	0.19*	0.13

CONCLUSION

It was of great scientific and practical interest to identify the characteristics of the reaction of blood corpuscles, considered as cellular components of a liquid medium that integrates the body to the introduction of the multivitamin remedy eleovit, taking into account the type of higher nervous activity of the mumps. The highest level of blood cells in pigs before exposure to eleovitis was recorded in animals with a strong balanced mobile type, and the lowest in animals with a weak type of higher nervous activity.

This picture persisted against the background of the use of the tested drug, demonstrating the greatest dynamics of the considered indicators in animals with a strong balanced mobile type of higher nervous activity. This pattern was true for the level of erythrocytes, leukocytes and platelets. The results obtained indicate the need to correct the doses of biological stimulants used in animals with different types of higher nervous activity. Based on the results obtained, there is reason to recommend assessing the severity of the effects of biostimulants with an increase in their dose in comparison with that traditionally accepted in gilts with a strong unbalanced type and a weak type of higher nervous activity.

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Karyotypic Analysis and Chromosome Banding in Freshwater Prawn Macrobrachium dayanum from Jammu and Kashmir, India

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ABSTRACT

The Union Territory of Jammu & Kashmir have rich faunal diversity in its aquatic resources. Shellfishes (such as prawns and crabs) together with finfishes are contributing significantly to meet the nutritional requirements of natives. The local prawns have greater potential to raise the economic standard of Jammu region if cultured extensively on commercial scale. In this regard, they need to be analysed at chromosomal and molecular level. In the present study, the chromosomes of Himalayan prawn (*Macrobrachium dayanum*) were characterized by means of conventional Giemsa staining, Ag-NOR and G-banding techniques. It is one of the most abundant shellfishes in water bodies of Jammu region having high protein and mineral content. The diploid chromosome number (2n) and fundamental number (NF) were found to be 100 and 176 respectively. The karyotype comprised of 60 metacentric, 16 submetacentric, 12 subtelocentric and 12 telocentric chromosomes. Idiograms were constructed on the basis of morphometric details of the chromosomes. Allosomes (sex chromosomes) remained indistinguishable. NORs were located on two submetacentric pairs of the complement. Results of G-banding provided the heterochromatin and euchromatin patterns of *M. dayanum*. Several meiotic stages such as leptotene, zygotene, pachytene, diplotene, diakinesis, metaphase I and metaphase II from testes were also observed. Karyological studies aid in exact taxonomic identification and understanding of the phylogeny of an organism. The data obtained in present work would serve the basis of stock improvement, future cross breeding and chromosomal manipulation experiments such as induction of polyploidy etc. Through this analysis we have concluded the results which can support future cytogenetic research in crustaceans by acting as a credible milestone.

KEY WORDS: CHROMOSOMES, G-BANDING, AG-NOR, KARYOTYPE, *M. DAYANUM*, METAPHASE.

INTRODUCTION

Macrobrachium dayanum belongs to family Palaemonidae of decapod crustaceans. It is broadly distributed in Northern India, Southern Nepal and Myanmar (Jayachandran, 2001; Cai and Ng, 2002). It is commonly available prawn in stream ecosystems of Jammu region and its nutritional value stands at par with culturable fish species (Langer et al., 2004; Jasrotia et al., 2017; Jasrotia and Langer, 2019). The identifying features of the species are: Rostrum straight or slightly upturned at distal half, reaching almost equal to the length of antennal scale or extending a little beyond it. Dorsal or upper surface of the rostrum bears 5-11 teeth of which 1-2 are post orbital and the ventral or lower surface possess 4-7 teeth (Paul, 1991; Sharma, 2015). Sexual dimorphism is quite distinct in *M. dayanum*. Second pair of walking leg is stout and more robust with sharp pincers in males as compared to females.

The second pair of swimmerets bears an additional structure called appendix masculina in males. The size of the specimen ranged from 5.0±0.10 to 6.3±0.38 cm

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in males and 4.9±0.12 to 5.9±0.36 cm in females. The females carry green coloured eggs in the brood chamber during the breeding season.

Despite having greater economic and commercial importance, the cytogenetic reports of family Palaemonidae in general and genus *Macrobrachium* in particular are very few. The reason for this is attributed to the technical difficulties associated with their highly condensed numerous chromosomes (Chow et al., 1990; Nagashree, 1993; Gonzalez-Tizon et al., 2013; Phimphan et al., 2018). There is no previous record of the karyotype of *M. dayanum*. The present study was thus undertaken to document the chromosome number, analysis of meiotic stages, development of karyotype and chromosomal banding of this species for the first time. It is pertinent to mention that karyomorphological information contributes to better understanding of systematics and genealogy. Moreover, it would help in analysing the course of evolution in family Palaemonidae.

MATERIAL AND METHODS

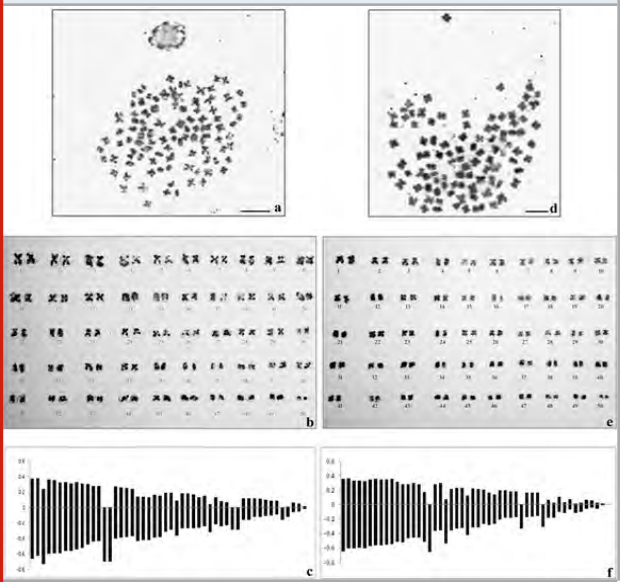
Live specimens of *M. dayanum* were collected by using cast net from Gho-manhasan stream and Sai stream of Jammu district and brought to Animal Cytogenetics lab, Department of Zoology, University of Jammu in the plastic containers ((Jayachandran, 2001; Cai and Ng, 2002; Sharma, 2015). The taxonomic identification of specimens is based on the standard keys. Before dissection, the animals were maintained in clean water in glass troughs equipped with aerators and thermoregulators. Adult specimens were injected intramuscularly with 0.05 % colchicine solution and were maintained for a period of 5 hours before sacrifice. Apart from this, dip treatment of 0.1 % colchicine solution for 10-12 hours was also applied on some specimens. Gonadal tissues, hepatopancreas and fertilized eggs were used for chromosomal preparations by following air-drying Giemsa staining technique with some modifications (Choudhary et al., 2013; Hassan et al., 2015). After colchicine treatment, the prawns were dissected and the required tissues were placed in hypotonic solution (0.9 % sodium citrate) for 50 minutes.

Fixation of the tissue was done in 3:1 methanol-acetic acid fixative (Carnoy's fixative) for 60 minutes (with three changes of fixative after every 20 minutes). The material was then minced in 45% acetic acid for 10-15 minutes. The suspension was dropped on the clean and pre-warmed slides and air dried. The conventional method of dabbing the fixed tissue material on clean slides followed by air drying was also used. After air-drying, the slides were stained with 4% Giemsa phosphate buffer solution (pH 6.8) for 30- 35 minutes. Ag-NOR and G-banding were done following standard protocols with certain modifications (Howell and Black, 1980; Sumner et al., 1971). The prepared slides were scanned under Olympus camera aided microscope and metaphase spreads as well as meiotic stages were photographed using Sony SSC-DC378P camera under 1000x magnification. For karyotyping, best metaphase spreads were selected and chromosomes were classified following internationally accepted standard classification (Levan et al., 1964). The chromosomal pairs were arranged in the decreasing order of their size in the karyogram. Morphometric measurements were done by using occulometer.

RESULTS AND DISCUSSION

The spermatogonial metaphase (Fig.1a) in male and somatic metaphase complement in female (Fig.1d) comprised of 50 chromosome pairs in each showing basic chromosome number to be 2n=100 in this species. The chromosome type and form were found to be similar in both the sexes and most of the chromosomes were metacentric and sub-metacentric. The diploid chromosome formula was determined as 2n=60m+16sm+12st+12t. Sex chromosomes were not morphologically differentiated from the autosomes in male and female karyotypes (Fig. 1b and 1e respectively). The average lengths of each chromosome including short and long arm length, total length, arm ratio, relative length percentage and centromeric index were calculated for both the sexes and presented in Table 1 and 2. The diagrammatic summary of male and female karyotype was shown by constructing the idiograms (Fig.1c and 1f).

Figure 1: Metaphase complements (2n=100), karyotypes and idiograms of *Macrobrachium dayanum* a. Spermatogonial metaphase (male) b. Karyotype of male c. Idiogram of male d. Metaphase plate (female) e. Karyotype of female f. Idiogram of female, Bars=5 µm



Morphometric measurement of the chromosomes showed mean haploid length to be 25.82 µm and 25.79 µm in male and female respectively. The total complement length was recorded as 51.64 µm in male and 51.58 µm in female.

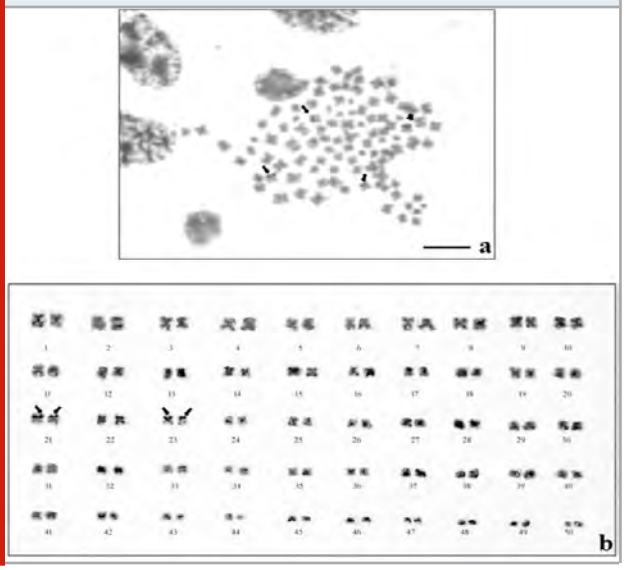
Table 1. Karyomorphometric data of *Macrobrachium dayanum* (female)

Chromosome pair No.	Mean length of Short arm (p) (µm)	Mean length of Long arm (q) (µm)	Absolute length (p+q) (µm)	Arm ratio (q/p)	Relative length %age	Centromeric index	Nomenclature
1.	0.35	0.65	1.0	1.85	3.87	35	Sub-metacentric
2.	0.36	0.61	0.97	1.69	3.76	37.1	Metacentric
3.	0.33	0.61	0.94	1.84	3.64	35.1	Sub-metacentric
4.	0.33	0.60	0.93	1.81	3.60	35.4	Sub-metacentric
5.	0.32	0.61	0.93	1.90	3.60	34.4	Sub-metacentric
6.	0.34	0.58	0.92	1.70	3.56	36.9	Metacentric
7.	0.35	0.57	0.92	1.62	3.56	38.04	Metacentric
8.	0.34	0.57	0.91	1.67	3.52	37.3	Metacentric
9.	0.34	0.56	0.90	1.64	3.48	37.7	Metacentric
10.	0.35	0.55	0.90	1.57	3.48	38.8	Metacentric
11.	0.31	0.52	0.83	1.67	3.21	37.3	Metacentric
12.	0.28	0.53	0.81	1.89	3.14	34.5	Sub-metacentric
13.	0.28	0.47	0.75	1.67	2.90	37.3	Metacentric
14.	0.29	0.46	0.75	1.58	2.90	38.6	Metacentric
15.	0.28	0.46	0.74	1.64	2.86	37.8	Metacentric
16.	0.17	0.52	0.69	3.05	2.67	24.6	Sub-telocentric
17.	-	0.66	0.66	-	2.55	-	Telocentric
18.	0.28	0.37	0.65	1.32	2.52	43.07	Metacentric
19.	0.29	0.36	0.65	1.24	2.52	44.61	Metacentric
20.	0.07	0.54	0.61	7.71	2.36	11.4	Telocentric
21.	0.21	0.36	0.57	1.714	2.21	36.8	Sub-metacentric
22.	0.23	0.34	0.57	1.47	2.21	40.3	Metacentric
23.	0.23	0.33	0.56	1.43	2.17	41.07	Metacentric
24.	0.12	0.43	0.55	3.58	2.13	21.8	Sub-telocentric
25.	0.22	0.32	0.54	1.45	2.09	40.7	Metacentric
26.	0.21	0.32	0.53	1.52	2.05	39.6	Metacentric
27.	0.20	0.27	0.47	1.35	1.82	42.5	Metacentric
28.	0.16	0.29	0.45	1.81	1.74	35.5	Sub-metacentric
29.	0.14	0.27	0.41	1.92	1.58	34.1	Sub-metacentric
30.	0.19	0.21	0.40	1.10	1.55	47.5	Metacentric
31.	0.19	0.19	0.38	1	1.47	50	Metacentric
32.	0.18	0.19	0.37	1.05	1.43	48.6	Metacentric
33.	0.18	0.18	0.36	1	1.39	50	Metacentric
34.	-	0.34	0.34	-	1.31	-	Telocentric
35.	0.16	0.18	0.34	1.12	1.31	47	Metacentric
36.	0.16	0.17	0.33	1.06	1.27	48.4	Metacentric
37.	0.16	0.16	0.32	1	1.24	50	Metacentric
38.	-	0.31	0.31	-	1.20	-	Telocentric
39.	0.06	0.19	0.25	3.16	0.96	24	Sub-telocentric
40.	0.02	0.19	0.21	9.5	0.81	9.52	Telocentric
41.	0.10	0.10	0.20	1	0.77	50	Metacentric
42.	0.03	0.12	0.15	4	0.58	20	Sub-telocentric
43.	0.07	0.07	0.14	1	0.54	50	Metacentric
44.	0.01	0.12	0.13	12	0.50	7.69	Telocentric
45.	0.02	0.10	0.12	5	0.46	16.6	Sub-telocentric
46.	0.06	0.06	0.12	1	0.46	50	Metacentric
47.	0.05	0.05	0.10	1	0.38	50	Metacentric
48.	0.019	0.06	0.079	3.15	0.30	24	Sub-telocentric
49.	0.015	0.015	0.03	1	0.11	50	Metacentric
50.	0.005	0.005	0.01	1	0.03	50	Metacentric

The absolute length of the largest chromosome was 1.03 µm and that of the smallest chromosome was 0.04 µm in male whereas absolute length of the largest chromosome was 1.0 µm and that of the smallest chromosome was 0.01 µm in female. Centromeric index for the largest and the smallest chromosome in male was calculated as 35.9 and 50 respectively. However, the CI for the largest and the smallest chromosome in female was found to be 35and 50 respectively.

The results of NOR- banding revealed the presence of NORs on two submetacentric pairs of NOR banded complement (Fig. 2a). NORs are associated with gene expressions. The NOR-banded karyotype is represented in figure 2b. By G-banding, a series of light and dark bands were produced that allow for the positive identification of each chromosome in the complement (Fig. 3a). The dark bands are A–T rich, heterochromatic regions of the chromosomes, while the light bands are C–G rich, euchromatic regions. The G-banded karyotype is respresented in figure 3b. Among meiotic stages (Fig.4a-h) from testes, leptotene (characterized by network of chromosomes), zygotene (chromosomes with free ends and synapsis of homologous chromosomes was observed), pachytene (chromosomes were slightly more condensed than in zygotene), diplotene (chromosomes with morphology of number eight and plus shaped indicating the places of cross over exchanges), diakinesis (chromosomes were further condensed and have assumed morphology of rings marking the chiasmata terminalisation), , metaphase I (with 50 bivalents) were clearly visible.

Figure 2: NOR-banding in *M. dayanum* a. NOR-banded metaphase complement (Arrows indicating the NOR regions) b. NOR-banded karyotype of *M. dayanum* (NOR bands on two sub-metacentric pairs)



The chromosomes of prawns of family Palaemonidae are not only very small in size and large in number but also showed a wide range of variations from species to species. The diploid number ranges from 56 in *Palaemon serratus* to 124 in *Macrobrachium villosimanus* (Chaudhary et al., 2013; Gonzalez-Tizon et al., 2013). However, except for *M. carcinus* (2n = 94), most *Macrobrachium* species possessed a diploid number either equal to or higher than 100 as *Macrobrachium siwalikensis* (2n = 100), *M. nipponense* (2n = 104), *M. idella* (2n = 104) and *M. scabriculum* (2n = 104), *Palaemon lamarrei* (2n = 118), *M. rosenbergii* (2n = 118), *Macrobrachium villosimanus* (2n = 124) (Mittal and Dhall, 1971; Vishnoi, 1972; Damrongphol et al., 1991; Qiu et al., 1994; Lakra and Kumar, 1995; Indy et al., 2009; Choudhary et al., 2013).

Figure 3: G-banding in *M. dayanum a.* G-banded metaphase complement b. G-banded karyotype

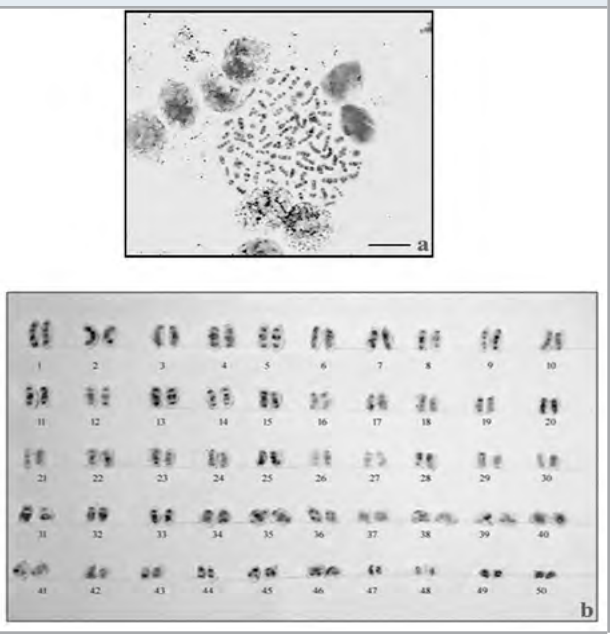
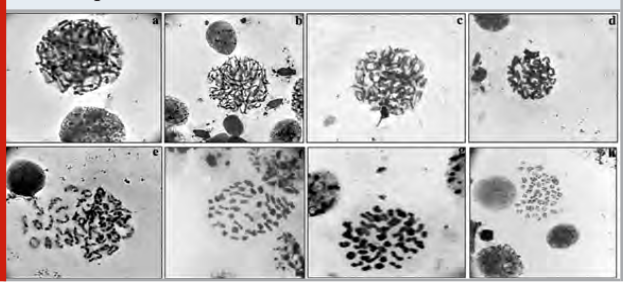


Figure 4: Meiotic stages observed in testicular tissue of *M. dayanum* a. Leptotene b. Zygotene c. Early Pachytene d. Late Pachytene e. Diplotene f. Diakinesis g. MetaphaseI h. MetaphaseII



The diploid number 2n=100 found in *M. dayanum* is consistent with the diploid number found in other congeneric species. NOR- and G-banding results of present study are found to be in accordance with the chromosomal banding analysis in *Macrobrachium villosimanus* and *Macrobrachium lanchesteri* (Choudhary et al., 2013; Phimphan et al., 2018).

Table 2. Karyomorphometric data of *Macrobrachium dayanum* (male)

Chromosome pair No.	Mean length of Short arm (p) (µm)	Mean length of Long arm (q) (µm)	Absolute length (p+q) (µm)	Arm ratio (q/p)	Relative length %age	Centromeric index	Nomenclature
1.	0.37	0.66	1.03	1.78	3.98	35.9	Sub-metacentric
2.	0.38	0.62	1.0	1.63	3.87	38	Metacentric
3.	0.24	0.73	0.97	3.04	3.75	24.7	Sub-telocentric
4.	0.36	0.60	0.96	1.66	3.71	37.5	Metacentric
5.	0.35	0.59	0.94	1.68	3.64	37.2	Metacentric
6.	0.32	0.59	0.91	1.84	3.52	35.16	Sub-metacentric
7.	0.33	0.56	0.89	1.69	3.44	37.07	Metacentric
8.	0.31	0.56	0.87	1.80	3.36	35.63	Sub-metacentric
9.	0.33	0.54	0.87	1.63	3.36	37.9	Metacentric
10.	0.31	0.52	0.83	1.67	3.21	37.3	Metacentric
11.	0.30	0.48	0.78	1.6	3.02	38.4	Metacentric
12.	0.28	0.44	0.72	1.57	2.78	38.8	Metacentric
13.	0.28	0.43	0.71	1.53	2.74	39.4	Metacentric
14.	-	0.70	0.70	-	2.71	-	Telocentric
15.	-	0.70	0.70	-	2.71	-	Telocentric
16.	0.27	0.40	0.67	1.48	2.59	40.29	Metacentric
17.	0.26	0.39	0.65	1.5	2.51	40	Metacentric
18.	0.25	0.38	0.63	1.52	2.43	39.6	Metacentric
19.	0.24	0.37	0.61	1.54	2.36	39.3	Metacentric
20.	0.14	0.43	0.57	3.07	2.20	24.5	Sub-telocentric
21.	0.14	0.42	0.56	3.0	2.16	25	Sub-metacentric
22.	0.13	0.42	0.55	3.23	2.13	23.6	Sub-telocentric
23.	0.16	0.39	0.55	2.43	2.13	29.09	Sub-metacentric
24.	0.15	0.38	0.53	2.53	2.05	28.3	Sub-metacentric
25.	0.19	0.31	0.50	1.63	1.93	38	Metacentric
26.	0.19	0.29	0.48	1.52	1.85	39.5	Metacentric
27.	0.09	0.36	0.45	4	1.74	20	Sub-telocentric
28.	0.18	0.27	0.45	1.5	1.74	40	Metacentric
29.	0.18	0.27	0.45	1.5	1.74	40	Metacentric
30.	0.17	0.27	0.44	1.58	1.70	38.6	Metacentric
31.	0.13	0.26	0.39	2.0	1.51	33.3	Sub-metacentric
32.	0.15	0.24	0.39	1.6	1.51	38.4	Metacentric
33.	0.04	0.32	0.36	8	1.39	11.12	Telocentric
34.	0.13	0.22	0.35	1.69	1.35	37.14	Metacentric
35.	0.08	0.24	0.32	3	1.23	25	Sub-metacentric
36.	0.07	0.23	0.30	3.2	1.16	23.3	Sub-telocentric
37.	-	0.29	0.29	-	1.12	-	Telocentric
38.	-	0.29	0.29	-	1.12	-	Telocentric
39.	0.12	0.16	0.28	1.33	1.08	42.8	Metacentric
40.	0.12	0.16	0.28	1.33	1.08	42.8	Metacentric
41.	0.12	0.12	0.24	1	0.92	50	Metacentric
42.	0.11	0.12	0.23	1.09	0.89	47.8	Metacentric
43.	0.10	0.11	0.21	1.1	0.81	47.6	Metacentric
44.	0.09	0.10	0.19	1.1	0.73	47.3	Metacentric
45.	0.09	0.09	0.18	1	0.69	50	Metacentric
46.	-	0.16	0.16	-	0.61	-	Telocentric
47.	0.01	0.12	0.13	6	0.50	7.69	Sub-telocentric
48.	0.06	0.06	0.12	1	0.46	50	Metacentric
49.	0.05	0.05	0.10	1	0.38	50	Metacentric
50.	0.02	0.02	0.04	1	0.15	50	Metacentric

CONCLUSION

The present study is the first report on karyotype and chromosomal banding in *Macrobrachium dayanum* from UT of J&K. The diploid number observed to be 100 with the karyotypic formula 60m+16sm+12st+12t. Numerous gene expressions regions i.e. NORs were located on two submetacentric pairs by silver staining. Alternate light and dark bands on chromosomes depicting GC and AT rich regions were revealed by G banding. The present work will serve as baseline for the genetic improvement, hybridisation experiments, conservation and management programmes for *Macrobrachium dayanum*. The data obtained in current study will help the researchers in prawn systematics for the valid species identification. Further fluorescence in situ hybridisation and molecular studies like mitochondrial DNA analysis, 16S rRNA analysis, microsatellite analysis and DNA sequencing would strengthen the field of prawn genetics in Jammu region.

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Conflict of Interest: The authors declare no conflict of interest.

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Isolation and Characterization of Escherichia coli from Rivers of Trivandrum City and Assessment of its Antibiotic Sensitivity

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ABSTRACT

Water pollution is a major problem which arises due to different human activities and the availability of pure water for human consumption is decreasing gradually. One of the predominant causes of water pollution is the deposition of fecal matters and thereby spreading microorganisms which can cause serious water borne diseases. Enterobacteria like *E. coli* are the major indicators of fecal contamination in the water bodies. The present study was conducted to explore the presence of *E. coli* from the two major rivers, located in two different places of the Trivandrum city, which are highly depended by the citizens. The presence of *E. coli* in the water samples were confirmed by several biochemical tests and the molecular confirmation of *E. coli* was done through polymerase chain reaction for 16S rRNA. Antibiotic sensitivity of *E.coli* against the commonly used antibiotics was also determined in this study. This study of isolation and characterization of *E. coli* from the two major rivers in Trivandrum and comparing their antibiotic sensitivity is first of its kind. Although water quality analysis of the water samples from these rivers have been done to study the physio-chemical parameters and total coliforms present, no attempts have been made to assess the antibiotic sensitivity of *E. coli* isolated from the samples. Our study was carried out to see the antibiotic sensitivity of the *E. coli* isolated and future studies could confirm if these can act as the indicators of water quality. Through this analysis we have concluded the results which can support future research by acting as a credible milestone.

KEY WORDS: ANTIBIOTIC SENSITIVITY, DRINKING WATER, *E. COLI*, INDICATOR, MICROBIOLOGICAL QUALITY, TRIVANDRUM RIVER

INTRODUCTION

Water is an essential component consumed in the greatest quantity around the world. It is the most vital element for life, procured from natural sources such as rivers, underground water, and lake water. Consequently, large number of health risks are associated with consumption of contaminated water. Drinking water should be safe and free from chemical toxins and pathogenic microorganisms. Accessibility and availability of fresh clean water not only plays a crucial role in economic development and social welfare, but also it is an essential element in health, food production and poverty reduction (Eckner, 1998; Odonkor and Ampofo., 2013). Methods had been being methods have been developed since 1900s to assess water quality regarding public health by enumerating coliforms and *Escherichia coli* cells in water as indicators of water purity. *E. coli* are widely

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distributed in the gastro-intestinal tract of humans, pests, ruminants, and wild animals, where they are known to live as commensals (Eckner, 1998; Feng et al, 2009). In normal habitat, *E.coli* is beneficial for digestion. Beyond certain limit or by ingestion of contaminated food and water, toxin produced by the bacteria cause infection in the cells of intestinal tract, enter into the blood and finally leads to many diseases (Adzitey et al., 2015).

The presence of *E. coli* in food or water indicates that there is an elevated risk of the presence of other enteric bacteria and viruses, such as Salmonella spp. Shigella or hepatitis A virus, etc. Therefore *E. coli* is universally considered as an indicator organism of fecal contamination in food and water samples and to compare the degree of contamination (Odonkor and Ampofo., 2013). *E.coli* O157:H7 was first human pathogen. Some of the pathogenic strains includes Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Enterohemorrhagic *E. coli* (EHEC) etc (Ebomah et al., 2018; Rayasam et al., 2019). *Escherichia coli* is a useful enteric bacterium in the study of waterborne transfer of antibiotic resistance. A lot of studies have brought out the existence of antibiotic resistant microorganisms and their prevalence in aquatic bodies (Nahar et al., 2019). Among such antibiotic resistant organisms, *E. coli* is a major candidate. Antibiotic resistance is a worldwide obstacle in therapeutics and new forms of antibiotic resistance are arising which can spread all over the world easily (Dhawde et al., 2018; Bong et al, 2020).

The use of antibiotics to combat infections in humans and other animals is a common practice, but indiscriminate use of antibiotics leads to drug resistance in these microbes, which warrants the initiation of steps to prevent public health hazards (Rather et al., 2012). Antibiotic sensitivity test is used to help to choose the antibiotics effective against the specific types of bacteria. The disk diffusion method is the gold standard for confirming the susceptibility of bacteria to various antibiotics. Some types of bacteria are resistant to certain antibiotics because of their genetic material. Infection caused by the resistant bacteria is not cured by treatment with those antibiotics. Polymerase Chain Reaction (PCR) is a molecular biology technique used for enzymatically replicating DNA of organisms (Rahman et al., 2013).

Molecular methods for the detection of *E. coli* in food and water have mainly concentrated on the use of PCR gene probe technology. However, there are a few reports on the potential use of 16s rRNA gene target method for the detection of *E. coli* (Bej et al., 1991; Fattahi et al., 2013). Using conserved sequences, flanking variable region as primers, the sequence of the variable region of the 16s rRNA gene could be amplified. Several studies have been carried out to isolate and characterize *E. coli* from major rivers on a global scenario (Tsen et al., 1998; Bong et al., 2020; Praveenkumarreddy et al., 2020). Most of these studies would confirm the presence of *E. coli* as indicator of the water quality and thus an indicator organism. Some of these studies would also focus on analyzing the antibiotic sensitivity of the isolates as indexing antimicrobial resistance has significance in clinical domain (Dhawde et al., 2018; Odonkor and Addo., 2018; Nahar et al., 2019; Purohit et al., 2020).

MATERIAL AND METHODS

The water samples were collected from selected stations from Killiyar river and the Vamanapuram river located near to the college where the present study was conducted. The water samples were collected and stored in sterile screw capped containers and transported to lab. For the isolation and biochemical analysis for identification of *E. coli*, the water samples were serial diluted in lactose broth to reduce the density of the culture to more usable concentrations to carry out MPN technique to estimate the viable number of organisms in the sample. The diluted samples were incubated for 24 hours at room temperature. After incubation, a loopful of the enriched culture from lactose broth of the presumptive tests was streaked onto EMB Agar and incubated at 37°C for 24 h. For the completed test, the pure colonies from the incubated EMB plates were cultured in lactose broth or nutrient agar plates and, gram staining and motility of the organism was performed.

The microbial motility was checked by hanging drop method and agar stab method. Biochemical tests are performed for the further identification and confirmation of the organism. The biochemical test performed were: IMViC Test (Indole test, Methyl Red test, Voges-Proskauer test and citrate test), Catalase test, Urease test, Motility Indole Urease test (MIU), Triple sugar iron test. To determine the antibiotic sensitivity by disc diffusion method, the Kirby-Bauer disc diffusion method was used. It was performed in Muller Hinton agar plates. Six antibiotics were tested: Tetracycline (10 µg), Gentamycin (10 µg) Ciprofloxacin (5 µg), Amoxicillin (10 µg), Ampicillin (10 µg), Cefixime (5 µg). A Muller-Hinton medium plate was swabbed with LB broth inoculated with *E. coli* overnight. Sterile discs were impregnated with each of the antibiotics and later, the antibiotic impregnated discs were placed properly above the uniformly spread inoculum containing plates with sterile forceps under aseptic conditions. The plates were incubated for 48 hours at room temperature. By using a scale, the zone of inhibition was measured after incubation.

PCR was conducted after the isolation of DNA from the bacterial samples. The latter was performed using phenol: chloroform extraction method. The *E. coli* cells which were cultured overnight in LB broth was selected for DNA extraction. DNA was extracted from exponential cultures by alkaline lysis with 0.5% of sodium dodecyl sulphate treatment, followed by alkaline lysis. The impurities were removed by the treatment with phenyl chloroform - isoamyl alcohol (24:24:2) extraction. DNA was then precipitated by 2.5 volume of isopropyl alcohol and pelleted by centrifugation. The DNA pellet was washed

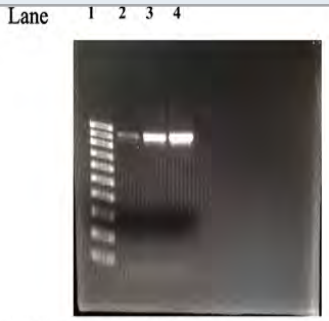
once with 70% alcohol and dried under vacuum. After centrifugation, the DNA was resuspended in TE buffer (Bej et al., 1991).

For the PCR reaction, the primers targeting variable regions of the *E. coli* 16S rRNA gene were developed by Indigenous DNA pvt. Ltd., and the primers were BGT24238 (*E. coli* forward -5'AGAGTTTGATCCTGGCTCAG3') and BGT24239 (*E. coli* reverse- 5'CTTGTGCGGGCCCCCGTCAATTC3'). The PCR solution contained 1X PCR buffer (10X PCR reaction buffer contains 500 µM KCl, 500 µM tris-HCl, (pH 8.3) and 25 µM $MgCl_2$), 200 µM each of the dNTPs, (Perkin- Elmer Cetus) 0.2-0.6 µM each of the primers, 2.5 U of DNA Taq polymerase and the template DNA. The total volume of PCR reaction was 100 µL. For each PCR cycle, the denaturation temperature was 94°C for 1 minute, annealing and extension temperatures 56°- 60°C and 70°C for 30 seconds respectively. The PCR products were examined by agarose gel electrophoresis using ethidium bromide dye.

Table 1. Characteristics of bacteria isolated from water samples

	TEST	OBSERVATION	RESULTS
	Indole production	Appearance of red color band at the junction of medium and reagent	Positive
Water samples from Killiyar and Vamanapuram rivers	Methyl Red	Appearance of red color	Positive
	Voges-Proskauer	No color change	Negative
	Simmon's citrate	No color change	Negative
	Catalase test	Production of gas bubbles	Positive
	Motility	Motile	Motile
	Gram's staining	Appearance of pink color	Gram negative
	MPN	Gas production	Positive
	Urease	No colour change	Negative
	TSI Agar	Yellow slant and yellow butt	A/A
	MIU Test	Bacterial growth occurs throughout the agar	Positive

Figure 1: Agarose gel electrophoresis image of PCR products after amplification of 16S rRNA for E. coli.
Lane 1 : 100 bp DNA ladder Lane 2 : positive control
Lane 3 : Amplicon from sample A (Killiyar river)
Lane 4 : Amplicon from sample A (Vamanapuram river)



Agarose gel electrophoresis (1.5%) of PCR products after amplification of 16S rRNA for E. coli. Lane 1: 100 bp DNA ladder, Lane 2: positive control Lane 3: E coli isolated from sample A Lane 4: E coli isolated from sample B

RESULTS AND DISCUSSION

Coliform such as *E. coli* have been widely used as indicator of the microbiological quality of surface and ground water. Thus, the presence of coliform is an index of bacteriological quality of water. So, in the present study, water samples were collected from two major rivers (i.e., Killiyar and Vamanapuram) in the Trivandrum City and analyzed for the presence of coliforms isolated and the antibiotic sensitivity of the samples were investigated. In the standard MPN test, the presumptive tests showed the presence of gas production in the tubes containing lactose broth with inverted Durham tube, inoculated with the water samples. It indicates the presence of lactose fermenting coliforms in both of the water samples. After incubation, the sample showed turbidity indicating the growth of coliforms. The confirmed test showed small colonies with green metallic sheen on EMB agar which confirms the presence of *E. coli* bacteria. The completed test gave final confirmation that the organism is Gram-negative, non-spore forming, rod shaped, lactose fermenting coliforms. Both hanging drop method and agar stab method showed high bacterial motility of the microbes in the sample (Sreelekshmi et al., 2020).

Upon Gram staining, the colonies showed pink coloration which is a characteristic of gram-negative bacteria. Biochemical analysis also helped to conclude that the water samples from the Killiyar river and the Vamanapuram river contained Coliform bacteria (Table 1). The isolates were confirmed to be *E. coli* by molecular analysis by the amplification of 16S rRNA. The antibiotic sensitivity of *E. coli* against some commonly used antibiotics such as Cefixime, Ciprofloxacin, Tetracycline, Gentamycin, Ampicillin and Amoxycillin was checked by the Kirby-Bauer disc diffusion method. The sensitivity range was observed by analyzing the diameter of Inhibition zone (in mm) on the 48 hours incubated MHA plates (Fig.1). The range of

inhibition zones are shown in table 2. The values clearly indicate that these *E. coli* isolates are highly sensitive to Cefixime, Ciprofloxacin, Gentamycin and least sensitive to Ampicillin and Amoxycillin. From these observations it was confirmed that the antibiotic sensitivity range of the isolated *E. coli* from both the rivers against the above antibiotics were almost similar indicating the strain similarities of both of the isolates (Sreelekshmi et al., 2020).

Figure 2: Antibiotic sensitivity test by Kirby-Bauer disk diffusion method using MHA Agar plates, showing antibiotic sensitivity pattern of *E. coli* isolates from Samples A and B (Killiyar and Vamanapuram rivers).

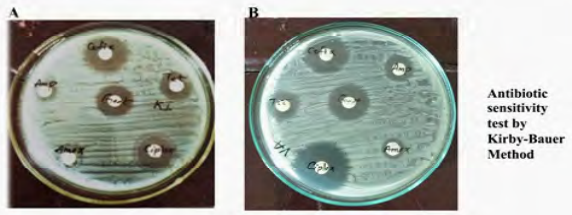


Table 2. The Zone of inhibition measurements

ANTIBIOTIC	ZONE OF INHIBITION	
	WATER SAMPLEFROM KILLIYAR RIVER	WATER SAMPLE FROM VAMANAPURAM RIVER
Cefixime (5µg)	22 mm	23 mm
Ciprofloxacin (5 µg)	20 mm	24 mm
Tetracycline (10 µg)	17 mm	16 mm
Gentamycin (10 µg)	16 mm	19 mm
Ampicillin (10 µg)	16 mm	15 mm
Amoxycillin (10 µg)	8 mm	10 mm

Previous studies have looked at the water quality of rivers like Vamanapuram and Karamana with emphasis to the physio-chemical parameters and presence of total coliforms, but no attempts were done to isolate or characterize *E. coli* from the samples and perform a comparative analysis. Although certain studies have shown the En-Antimicrobial resistance of bacteria isolated from various stations at Karamana river, the sites of present study were not included. Thus, the present study characterizes the *E. coli* isolated from two major rivers in Trivandrum city which is highly depended by the citizens and looks at the antibiotic resistance of the bacteria (Athira, 2019; Sreelekshmi et al., 2020).

CONCLUSION

The water samples were collected from selected stations on Killiyar and Vamanapuram rivers in Trivandrum city where anthropogenic activity is remarkably high. A major population in the city is depending upon these rivers for drinking water. Several biochemical analyses of the river samples revealed the presence of *E. coli.* which could be an indicator of poor water quality of the samples. The presence of *E. coli* was also confirmed by 16S analysis. Both the water samples tested had *E. coli* that were sensitive to the antibiotics with maximum sensitivity towards cefixime when compared to other antibiotics and most resistant to Amoxyllin, which is alarming as it is a commonly used antibiotic (Table 2). The antibiotic sensitivity range of the isolated *E. coli* from both the rivers against the tested antibiotics were almost similar indicating the possibility of strain similarities between the isolates. This study is the first of its kind which characterizes and compares the antibiotic sensitivity of *E. coli* in the heart of Trivandrum city isolated and characterized from these two major rivers. Future studies could confirm the possibility of choosing these strains as an indicator organism of water quality analysis. Further this study cautions the use of water from these rivers as they are subjected to contamination by coliforms

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Conflict of Interest: The authors declare no conflict of interest.

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Assessment of Treatment Needs in Orthognathic Patients in a Dental University Hospital in Saudi Arabia

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ABSTRACT

The present study was aimed to assess the treatment needs amongst orthognathic patients attending the Dental University Hospital at King Saud University using the Index of Orthodontic Treatment Need, Dental Health Component (IOTN-DHC) and the Index of Orthognathic Functional Treatment Need (IOFTN). A retrospective study was conducted on records of subjects who had been attending the Dental University Hospital at King Saud University, Riyadh Saudi Arabia, seeking orthodontic/surgical treatment in the period from 2000 to 2017. The pre-treatment sets of study models with their correspondent clinical photographs and radiographs were graded using the IOTN-DHC and the IOFTN. These assessments were undertaken by two calibrated dentists. The Class III skeletal pattern was the most prevalent type of malocclusion (54.5%). In total, 78.2% of the sample was classified by the IOFTN as having great and very great functional needs, as opposed to 91% classified by the IOTN. The most prevalent IOFTN score was 5.4 (open bite ≥ 4 mm, 25.4%), followed by 5.3 (reverse OJ ≥ 3 mm, 18.2%) and 4.2 (increased OJ ≥ 6 mm and ≤ 9 mm, 11%). The IOTN and IOFTN indices were highly correlated in assessing treatment needs for craniofacial problems. The IOFTN is a valid and reliable tool to prioritize treatment addressing functional needs. It is highly correlated with the IOTN in the prioritization of healthcare. The vast majority of patients undergoing orthognathic surgical procedures at the dental university hospital were in the great and very great need categories, and the Class III pattern was the most common type of malocclusion to be addressed by an orthognathic approach.

KEY WORDS: INDICES, IOTN, IOFTN, FUNCTIONAL NEEDS.

INTRODUCTION

The role of indices in healthcare includes classification of diseases, which can aid in understanding etiology, determining prognosis and possible treatment options, measuring the prevalence and incidence of a disease within a population, and prioritizing healthcare among individuals. Regardless of their purposes, simplicity and clarity, accessibility and feasibility, objectivity, amenability to statistical analysis, sensitivity, reliability, and validity, which is measuring what is supposed to be

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measured , are the key requirements for developing an ideal index of health, (Waring 2003, Barber 2017).

The word malocclusion lacks an adequate definition because of the wide variations among individuals in the perception of what constitutes an occlusal problem (Bellot-Arcís 2012). Hence, a number of indices are used to prioritize treatment in those with occlusal disorders and monitor the quality of their treatment outcomes, including the Index of Orthodontic Treatment Need (IOTN) (Brook 1989), the Dental Aesthetic Index (DAI) (Cons 1986), the Index of Complexity, Outcome and Need (ICON) (Daniels 2000), the Peer Assessment Rating (PAR) (Richmond 1992), and the Occlusal Index (OI) (Summers 1971).

Dentofacial deformity describes a condition in which there are significant deviations in the maxillo-mandibular complex from normal proportions that also negatively affect the intra-arch and inter-arch relationships. Furthermore, breathing, speech, swallowing, chewing, lip closure, and psychosocial health can be adversely affected (Posnick 2013). Consequently, subjects with dentofacial deformities usually require a combination of orthodontic treatment and orthognathic surgery as part of an interdisciplinary approach to reposition the jaw to achieve a normalized and functional relationship. This may involve surgical procedures on the maxilla, mandible, or both jaws, as well as their dentoalveolar segments. Reports indicate that approximately 19% of individuals who attend an orthodontic assessment ideally require orthognathic procedures (Posnick 2013, Olkun et al 2019 Eslamian 2019).

The IOTN is the index most commonly used for prioritizing treatment. It has a dental health component (DHC), which is a modification of the index of treatment priority developed by the Swedish Dental Health Board (Linder-Aronson 1974), and an aesthetic component (AC) that was adapted from the Standardized Continuum of Aesthetic Need (SCAN) index (Evans 1987), both of which record the need for treatment based on dental health, functional grounds, and social-psychological grounds (Brook 1989). The IOTN has been widely applied in the UK National Health Services (NHS) primary care since 2006 (Ireland 2014). It has obtained a high level of agreement amongst examiners compared to different occlusal orthodontic indices (Brook 1989).

Table 1. Inter-operator agreement for the major categories.

IOTN			IOFTN		
Examiner	Intraclass correlation	95% confidence interval	Examiner	Intraclass correlation	95% confidence interval
1	0.95	0.83 to 0.99	1	0.92	0.70 to 0.98

Table 2. Intra-operator agreement for the major categories.

IOTN			IOFTN		
Examiner	Intraclass correlation	95% confidence interval	Examiner	Intraclass correlation	95% confidence interval
1	1.00	1.00 to 1.00	1	0.98	0.94 to 0.99
2	0.96	0.83 to 0.99	2	0.93	0.73 to 0.98

Table 3. Sagittal skeletal pattern distribution in the study sample.

Skeletal Discrepancies	Frequency	Percent
Class I	9	16.4
Class II	16	29.1
Class III	30	54.5
Total	55	100

Moreover, the DHC also shows strengths in the aspects of both time and ease of use (Cardoso 2011). This might be related to the acronym MOCDO (missing, overjet, crossbite, displacement of contact points, and overbite), which is used as a hierarchical scale to grade malocclusion (Richmond 1994). Therefore, the IOTN is the most frequently used index in orthodontic research (Bellot-Arcís 2012). In terms of grading, the DHC appears more reliable in providing constant grading over time, while the AC typically shows improvements during adolescence (Cooper 2000). The purpose of the IOTN-

DHC is to assign a score to the occlusal traits that make up a malocclusion. The grading process categorizes the severity and need for treatment from 1 to 5, with grade 1 representing no need for treatment and grade 5 representing a significant need for treatment (Appendix, Table A).

Table 4. Distribution of the IOFTN functional need scores categories in the study sample.

IOFTN	Gender		Total (%)
	Male	Female	
1.14	2	2	4 (7.3)
2.9	0	1	1 (1.8)
2.11	0	1	1 (1.8)
3.3	2	3	5 (9.1)
3.4	0	1	1 (1.8)
4.1	2	1	3 (5.5)
4.2	2	4	6 (10.9)
4.4	1	3	4 (7.3)
4.8	0	1	1 (1.8)
5.2	2	2	4 (7.3)
5.3	8	2	10 (18.2)
5.4	5	9	14 (25.4)
5.7	0	1	1 (1.8)
Total	24	31	55

Table 5. Distribution of the IOTN Dental Health Components in the study sample.

IOTN (DHC)	Gender		Total (%)
	Male	Female	
2b	0	1	1 (1.8)
3b	2	0	2 (3.6)
3d	0	1	1 (1.8)
3e	0	1	1 (1.8)
4a	1	1	2 (3.6)
4b	2	0	2 (3.6)
4c	0	2	2 (3.6)
4d	1	2	3 (5.5)
4e	2	3	5 (9.1)
4h	7	9	16 (29.1)
5a	2	3	5 (9.1)
5h	5	4	9 (16.4)
5i	0	3	3 (5.5)
5m	2	1	3 (5.5)
Total	24	31	55

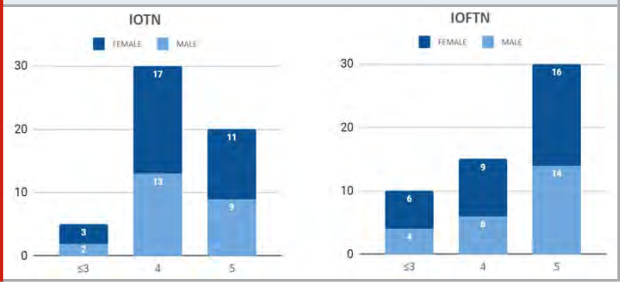
However, there are some limitations in the use of the IOTN. In cases of the DHC, those with functional or facial concerns arising from dentofacial deformities and those not amenable to orthodontic treatment alone are not included. As a result, Ireland et al. recently established a new index, known as the Index of Orthognathic Functional Treatment Needs (IOFTN). Similar to the IOTN-DHC, the IOFTN has five grades –grade 1 shows no need for treatment and grade 5 shows a significant need for treatment (Appendix, Table B). Modifications and additions to the subcategories within the major categories were introduced to reflect the functional need for treatment indicated for orthognathic patients. Generally, the index will be applied to those with complete facial growth (Ireland 2014).

Up to our knowledge there has been no attempt to evaluate the need and complexity of individuals undergoing orthodontics with surgical approach in a university setting in Saudi Arabia. Hence, the aim of this retrospective study was to assess the treatment needs among orthognathic patients attending the Dental University Hospital at King Saud University using the IOTN-DHC and the IOFTN.

MATERIAL AND METHODS

This retrospective study was conducted on records of subjects who had been attending the Dental University Hospital at King Saud University, Riyadh Saudi Arabia seeking orthodontic/surgical treatment in the period from 2000 to 2017. Ethical approval for this study was obtained from the Institution Review Board at the College of Medicine, King Saud University, Riyadh KSA (E-17-2644; 06/11/2017). The collected records included pre-treatment study models, photographs and orthopantomographs (OPGs), lateral cephalometric radiographs, and relevant demographic information. Incomplete records, such as missing or damaged study models, missing or poor-quality photographs, and missing or poor-quality radiographs, were excluded from the study.

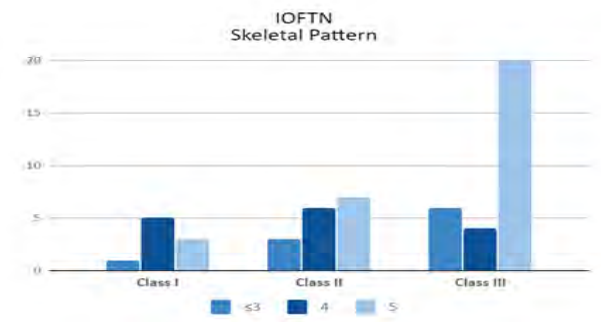
Figure 1: Distribution of IOTN (DHC) and IOFTN scores between genders.



For all selected samples, demographic characteristics, including age and gender, were recorded. The pre-treatment sets of the study models, with their correspondent clinical photographs, were graded using the IOTN-DHC and the IOFTN. OPG radiographs were

used to assess relevant clinical information, such as impacted teeth, missing teeth, and supernumerary teeth. The pre-treatment cephalometric radiographs were used to assess the anteroposterior skeletal relationship. Measurements and assessments were performed by two calibrated dentists. These measurements were done twice over a 10 day interval to assess the inter- and intra-operator agreement.

Figure 2: Relationship between sagittal skeletal patterns and IOFTN grades.



Statistical Analysis: Descriptive statistics (mean, standard deviation, frequencies) were used to describe the quantitative and categorical variables. Intraclass correlation was used to quantify the inter-operator and intra-operator consistency in the assessment of the IOFTN and IOTN scale levels. Spearman's rank correlation was used to quantify the relationship between the two indices. The frequencies of the different components of the IOFTN and IOTN between different genders and malocclusions were compared using Pearson's chi-square test. Data were calculated using IBM® SPSS® Statistics, Version 22 (International Business Machines Corporation; Armonk, New York, USA) at a predetermined significance level of $p < 0.05$.

RESULTS AND DISCUSSION

In total, 80 subjects were part of the study; 25 subjects were excluded from the study because of incomplete records. Thus, 55 participants were included in this study. Among these subjects, there were 31 females (56.4) and 24 males (43.6). The age ranged from 18–39 years, with a mean age of 21.3 years and a standard deviation 4.6.

Table 6. Spearman's Correlation between the two indices.

			IOFTN Examiner 1	IOFTN Examiner 1	P Value
Spearman's rho	IOFTN Examiner 1	Correlation Coefficient	1	0.328	0.05
		Significance (two-tailed)	-	0.014	
	IOTN Examiner 1	Correlation Coefficient	0.328	1	
		Significance (two-tailed)	0.014	-	
		IOFTN Examiner 2	IOFTN Examiner 2		
	IOFTN Examiner 2	Correlation Coefficient	1	0.408	
		Significance (two-tailed)	-	0.002	0.01
	IOTN Examiner 2	Correlation Coefficient	0.408	1	
		Significance (two-tailed)	0.002	-	

Inter-operator agreements for the major categories of the IOTN and the IOFTN were highly correlated (Table 1); the intra-operator agreement for the IOFTN and IOTN was very good (Table 2).The Class III skeletal pattern was the most prevalent type of malocclusion (54.5%) (Table 3). According to Table 4, the most prevalent IOFTN score was 5.4 (open bite ≥ 4 mm, 25.4%), followed by 5.3 (reverse OJ ≥ 3 mm, 18.2%) and 4.2 (increased OJ ≥ 6 mm and ≤ 9 mm, 11%). Overall, the percentage of patients who underwent orthognathic surgery scoring grade 4 and grade 5 functional needs was 78.2% according to the IOFTN. The distribution of IOFTN grades is shown in Table 4, while the distribution of IOTN scores is shown in Table 5. In addition, 91% of the patients had great and very great needs, according to the IOTN-DHC.

Overall, Class III sagittal skeletal pattern subjects showed a higher percentage (63.3%) of IOFTN grade 5 (very great need); however, subjects with Class II skeletal patterns demonstrated a higher percentage of grade 4 (great need) (Figure 2). Spearman's correlation between the two indices revealed a highly significant correlation by the two examiners (Table 6). This level of significance is evidence of a sufficient sample size.

According to Ireland et al., the IOFTN was developed to overcome the limitations of the IOTN's DHC, which does not account for the skeletal components of malocclusion, as well as to assist in prioritizing public resources for orthognathic surgery (Ireland 2014). Reliability is an important requirement for an index. The present study

established a very good inter-operator agreement with the IOFTN, as in the results reported by Ireland et al. (0.64–0.88) (Ireland 2014). The inter-operator agreement for the IOTN demonstrated a very good agreement in contrast to the kappa scores reported by Brook and Shaw (0.73–0.79) (Brook 1989). The study showed a very good intra-operator agreement for the IOFTN, in contrast to the findings by Ireland et al. (0.53–0.80) (Ireland 2014). The IOTN intra-operator agreement ranged from a good to a very good agreement, which is comparable to the results reported by Brook and Shaw (0.75–0.84) (Brook 1989).

Appendix THE IOTN INDEX

Table A. The dental health component of the IOTN, adapted from Brook and Shaw (4).

Grade 1 (None)	
1	Extremely minor malocclusions including displacements less than 1 mm.
Grade 2 (Little)	
2a	Increased overjet 3.6–6 mm with competent lips.
2b	Reverse overjet 0.1–1 mm.
2c	Anterior or posterior crossbite with up to 1 mm discrepancy between retruded contact position and intercuspal position.
2d	Displacement of teeth 1.1–2 mm.
2e	Lateral or anterior open bite 1.1–2 mm.
2f	Increased overbite 3.5 mm or more, without gingival contact.
2g	Prenormal or post.
Grade 3 (Moderate)	
3a	Increased overjet 3.6–6 mm with incompetent lips.
3b	Reverse overjet 1.1–3.5 mm.
3c	Anterior or posterior crossbites with 1.1–2 mm discrepancy.
3d	Displacement of teeth 2.1–4 mm.
3e	Lateral or anterior open bite 2.1–4 mm.
3f	Increased and complete overbite without gingival trauma.
Grade 4 (Great)	
4a	Increased overjet 6.1–9 mm.
4b	Reverse overjet greater than 3.5 mm with no masticatory or speech difficulties.
4c	Anterior or posterior crossbites with greater than 2 mm discrepancy between retruded contact position and intercuspal position.
4d	Severe displacements of teeth, greater than 4 mm.
4e	Extreme lateral or anterior open bites, greater than 4 mm.
4f	Increased and complete overbite with gingival or palatal trauma.
4h	Less extensive hypodontia requiring pre-restorative orthodontic space closure to obviate the need for a prosthesis.
4l	Posterior lingual crossbite with no functional occlusal contact in one or both buccal segments.
4m	Reverse overjet 1.1–3.5 mm with recorded masticatory and speech difficulties.
4t	Partially erupted teeth, tipped and impacted against adjacent teeth.
4x	Supplemental teeth.
Grade 5 (Very Great)	
5a	Increased overjet greater than 9 mm.
5h	Extensive hypodontia with restorative implications (more than 1 tooth missing in any quadrant) requiring pre-restorative orthodontics.
5i	Impeded eruption of teeth (with the exception of third molars) due to crowding, displacement, the presence of supernumerary teeth, retained deciduous teeth and any pathological cause.
5m	Reverse overjet greater than 3.5 mm with reported masticatory and speech difficulties.
5p	Defects of cleft lip and palate.
5s	Submerged deciduous teeth.

THE IOFTN INDEX

Table B. The scoring system of the IOFTN, adapted from Ireland et al. (14).

Grade 1 (None)	
1.12	Speech difficulties.
1.13	Treatment purely for TMD.
1.14	Occlusal features not classified above.
Grade 2 (Little)	
2.8	Increased overbite, but no evidence of dental or soft tissue trauma.
2.9	Upper labial segment gingival exposure < 3 mm at rest with no evidence of gingival/periodontal effects.
2.11	Marked occlusal cant with no effect on the occlusion.
Grade 3 (Moderate)	
3.3	Reverse overjet ≥ 0 mm and < 3 mm with no functional difficulties.
3.4	Open bite < 4 mm with no functional difficulties.
3.9	Upper labial segment gingival exposure < 3 mm at rest, but with evidence of gingival/periodontal effects.
3.10	Facial asymmetry with no occlusal disturbance.
Grade 4 (Great)	
4.2	Increased overjet ≥ 6 mm and ≤ 9 mm.
4.3	Reverse overjet ≥ 0 mm and < 3 mm with functional difficulties.
4.4	Open bite < 4 mm with functional difficulties.
4.8	Increased overbite with evidence of dental or soft tissue trauma.
4.9	Upper labial segment gingival exposure ≥ 3 mm at rest.
4.10	Facial asymmetry associated with occlusal disturbance.
Grade 5 (Very Great)	
5.1	Defects of cleft lip and palate and other craniofacial anomalies.
5.2	Increased overjet > 9 mm.
5.3	Reverse overjet ≥ 3 mm.
5.4	Open bite ≥ 4 mm.
5.5	Complete scissors bite affecting whole buccal segment(s) with signs of functional disturbance and or occlusal trauma.
5.6	Sleep apnoea not amenable to other treatments such as MAD or CPAP (as determined by sleep studies).
5.7	Skeletal anomalies with occlusal disturbance as a result of trauma or pathology.

In the present sample, the most prevalent IOFTN score was 5.4 (open bite ≥ 4 mm, 25.4%), followed by 5.3 (reverse OJ ≥ 3 mm, 18.2%), and 4.2 (increased OJ ≥ 6 mm and ≤ 9 mm, 11%). The findings have been different in the other studies as a study conducted in a University Hospital in Iran and found that the most prevalent score was 5.3, followed by 4.2 and 4.3 (reverse overjet ≥ 0 mm and < 3 mm with functional difficulties) (Borzabadi-Farahani 2016). Harrington et. al.(2017) conducted a study in UK and reported that the most prevalent score was 5.2 (increased overjet > 9 mm), followed by 5.3 and 4.2. In Turkey, Olkun et. al.(2019) conducted study and found that the most prevalent score was 5.3, followed by 4.3 and 5.4 Another study in Iran (Eslamian 2019) reported that the most IOFTN score was 4.3, followed by 5.3 and 5.4.

The Class III skeletal pattern was the most prevalent (54.5%) sagittal skeletal relationship, which is similar to the findings of earlier workers, (Eslamian 2019, Olkun 2019, Harrington 2017, Lee 2014, Al-Deaiji 2001) and in contrast to (Borzabadi-Farahani 2016). These variations are most notably attributed to the different ethnic backgrounds of the samples. More than half of the subjects with the Class III skeletal pattern were categorized as having a great to very great functional need for orthognathic surgery, justifying the proposed treatment offered to these patients. According to the IOFTN, 78.2% of the patients were classified as having great or very great functional needs. This is dissimilar to previous findings in the UK, Iran and Turkey, reporting 88–95% of patients as having great or very great functional needs (Howard-Bowles 2017, Borzabadi-Farahani 2016, Harrington 2017, Olkun 2019, Eslamian 2019).

As stated in Howard-Bowles' study, the definition of occlusal traits within the major categories of the

IOFTN needs to be improved; moreover, a calibration course similar to that for the IOTN is required to reduce ambiguous interpretations of the traits described. Suggestions were made to propose a system resembling that of the IOTN (MOCDO) to ensure efficiency in scoring patients; hence, the acronym OOSGA would follow the hierarchy (overjet, overbite, scissor bite, gingival exposure, and asymmetry) (Howard-Bowles 2017). However, the IOFTN mostly assesses occlusal traits, ignoring the skeletal component of malocclusion. This is particularly important when assessing subjects with well compensated malocclusion, those who have had previous orthodontic treatment, or those who do not necessarily score high using the IOFTN but have severe sagittal, vertical, or transverse skeletal discrepancies.

There are shortcomings in the present study, one of which is that it is retrospective, cross-sectional, and single center in nature. Another limitation lies in the lack of skeletal discrepancy consideration in the use of the IOTN and IOFTN indices. It is imperative to consider that scoring with the IOFTN from study models will require additional information, mainly the presence of the patient to address some subcategories, such as facial asymmetry, upper labial gingival exposure, soft tissue trauma due to excessive overbite, sleep apnea, and any trauma or pathology causing skeletal anomalies with occlusal discrepancy.

CONCLUSION

The IOFTN is a valid and reliable tool for prioritizing treatment addressing functional needs. It is highly correlated with the IOTN in prioritizing healthcare. The vast majority of patients undergoing orthognathic surgical procedures at the Dental University Hospital at King Saud University were in the great and very great need categories. The most common type of malocclusion to be addressed through an orthognathic approach was the Class III pattern. These findings shed a light on the complexity of skeletal malocclusions undergoing orthognathic surgery. A comprehensive nationwide study evaluating the need and complexity of orthognathic surgeries are required, to support in legislations governing health services in the Kingdom.

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Paleo Environmental Analysis of Cretaceous Inoceramid Fossils of Bagh Beds from Eastern Region of India

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ABSTRACT

The present paleo environmental study has been performed with respect to bivalve fossil assemblages, explored in and around Bagh at tehsil Kukshi and tehsil Manawar of district Dhar of Madhya Pradesh, India. In mollusca, *Inoceramid* bivalves dominate in Bagh beds, they are sessile benthos so, the study of paleoenvironment of the Bagh beds can be figured out from them, particularly their abundant diversity and number may be due to the variation in the sea level and other oceanographic conditions. Their large number and varieties are found embedded in different levels of Nodular limestone. This study will help to reconstruct the idea about ecosystem of past time. The present approach is to step up paleoecology of excavated *Inoceramid* fossils of class bivalve of Cretaceous period obtained from different vicinities of Bagh beds of Dhar district. The collected specimens have been compared with previously collected specimens of bivalves from different parts of the world by many paleontologists to get more appropriate results. The intense and passionate examination of morphology and systematic study of *Inoceramid* fossils was needed for paleoecological study. Three species viz. *Inoceramus concentricus*, *Inoceramus concentricus var. baghensis* and *Inoceramus concentricus* var. *subsulcatus* of bivalves were excavated, their paleontology have also been described in the present paper. These explored species were also studied systematically and paleoecologically. The study also deals about the mode of life and the environment in which they lived. *Inoceramid*s are extinct bivalves but by homoplassic approach, it can be said that they were essentially sessile benthos and probably used their byssus for anchoring in high energy environment. In nature they are gregarious and independent of any sedimentary facies. They thrived on the shallow marine continental shelf as well as in estuarine conditions. They are cosmopolitan, may be due to their planktotrophic larval stage. The Bagh beds being a product of epicontinental Cretaceous sea, provide congenial environment for flourishing the *Inoceramid* bivalves.

KEY WORDS: BAGH BEDS, CRETACEOUS, FOSSILS, *INOCERAMID*, PALEOECOLOGY.

INTRODUCTION

Paleoenvironmental or paleoecology is the study of ecology in regards to fossil assemblages of the inhabitants, which lived in the past. It utilizes the details of collected fossils which can rebuild the ecosystems of the past. Eventually these studies may offer information on essential biological questions as the growth and reason of adaptive morphological alteration observed today with paleoenvironment. The Bagh bed is a noteworthy paleontological unit of the Narmada valley, which was formed by invading water of transgressing arm of Tethys Ocean.

The name "Bagh beds" comes from the type locality Bagh. Many macro invertebrate fossil fauna exists in Bagh beds including bivalves. The Paleoecology of bivalves of Bagh beds during Cretaceous period has been

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focused in the present research, which is illustrated by assorted assemblage of bivalves excavated from these beds.Currently the deposit from Bagh beds is dispersed sporadically, which was spread about 345 km away from Barwah (M.P.), east to Rajpipla (Gujarat) in the west. The Bagh beds can be discriminated in Eastern region which comprises the region of Barwah (Man valley), Bagh in the Dhar district and is extended upto Jobat in the Alirajpur - Jhabua district of M.P and Western region which covers the area from west of Alirajpur (MP India), all the way through Kawant up to Rajpipla in Baruch district, Gujarat India.

The Bagh beds comprise of Nimar Sandstones, Nodular Limestone and Bryozoan Limestone. They first appeared about 300 million years ago, in the middle Cambrian and were plentiful during the Mesozoic and Cenozoic eras, deep in the sea, ocean and streams. However, they were ample in the Silurian and Devonian period. One of the valuable biostratigraphic group of bivalves is *Inoceramids*,that disappeared at the end of the Cretaceous period. *Inoceramids* had a comparatively wide ecological tolerance at the genus and species level. This was more prominent group and abundant in quantity and diversity than any other group of bivalves in the study area. *Inoceramid* bivalves first existed in the Permian and became dominant during the Jurassic and Cretaceous period.

The *Inoceramid* are good bioindicators for considering the stratigraphy and age of rock formation. Kumar et al (2018) tried to solve confusion the age of the Nodular Limestone formation (Late Cretaceous) at sub stage level throughout ammonoid and Inoceramid index taxa. They also agreed to differentiate the three divisions (Early, Middle and Late) of the Turonian stage in the Narmada basin, Central India. Extensive *Inoceramus* fossil collection from late Cretaceous at Bagh beds has been done to construct lithological, biostratigraphic and chronostratigraphic framework. Even though among widespread information accessible concerning the entire fauna of the Bagh beds, it has become possible to interpret paleoecology with special reference to bivalves. Foremost involvement on invertebrate remnants of Bagh were studied by various paleontologists viz. Chiplonkar (1937-1942), Badve (1972), Ghare (1974), Dassarma and Sinha (1975), Nayak, (1983), Gangopadhyay & Bardhan (2007),Smith (2010), and Gangopadhyay & Maiti (2012), Pathrade et al. (2012), recently, Khatri and Pathrade (2016), and Kumar et al. (2018).

Further significant work on paleoecology on Bagh beds of Eastern Indian region has not been reported recently . In this part of research, the bivalve fossil fauna was collected and identified. They were then verified from Geological Survey of India" CHQ, Curatorial division, Kolkata, West Bengal. Many paleontologists worked on different aspects of paleontology, but work on paleoecology of bivalves (*Inoceramids*) of Bagh beds is very scanty, so this Paleoecological study of the collected bivalve fossil fauna has been explored to know their past environment. The objective was chosen to unknot the facts of their habitats and climate in the past.

MATERIAL AND METHODS

Location of the Study Area: The present paleontological research was performed in Dhar district of Madhya Pradesh. The most important outcrop of invertebrate fossils was noticed in and around Bagh of tehsil Kukshi and tehsil Manawar of district Dhar (Fig. 1, 2, and 3).

Figure 1: InferredseawayalongtheNarmada–Taptiriftint heDeccanvolcanicProvince,peninsularIndia (after Keller etal.,2009).

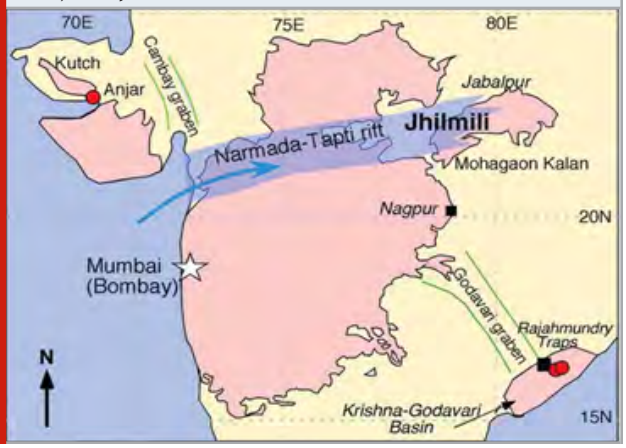


Figure 2: Field Photograph: Bagh Beds exposed near Gandhwani village

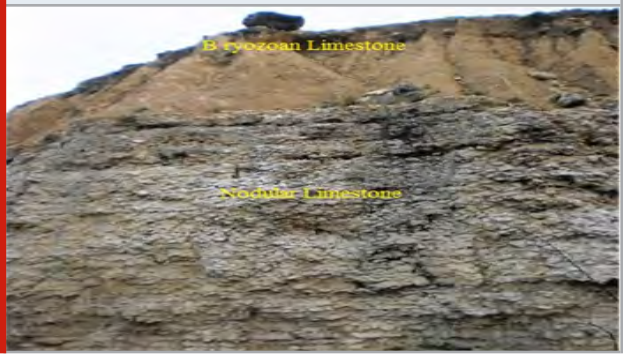
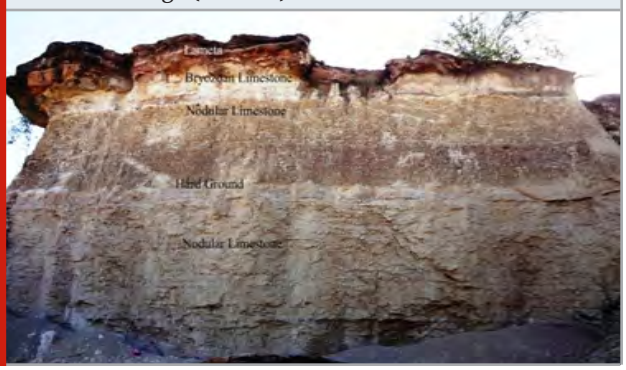


Figure 3: Field Photograph: Bagh Beds exposed near Khandlai village (Kukshi)



Survey of the fossiliferous areas in different villages has been done by land records. Stratigraphical and geological study of the fossiliferous rocks had also been done. The fossils were collected by excavating the area of Bagh beds up to depth of two to three meters. Some geological tools like hammer, variety of chisels and mallet were used to split and break rocks for excavating fossils. The extra matrix was then removed by using hand tools. Magnifying lens was used for the field study and identification of the collected fossils. Fossil specimens were numbered and their details were recorded in the field note book. The accurate site and the course of each fossil and position of every sediment layer in the stratigraphical sequence were noted. For detailed morphological information the fossil specimens were measured and photographed to the scale in various postures- dorsal, ventral, lateral etc. These fossils were identified and classified according to the Treatise on Invertebrate Paleontology by Moore, (1969) pt. N –Bivalvia vol. 1 and 2 Geol. Soc. Amer. and Univ. Kansas press.

RESULTS AND DISCUSSION

Assemblage Study of Bivalves: Few paleontologists have endorsed superficial oceanic/estuarine surroundings of depositions, while others have preferential of non-marine interpretation for its sediments. Only vigilant examination of morphology of fossils and information reported by many paleontologists help to judge the paleoecological nature of the region from where the fossils were collected. Three species of Bivalves have been excavated during the present research. These explored species were studied thoroughly and described here.

1. Inoceramus concentricus Parkinson, 1910 (Figure 4).

Figure 4: Inoceramus concentricus Parkinson, Right valve

Systematic Palaeontology: Class: Bivalvia Linne, 1758; Subclass: Pteriomorphia Beurlen, 1944; Order: Pterioida Newell, 1965; Sub Order: Pterriina Newell, 1965; Super Family: Pteriacea Gray, 1847; Family :*Inoceramidae Giebel*, 1852; Genus: *Inoceramus* Sowerby, 1814; Species: *Inoceramus concentricus* Parkinson, 1910.

Synonymy: 1822 *Inoceramus concentricus* Parkinson; Sowerby, p. 183, pl. 8CCV, figs. 1-6; 1828 *Inoceramus gryphaeoides*; Sowerby, p. 161, pl. dl xxxiv, fig. 1. 1846 *Inoceramus concentricus* Park. d' Orbigny, p. 566. 1846 *Inoceramus concentricus* Park.; Leymerie, pl. V, fig. 12.1877 *Inoceramus concentricus* Park.; Schliiter, p. 255.1911 *Inoceramus concentricus* Park.; Woods, p. 265, pl. XLVI, figs. 1-10.1912 *Inoceramus concentricus* Woods, p. 4, figs. 5-9. 1972 *Inoceramus concentricus* Park.; Badve, p. 235, pl. XXIII, figs. 1,8,9.1975 *Inoceramus concentricus* Park.; Dassarma and Sinha, p. 23, pl. I, fig. 4.

Material: One specimen, **Dimensions:** Length- 52 mm; Height-70 mm

Description: This species of *Inoceramus* is perceptibly high and apparent. It holds umbones which are bent and pointed anteriorly. It is up to 3/5th of the height from the umbones, the anterior is a slightly concave and then it merges with the ventral margin, comprehensive with a convex outline. The posterior side is extended out in the form of an ear and has a quite broad outline. Its dorsal side has a quite broad outline, at 1/3 rd of the height, the shell is tumid and thick. The valves have unequal convexity; as the left valve is slightly more convex. Prevailing depressions are visible on the surface which is also bowed with low folds. The one on the left valve is weaker and meager. The folds have about 4-5 concentric ribs and they finish posteriorly on the ear. The concave anterior region of the valves makes a right angle with the plane found in between the valves. The posterior part of the valve is slightly extended and is not as convex as the anterior portion under the umbones.

Discussion: The excavated species shows similarity with *Inoceramus concentricus* (Woods, 1911) from the upper Greensand. The only remarkable difference that can be seen in explored specimen has less convexity than other specimens. However its valve is considered as having more local disparity by Woods and hence this specimen could not be placed under different category.

Occurrence: Nodular Limestone at Chirakhan of district Dhar, Madhya Pradesh.

2. Inoceramus concentricus Parkinson var. baghensis, 1975 (Figure 5).

Systematic Palaeontology: Class: Bivalvia Linne, 1758; **Subclass:** Pteriomorphia Beurlen, 1944; Order: Pterioida Newell, 1965; Sub Order: Pterriina Newell, 1965; Super: Family: Pteriacea Gray, 1847; Family: *Inoceramidae Giebel*, 1852; Genus: *Inoceramus* Sowerby, 1814; Species: *Inoceramus concentricus* Parkinson var. *baghensis*, 1975

Synonymy: 1975 *Inoceramus concentricus* Parkinson var. baghensis; Dassarmaand Sinha, p. 24, Pl. I, fig. 5.

Material: Two specimens **Dimensions:** Length - 79 mm; Height- 94 mm; Thickness -32 mm.

Figure 5: Inoceramus concentricus Parkinson var. baghensis

Description: The shell has thin test and is of medium size. Its shape is ovate and inequilateral. Antero-posteriorly and dorso-ventrally the left valve is inflated and is convex. Postero-dorsal is broadly arched, ventral margin is narrowly curved, posterior broadly arched and postero-dorsal margin is almost straight, while the antero-dorsal margin area is slender and straight, antero-ventral is smooth and rounded. The surface is deliberated and adorned with a vast number of closely placed concentric rings and low broad concentric ridge. The ridges are still stronger, raised to a large extent, and set apart by narrow deep depressions ventrally.

Discussion: The collected specimen of *Inoceramus* showing resemblance in shape and size with the shell and the ornamentation of the Inoceramus concentricus Parkinson var. baghensis formerly illustrated by Dassarma and Sinha,(1975) from the Man valley, Dhar district of Madhya Pradesh. Occurrence: Nodular Limestone at Rampura and Bagh of Dhar district of Madhya Pradesh.

3. Inoceramus concentricus Parkinson var. subsulcatus Willshire, 1910 (Figure 6)

Systematic Palaeontology: Class: Bivalvia Linne, 1758; Subclass: Pteriomorphia Beurlen, 1944; Order: Pterioida Newell, 1965; Sub Order: Pterriina Newell, 1965; Super Family: Pteriacea Gray, 1847; Family: *Inoceramidae* Giebel, 1852; Genus: *Inoceramus* Sowerby, 1814; Species: *Inoceramus concentricus* Parkinson var. subsulcatus Willshire, 1910.

Synonymy: 1911 Inoceramus concentricus Parkinson var. subsulcatus Willshire; Woods, p. 262, pl. 47, figs. 3-14.1972 *Inoceramus* (Birostrina) subsulcatus Willshire; Badve, p. 238, pl. XXIV, fig. 3.1972Inoceramus (Birostrina) subsulcatus Willshire; Chiplonkar and Badve, p. 199-200, pl.1, fig. 4.1975 Inoceramus concentricus Parkinson var. subsulcatus Willshire; Dassarma and Sinha, p. 24, pl. III, fig.2.

Material: Two specimens. **Dimensions:** Length - 68 mm; Height- 62 mm.

Figure 6: Inoceramus concentricus Parkin sonvar. subsulcatus Willshire

Description: Its shell is elevated, thick and moderately tumid. Umbones are slightly incurved distinctly pointed and antero dorsal area is flat, straight and long while antero ventral region is rounded. The postero dorsal area is compressed and flattened. The surface ornamentation consists of weakly developed fine concentric lines which are becoming finer posteriorly. Two superficial ribs are also noticeable.

Discussion: In the present collection, specimens of Inoceramus, showing resemblance in shape and size of shell and ornamentation with the Inoceramus (Birostrina) subsulcatus Willshire described by Dassarma and Sinha (1975, p. 24, pl. III, fig. 2) from the Bagh area.

Occurrence: Nodular Limestone at Zirabad and Chirakhan of district Dhar, M.P. The Cretaceous bivalves collected from Bagh beds have the supremacy of Inoceramids which spot to a very superficial nature of the Bagh basin. In the exclusive group of bivalves, generic and species perceptions were discussed. Here morphological and morphometric factors are considered valuable for future efficient work, the ecosystem and life habit of the Inoceramidae is largely argued. Possibly most precious is the opportunity for the recognition of areas, which will encourage and guide further work.

Klinger and Kennedy (1989) opinioned that Zululand basin at that time was shallow protected epicontinental seaway. It has already been mentioned that after drifting apart of Madagascar and Seychelles from the Indian westcost, the marine transgression of upper Turonian found path along the Narmada rift to give rise and epicontinental Bagh basin. Nectobenthic swimmer ammonoid placenticeras entered the newly formed virgin basin and radiated exclusively. According to Westermann (1990) *Placenticeras* were in habitant of proximal sublittoral (<100m) marine environment but probably below wave base. On the other hand coronate form and

keeled ammonoid *Barroisiceras* lived in the shallowwest offshore region of warm epicontinental sea (30-50m). From this point view of ammonoids, the Bagh basin was suppose to be a shallow epicontinental sea. These coronate forms are absent in lower nodular limestone which host on placenticeratid ammonoid. Hence on the basis on ammonoid study it can be said that the Bagh basin progressively becoming shallow upwards, which has already been describe lithologicaly.

Inoceramids is possibly the most fascinating group of extinct bivalves. By homoplassic loom it can be said that they were basically sessile benthos and most likely used by their byssus for attaching in high energy surrounding. In nature, they are expressive and independent of any sedimentary facies. They succeded in the shallow marine continental shelf as well as in estuarine circumstances. They are cosmopolitan, perhaps because of their planktotrophic larval stage. The Bagh beds being a product of epicontinental Cretaceous sea, might have been provided congenial environment for the *inoceramid* bivalves. The *inoceramids* bear no predation mark. Cretaceous shallow epicontinental seas were dominated by Mosasaurs (speedy swimmers) where temperature was higher than normal, otherwise so much carbonates could not be found.

It may be that Mosasaurs swam in the upper part and placenticeratid ammonite and *Inoceramid* bivalve thrived in near basal and basal part respectively. It is believed that most *Inoceramids* appear to have lived a stable life on the sea bottom, attaching themselves to a stable surface by byssus. Cretaceous inoceramids were in general, better adopted. They colonized not only the dysaerobic zone, but found also in continental shelf as well as in estuaries, the Bagh basin was a hospitable area, suitable for their growth. Kumar et al.(2018) tried to resolve uncertainty about the age of the Nodular Limestone formation (Late Cretaceous) at sub stage level throughout ammonoid and Inoceramid index taxa but recent work on paleoecology of Bagh beds during Cretaceous period has not been reported.

So, the bivalves described above, were obtained during research work, includes the dominant inoceramids, indicating a very shallow nature of the Bagh basin. Bagh basin was already been noticed as an arm of Tethyan seaway paleobiogeographically, the Tethys was positioned within the tropical realm. Almost, fossils gathering from different geographical regions and at certain latitudes indicate temperature variation in the geologic past.

CONCLUSION

Bagh Beds fundamentally deals the environment in which the invertebrate organisms communally flourish in that shallow marine environment in the past. Inoceramids did well in the shallow marine continental shelf and also in estuarine circumstances. This research work involves various areas of paleontological and paleoecological investigations of Cretaceous period, which facilitate researchers to tie the past with present. It will go on so as to give importance outcome in the future by gathering and meticulous study of more fossils from Bagh beds. It is the truth that reminiscence of the past is related to the present and future background. Renovation in knowledge in sequence is needed as a basis for calculating the nature and rates of change in climatic conditions for predicting future weather for years to come.One can say that paleoecology formed a chief column for considering comparative sequences and evolutionary drift.

Future investigations are needed by discovering new fossils for the wholeness of the record which may disclose the secrecy, so that conclusion could be drained regarding evolutionary prototype and tendency. Actual facts about climatic and environmental disparity in past can be estimated by collecting more and more fossils from different areas of the world and associating them with continental drift. They also assist in understanding ecological association, the disputes of global warming and destruction dynamics. Roughly, fossils gathered from diverse geographical regions and at certain latitudes point towards temperature disparity in the geologic past.

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Biochemical Characterization and Probiotic Potential of Lactic Acid Bacteria Isolated from Camel Milk

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ABSTRACT

Lactic acid bacteria (LAB) play an important role in digestion of food material in the gut. Recent researches reveal that the signals from gut are sent to the brain which controls all body functions. LAB acts as probiotics and plays significant role in health of man and animals. LAB found in human and animal milk influence the health of its consumers and the taste of milk also depends upon the type of LAB found in the milk. Deficiency of probiotics is very commonly reported in the people of developing nations. Demand of naturally occurring good probiotic strains with ideal characteristics meeting the eligibility criteria framed by WHO is the necessity of time. Therefore, In vitro study on biochemical characterization of Lactic Acid Bacteria (LAB) was performed using 12 different camel milk samples collected from the Bagru village of Jaipur district (Rajasthan). The isolates were initially confirmed to be LAB using biochemical tests - catalase, oxidase, Gram's staining, MR-VP and Sugar Fermentation tests. The isolates were also checked for their probiotic potential by examining their growth at low pH, different temperatures and different bile salt concentrations. The isolates which gave satisfactory results were further examined for their resistance against antibiotics as well as their antimicrobial activity against common human pathogens. In view of high rise in demand of good probiotic supplements throughout the world, there is a need of suitable probiotic local strains. Five best species of LAB isolated from camel milk were characterized phenotypically and were evaluated for their probiotic potentials. The present study was carried out with the aim of phenotypic characterization of Lactic Acid Bacteria from camel milk and to evaluate their probiotic potential.

KEY WORDS: CAMEL MILK, PROBIOTICS, LACTIC ACID BACTERIA, ANTIMICROBIAL ACTIVITY.

INTRODUCTION

As per FAO report, the population of camels at global stage is estimated to be around 26.99 million which is spread over 47 different countries. It is also reported that about 83% of camel population is mainly found in Northern as well as Eastern part of Africa whereas the rest of the population inhabits in Middle East Asia and Indian subcontinent. Across the globe, Somalia is the only country having 7.10 million camels which is the highest amongst all. India ranks 10th in the world with a population of 0.38 million camels (FAOSTAT, 2015).

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Camels in India are mainly found in the states of Uttar Pradesh (2.0%), Bihar (2.2%), Haryana (4.7%), Gujarat (7.6%) and Rajasthan leading with 81.4% (DAHDF,2014). Camel is considered to be the ship of desert since years and the milk laid by camel is believed to be the white gold of desert. Camel milk has wide range of health benefits and believed to be a good source of probiotics (Seifu et al., 2012). Camel milk is reported to contain both Gram-positive as well as Gram-negative bacteria (Kumar et al., 2016). Past studies have mainly focused on the physiological adaptations, anatomic characteristics and bio-molecules present in camel milk (Benmechernene et al., 2014).

Large numbers of studies in the past have focused on the microbiology of cow, buffalo, goat and sheep milk, however, very little scientific information on the microbiology of camel milk is available till date. Very few researchers have tried to focus on the microbial population of camel milk and to find the differences in the comparison to milk of other animals (Fguiri et al., 2017). Camel itself is a unique animal having the ability of surviving in both the extreme heat as well as cold temperatures which may produce significant differences in microbial composition as well as its biological characteristics of milk (Vimont et al., 2017). Isolation, characterization and applications of Lactic acid bacteria in human colostrums, and from cow and buffalo milk have previously been studied by our group (Bisht 2019, 2020; Arya et al., 2020).

Large number of lactic acid bacteria (LAB) found in raw camel milk have been proven to be of great technical relevance in diary industry. Among all LAB, species of *Lactococcus* have shown to be the best starter culture for cheese manufacturing as well as in production of several flavor compounds (Ruggirello et al., 2016). *Lactobacillus* is another important genus of LAB which is very commonly found in all raw milk and has wide range of dairy applications. These have the ability of producing various aroma compounds and have been proven to be highly probiotic which contributes to the quality and nutrition of dairy products (Stefanovic et al., 2018). Besides these two, there are several other genera of LAB such as *Streptococci, Bifidobacterium, Aerococcus, Pediococcus* and *Enterococcus* are used in dairy.

Camel milk is also known for health promoting effects such as aiding digestion, reducing the risk of asthma, atopic diseases and several other allergies (Zibaee et al., 2015). Camel milk has evidently proven to be a safer consumption even after storage of several days without chilling it in refrigerator. That could be only possible if biological active bacteria produce antimicrobials such as bacteriocins, antifungal agents and other organic acids (Kumari et al., 2008; Omer et al., 2009). The production of antimicrobials by microbial species may act as bio-preservative agents which could increase the shelf life of camel milk. This brought us great interest for carrying out such a study on camel milk. The entire study will highlight the biochemical characteristics of LAB which were isolated from camel milk.

MATERIAL AND METHODS

Sample collection: All the camel milk samples were collected from the Bagru village of Jaipur District (Rajasthan, India). Nipples of camel were washed with sterile distilled water and were carefully cleaned with cotton dipped in alcohol. The tubes used for sample collection were autoclaved using standard procedure before collection. The first 5 mL of milk sample was discarded to avoid contamination from skin flora. The mid flow of milk was carefully aseptically collected in the sterile tubes and these were sealed capped immediately. About 10 to 15 mL of milk sample was collected from each camel. The data regarding the diet, age, habitat and its other physiological activities were recorded by consulting the owner of camels. The samples were immediately brought to the laboratory and processed as described earlier (Bisht 2019; Arya 2020).

Isolation of Probiotic Bacteria: MRS (de Man Rogosa and Sharpe agar) [Hi-Media] was used for isolating probiotic bacteria from camel milk. The milk samples were serially diluted up to 10-6in sterile peptone water and 0.1mL of inoculum from each dilution was aseptically inoculated on MRS agar plates using spread plate technique. The inoculated plates were placed in inverted position in desiccators using standard protocol and were incubated at 37°C for 48h in incubator (Bisht 2019; Arya 2020).

Characterization of Probiotic Bacteria: The colony characteristics (size, shape, texture, opacity, pigmentation, margins and color) of isolated bacteria were carefully noted down. The bacterial colonies were counted using digital colony counter and CFU/mL was calculated using standard protocol. The distinct colonies were further sub-cultured on MRS agar plates to obtain pure cultures for further studies. Gram's staining: A single pure colony was picked from the surface of plate and was gently mixed with a single drop of sterile distilled water on the surface of cleaned glass slide to prepare a smear which was further heat fixed carefully using Bunsen burner. The standard protocol of gram's staining was performed and the slide was observed under oil immersion lens of microscope. All the bacteria which were Gram positive in nature were further tested for other biochemical activities (Hammes et al., 2009). Catalase test: Catalase is an enzyme which break down hydrogen peroxide (H2O2) into water and oxygen gas is liberated. On basis of this principle, all isolates were examined for their catalase activity. A drop of 3% hydrogen peroxide was gently mixed with a single distinct colony on the surface of clean glass slide, and the production of bubbles was indicator of catalase activity (Kumar and Kumar, 2015). The bacteria which did not show catalase activity were further screened for its oxidase activity.

Oxidase activity: Cytochrome c oxidase is a type of enzyme which is found in bacteria as an electron transport chain. Presence of this enzyme oxidizes a reagent called tetramethylphenilindiamine and gives indophenols as an end product in blue color. All catalase negative isolates were examined for their oxidase

activity and all oxidase negative were further tested for their ability to ferment different sugars (Kumari et al., 2008; Bisht and Garg, 2019). Carbohydrate test: Bacteria ferment carbohydrates to form acid and gas or only acid as their end product. Different bacteria ferment different sugars or their combination which greatly help in the biochemical characterization of various species as described in Bergey's Manual of Systematic Biology, 9th edition. For this test, different sugars were prepared such as Glucose, Galactose, Maltose, Mannitol, Fructose, Sucrose, Xylose, Arabinose, Cellulose and Lactose using standard protocol [Hi-Media]. Durham's tube was added to each tube of sugar to which 0.1mL of pure overnight grown culture was inoculated and the tubes were allowed to incubate at 37°C for 48h. Results were recorded after incubation and were compared with Bergey's Manual for identification (Bisht and Garg, 2019).

Assessment of probiotic activity: For determining the probiotic activities of isolated bacteria, all the isolates were tested for their survival at low pH, their growth at different temperatures, tolerance again different bile salt concentrations, antimicrobial activity and their ability to resist against different common antibiotics (Bisht 2019; Kang et al.,2019). Survival at low pH: It is believed that the food eaten by us stays in our stomach for at least 3 h and according to the literature the pH of human stomach is found in between 2-3 in healthy human (Arya et al., 2020). Therefore, all the isolates of this study were checked for their growth at acidic pH values. For this test, desired MRS broth was prepared of different pH (1, 2, 3, 4, 5 and 6) by adding 0.1N HCL to which 0.1mL of overnight grown culture was added aseptically separately in each tube prepared at different pH. These were incubated at 37°c for 3 h on shaking incubator.

After incubation the absorbance was measured at 620 nm. The initial absorbance was measured at 0h. The survival rate of isolates was measured by plotting graph using standard protocol (Powthong and Suntornthiticharoen, 2015). Tolerance against different bile salt concentrations: To check the tolerance of isolates against different bile salt concentrations, desired MRS broth was prepared with concentration (0.3%) by adding Ox-bile. Overnight grown culture of isolates was inoculated to MRS broth prepared of 0.3% bile salt concentration and was incubated at 37°c for 6 h in shaking incubator. The absorbance (620 nm) of broth was measured at the regular interval of 1 hour to measure the growth curve of isolates (Goswami et al., 2017; Bisht and Garg, 2019). Growth at different temperatures: All the isolates which were able to tolerate bile salt concentration, were further examined for the growth at different temperatures. The pure culture of isolates was inoculated on MRS agar plate and were incubated at different temperatures (10°,20°,30°,37°,45°and 50°) in incubator (Bisht 2019; Kang et al., 2019).

Antibiotic susceptibility test: The isolates which showed good probiotic potentials were checked for their resistance against common antibiotics using Kirby Buayer's method. The overnight grown cultures were inoculated on Muller Hilton (MH) agar plates using spread plate technique. Wafers of different antibiotics (Penicillin G, Amoxicillin, Ciprofloxacin, Trimethoprime, Gentamycin, Erythromycin, Streptomycin and Tobramycin) of different concentrations were placed on the surface of agar and were gently pressed (Abdullah and Osman, 2010; Bisen et al., 2013; Bisht and Garg, 2019; Kang et al., 2019). The plates were allowed to incubate in upright condition at 37°C for 24-48h.

The zones of inhibition were observed and measured with scale after incubation. Antimicrobial activity against pathogens: The isolates which were found to be resistant against antibiotics were further tested for their antimicrobial activity against human pathogens. The pathogens used in the study were *Escherichia coli* (ATCC-35218), *Staphylococcus aureus* (ATCC-25923), *Salmonella typhi* (MTCC-733), *Pseudomonas aeruginosa* (ATCC-27853) and *Proteus vulgaris* (ATCC-33420) using Agar well diffusion method. The overnight grown cultures of pathogen were inoculated on MH plates using spread plate technique (Batdroj et al.,2006; Gaspar et al., 2018; Bisht 2019). A sterile cork borer of (diameter 6 mm) was used to puncture the agar surface to prepare wells. The overnight grown liquid cultures were filled in the wells using micropipette (Putra et al., 2017). The plates were allowed to incubate in upright position at 37°C for 24-48h. The zones of inhibition were observed and measured using scale (Bisht 2019; Kang et al., 2019).

Table 1. Some characteristics of presumptive LAB isolated from camel's milk

Characteristic of isolates	RR	SP76	LB005	SP53	DW2
Gram stain	+	+	+	+	+
Morphology	Cocci	Bacilli	Cocci	Bacilli	Cocci
Presence of spore	-	-	-	-	-
Catalase test	-	-	-	-	-
Oxidase test	-	-	-	-	-
Indole test	-	-	-	-	-
MR	+	+	+	+	+
VP	-	-	-	-	-
Gas from glucose	-	-	-	-	-
Citrate utilization	-	-	-	-	-
Gelatin hydrolysis	-	+	-	-	-
Starch hydrolysis	-	-	-	-	-

'+': Growth and '-': No Growth

RESULTS AND DISCUSSION

A total number of 12 camel milk samples were studied for the isolation and characterization of LAB and for the assessment of their probiotic potential. In the present study, 23 different species of LAB were isolated which were primarily identified on the basis of biochemical

characterization as per description given in Bergey's Manual of Systematic Bacteriology, 9th edition. All the isolates of the present study showed positive growth on MRS agar plates and were Gram's positive but catalase and oxidase negative in nature (Table 1). On examination of their probiotic potential, 5 best isolates were screened on the basis of their ability to grow at low pH, bile salt tolerance, growth at different temperatures, their antibiotic and antimicrobial activities against human pathogens.

Table 2. Antibiotic Susceptibility test of Isolates (zone of inhibition mm diameter).

Antibiotics	Symbol	µg/disc	Isolates				
			RR	SP76	LB005	SP53	DW2
Erythromycin	E	5	R	14	R	19	10
Trimethoprime	TR	30	12	18	20	R	15
Penicillin-G	P	1	R	R	16	18	20
Gentamycin	HLG	120	R	20	20	14	R
Streptomycin	HLS	300	20	24	R	20	16
Amoxicillin	AMX	10	R	18	27	R	15
Tobramycin	CAZ	30	R	25	R	R	20
Ciprofloxacin	CIP	5	22	R	14	12	R

Figure 1: Antibiotic Susceptibility

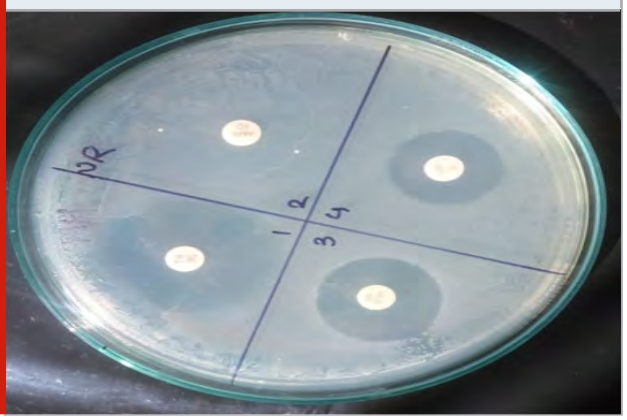
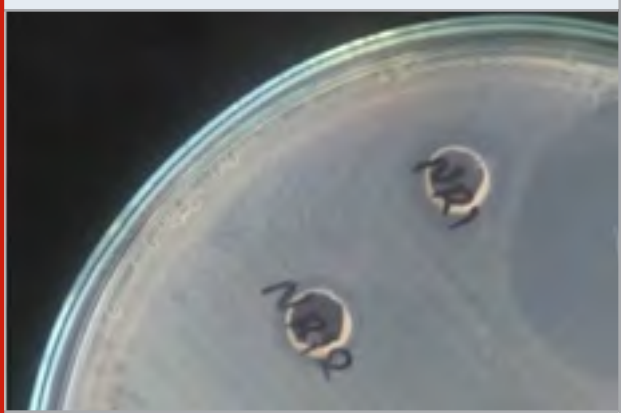


Figure 1: Antimicrobial Activity

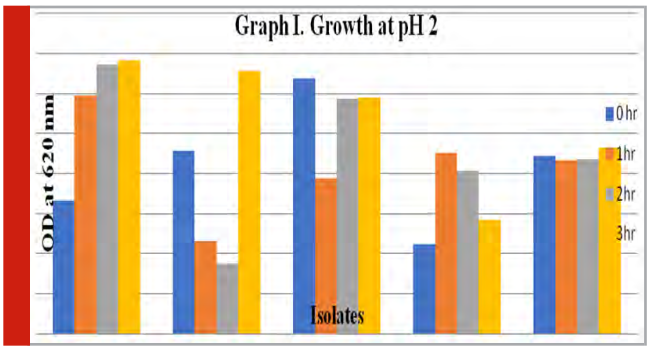


The study found that camel milk can be one of the good sources of quality LAB. Through, this study out of 23 isolates of LAB, 12 possessed the ability to survive at pH 2 which constitute about 52.17%. The ability of isolates to tolerate bile salt was found to be 39.13% which comes to 9 isolates. Seven of nine isolates had the potential to survive at both higher as well as lower temperatures which comes to about 30.43%. Seven isolates were checked for the resistance out of which 5 best were chosen for testing their antimicrobial activities against human pathogens. All the 5 best isolates were precisely identified on the basis of their biochemical characterization using Bergey's manual of Systematic Bacteriology (Olmo et al. 2020).

Antibiotic susceptibility pattern of LAB: Antibiotic susceptibility pattern of selected LAB isolates was observed using Kirby-Bauer disc diffusion method. The results are shown in Table 2. Isolate RR was sensitive to Trimethoprime (12mm), Streptomycin (20mm), Ciprofloxacin (22mm) but was resistant to Erythromycin, Penicillin-G, Gentamycin, Amoxicillin, Tobramycin. Isolate SP76 was only resistant to Penicillin-G, Ciprofloxacin but sensitive to Erythromycin (14mm), Trimethoprime (18mm), Gentamycin (20mm), Streptomycin (24mm), Amoxicillin (18mm), Tobramycin (25mm). Isolate LB005 was sensitive to Trimethoprime (20mm), Penicillin-G (16mm), Gentamycin (20mm), Amoxicillin (27mm), Ciprofloxacin (14mm) but resistant to Erythromycin, Streptomycin, Tobramycin. Isolate SP53 was sensitive to Erythromycin (19mm), Penicillin-G (18mm), Gentamycin (14mm), Streptomycin (20mm), Ciprofloxacin (12mm) but resistant to Trimethoprime, Amoxicillin, Tobramycin. Isolate DW2 was resistant to Gentamycin, Ciprofloxacin but sensitive to Erythromycin (10mm), Trimethoprime (15mm), Penicillin-G (20mm), Streptomycin (16mm), Amoxicillin (15mm), Tobramycin (20mm) (Fig I and Fig II).

Such resistance to a wide spectrum of antibiotics indicated that if isolated probiotics induced in patients treated with antibiotic therapy may be helpful in faster recovery of the patients due to rapid establishment of desirable microbial flora. Resistance of the probiotic

strains to some antibiotics could be used for both preventive and therapeutic purposes in controlling intestinal infections (EI-Naggar, 2004). Gad et al. (2014) isolated 244 LAB strains from dairy and pharmaceutical products and tested their antibiotic resistance against vancomycin, tetracycline, erythromycin and clindamycin and found that most LAB were within the normal range of susceptibility and 16 strains of Lactobacillus, 8 of *Lactococcus* and *Streptococcus* were resistant against tetracycline and/or erythromycin. PCR analysis showed that some strains harbor resistant genes. The antibiotic resistance of isolated LAB was assessed using antibiotic discs [Hi media] on MH agar plates against Erythromycin (5µg), Trimethoprime (30µg), Penicillin-G (1U), Gentamycin (120µg), Streptomycin (300µg), Amoxicillin (10µg), Tobramycin (30µg) and Ciprofloxacin (5µg) (EI-Naggar, 2004).



Probiotic activity of selected isolates: A variety of acid levels has been found in different regions of gastrointestinal tract. Stomach and the other regions of gastrointestinal tract have the highest acidity and these areas may fall to as low as pH 2 - 3. In order to be used as beneficial effect, LAB must be able to survive under these harsh conditions and colonies in the gut. In present research, the selected LAB isolates were able to grow in pH 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0. The survival rate of RR LB005 was maximum at pH 1-6 and showed the highest viability and showed moderate growth even at pH 2 (Fig I) (Powthong and Suntornthiticharoen, 2015).

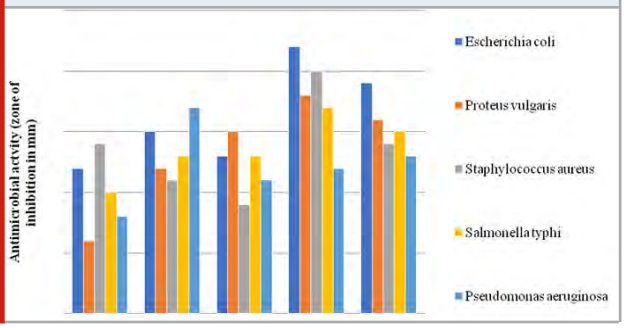
In general, the survival rate of three cultures RR, SP76 and DW2 during 3 h of incubation increased at all pH conditions. In present study, all the selected LAB isolates were able to survive at temperature10, 20, 30, 40 and 45°C. All the results are shown in table 3. Adamberg et al. (2003) have also evaluated the growth of LAB at various pH and temperature. Similar findings were shown by Powthong and Suntornthiticharoen, (2015). The change of pH and temperature is an effective method for determination of technological characteristics and comparative physiological study of LAB. The temperature is an important factor which can dramatically affect the bacterial growth. The reason for choosing this temperature range was to detect whether the isolated cultures were able to grow within range of normal body temperature or not.

Table 3. Probiotic activity of Isolates in term of growth at different temperatures, pH and bile salt tolerance

Isolates	Bile salt (%)	Growth at different temperature (°C)				Growth at different pH				
	0.3	10	20	40	45	1	2	3	4	6
RR	+	+	+	+	+	+	+	+	+	+
SP76	+	+	+	+	+	-	+	+	+	+
LB005	+	+	+	+	+	+	+	+	+	+
SP53	+	+	+	+	+	-	-	-	+	+
DW2	+	+	+	+	+	-	+	+	+	+

'+': Growth and '-': No Growth

Figure 3: Antimicrobial activity of 5 isolates against pathogenic bacteria



As if the isolates were not able to survive within the selected temperature range then they would not have been able to survive in the human gut, which is an essential factor of probiotics to show their effectiveness. The results obtained were positive for growth at chosen temperature range (Powthong and Suntornthiticharoen, 2015).

Adamberg et al. (2003) have studied the effect of pH and temperature on selected LABs and found that these factors influence the growth of most lactic acid bacteria. Mu and Ohegbu (2018) correlated the effect of pH and temperature with bacteriocin production by LAB. Somashekaraiah et al. (2019) have also evaluated

the probiotic activities 75 strains isolated from naturally fermented drink of coconut and found that 16 showed high probiotic activities in terms of antimicrobial and antibiotic resistance and concluded that they have good potential in functional fermented foods as bio-preservatives. Olmo et al. (2020) have reported that storage of foods with LAB at lower temperature increases the shelf life of the food stuff while at room temperature, it is not so effective. Roger et al. (2015) has reported that LAB inhibit the growth of Aspergillus fumigatus and also reduces the production of aflatoxins. It suggests that addition of LABs in the food can act as good bio-preservative agents and can increase the shelf life and can protect against food spoilage microbes. Our results show that the strains showing high antibiotic resistance have great potential to be used as probiotic (Roger et al. 2015; Somashekaraiah et al. 2019; Olmo et al. 2020).

Antimicrobial Activity of Isolates: 5 isolates exhibited inhibitory activity against several pathogenic bacteria, including *Escherichia coli* (ATCC-35218), Proteus vulgaris (ATCC-33420), Staphylococcus aureus (ATCC-25923), *Salmonella typhi* (MTCC-733) and *Pseudomonas aeruginosa* (ATCC-27853) (fig III).

CONCLUSION

Camel milk was found to be the good source of potential probiotic LAB. Large varieties of LAB were isolated in the present study. It was also found that few species of LAB are very difficult to sub-culture. On the basis of biochemical characterization, five good species of LAB with good probiotic potentials were identified. Further genomic studies are still required to be carried out for molecular characterization of these isolated species. LAB's have wide range of applications on human health and great potential to be used bio-preservatives. Therefore, further in vivo studies are required to be carried out for clear justification. The probiotic strains available and sold in the market have some or the other limitation with limited applications. A good strain of probiotic can be searched with wide range of applications which can solve the problem of deficiency of probiotics in humans. Even a mixture of two or more probiotic strains should be standardized and commercialized for human consumption.

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Ethical Clearance Statement: The Current Research Work Was Ethically Approved by the Institutional Review Board (IRB) of Deemed-to-be-University, Meerut, Uttar Pradesh, India.

Conflict of Interest: None

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Impact of the COVID-19 Pandemic on Physical, Psychological and Nutritional Characteristics of Elite Athletes: a Cross-Sectional Web Survey

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ABSTRACT

COVID-19 has upended sports and sporting calendars worldwide; causing postponement or cancellations of sports events globally. Amid the lockdown, most of the athletes are left on their own at their homes. This study investigates the impact of the ongoing Pandemic on physical, psychological and nutritional characteristics of elite athletes amid COVID-19 spread. A cross sectional web survey was carried out using a validated questionnaire comprising of total 19 questions regarding the demographic details, physical, psychological and nutritional characteristics of elite athletes before and after COVID-19 Spread. The normality of data was established using Kolmogorov-Smirnov test. The frequency and percentage n (%) of ordinal data of participant responses were calculated. A total of ninety four elite athletes voluntarily participated, out of which 73 (78%) were male athletes. 40% were professional cricketers followed by 10% badminton and 10% table tennis players and rest 40% belong to various other sports. 76% of total athletes played their sport at the national level The vigorous intensity training schedules were routinely adapted by 39 (42%) of total athletes before the lockdown phase amid COVID-19 spread which reduced to 9(10%) afterwards. 59(63%) of total athletes self reported being in relaxed mood. 37 (39%) felt disturbed about the cancellation of tournaments and their inability to practice. Daily calorie intake was increased among 26 (28%) of athletes. Covid-19 spread has significantly impacted training regimes, eating habits, and state of mind of elite athletes. Although majority of athletes reported being in a relaxed and happy state of mind, however long periods of re-training and psychological counseling would be required to reverse the effects of detraining caused due to the ongoing Pandemic crisis.

KEY WORDS: SPORTSPERSON; PSYCHIATRY; TRAINING; RECONDITIONING.

INTRODUCTION

The coronavirus disease 2019 (COVID-19) has upended sports and sporting calendars worldwide causing postponement or cancellations of sports events globally. On March 24, 2020 The Guardian reported that the 2020 Summer Olympics have been rescheduled to a date beyond 2020. Restriction on sporting events has put a hold on all sports and recreational activities which affect the rigorous training regimen of elite athletes, to enhance and maintain their peak sports performance. Amid the lockdown, most of the athletes are left on their own at their homes. Prolonged stay at home may lead to an increase in sedentary behavior of the athletes. Certain health problems can also occur due to the lack of strength and endurance training. The irregularity or cessation of high intensity aerobic or endurance training for more than 2 weeks can significantly affect the cardiovascular

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endurance and, in turn, the immunity of athletes as reported by Pedlar et al. (2018, 2020).

According to the Law of Reversibility Principle, a detraining phase extending till 2 weeks post a high intensity training regime triggers a vicious cycle of reduced lean mass and an increase in body fat, leading to a significant decrease in muscle strength and power (Lee et al., 2017). A similar or longer period of retraining is required to regain the earlier physical fitness levels. The continuation of same intensity and timely progression of training sessions is essential to maintain peak performance of athletes. Post lockdown, incomplete training regimens can make it difficult for athletes to reach their peak performance levels. Mental health is directly proportional to physical health; and any disproportion can affect an athlete's performance in sports to a great extent. Till date, to the best of our knowledge, there is no literature available on the lockdown phase impact on elite athletes. In view of the same, the present survey has been taken to find out the impact of COVID-19 Pandemic on elite athlete's physical health, nutrition and mental wellbeing (Lee et al., 2017).

MATERIAL AND METHODS

Institutional Ethics Committee (IEC) of Maharishi Markandeshwar Deemed to be University (IEC-114F) approved the study Protocol. This study protocol is in accordance with the National ethical guidelines for Biomedical and Health Research involving human subjects-ICMR guidelines (Revised 2017) and guidelines of Helsinki declaration 2013. The individual data was collected in the month of April and May 2020 from national and international level elite athletes. The e-survey was sent to 110 elite athletes from different zones of the country using chain-referral sampling.100 elite athletes voluntarily participated in the study (responses of 6 athletes were rejected later on due to incomplete form submission). To analyze the impact of COVID-19 Pandemic on physical, nutritional and psychological characteristics of elite Indian athletes, a self structured and validated questionnaire for a comprehensive survey was prepared. The questionnaire was validated using face validity and pilot testing of Questionnaire on 12 Participants.

Table 1. Structured Questionnaire

1.	Name:
2.	Which Sport are you playing?
3.	Age: (i) Less than 18 years (ii) 18-35 years (iii) Above 35 years
4.	Gender: (i) Male (ii) Female
5.	Which level of tournament did you play? (i) National (ii) International
6.	Before lockdown, the intensity of your daily workout/practice/ training was (i) Light (ii) Moderate (iii) Heavy Please specify your Daily Practice/Training:
7.	During lockdown, the intensity of your daily workout/practice/ training was (i) Light (ii) Moderate (iii) Heavy Please specify your Daily Practice/Training:
8.	Before lockdown, how much time you spend on your workout in a day? (i) Less than 2 hour (ii) 2-4 hours (iii) More than 4 hours
9.	During the lockdown, how much time you spend on your workout in a day? Less than 2 hour (ii) 2-4 hours (iii) More than 4 hours
10.	What is the impact of lockdown on your daily food intake? (i) Increased (ii) Decreased (iii) Unchanged
11.	Do you feel lockdown will affect your sports performance in future? (i) Yes (ii) No
12.	Do you feel Happy/Relaxed/Motivated that you can improve your game for future competitions (i) Yes (ii) No
13.	Do you feel Worry/ Tension /Anxiety/Stress about your performance in future competitions? (i) Yes (ii) No
14.	Are you suffering from any financial problem? (i) Yes (ii) No
15.	'You are not able to play your sport' what are you feeling? (i) Aggressive (ii) Irritated (iii) Depressed (iv) None of the above
16.	How is your mood now days? (i) Relaxed (ii) Stressed
17.	Do you have any thought that you might quit your game? (i) Yes (ii) No
18.	Are you practicing Yoga/Meditation/Breathing exercise/or other relaxation techniques daily? (i) Yes (ii) No
19.	Are you enjoying your increased family time at home? (i) Yes (ii) No

It consisted of a total of 19 questions, which included description of the survey, their consent to participate, demographic data of the athletes, details about their training regimes, changes in physical activity, diet and weight modifications, mental well-being, performance issues, and social and family interactions (Table 1). The structured questionnaire had been copyrighted under all the author names with registration number L-96011/2020. Open and close- ended questions were formulated to access all the items in the questionnaire. The questionnaire was included in the Google® form link and sent by various social media applications (Facebook, Whatsapp and Messenger). Participants were asked to answer the question by themselves.

Table 2. Participant's characteristics (n= 94)

S. No.	VARIABLE		Frequency (Percentage)
1.	Type of sport	Cricket	38 (40.4)
		Badminton	10 (10.6)
		Table tennis	10 (10.6)
		Football	5 (5.3)
		Volleyball	5 (5.3)
		Basketball	07 (7.4)
		Other	19 (20.2)
2.	Gender	Female	21 (22.3)
		Male	73 (77.7)
3.	Age (In years)	Less than 18 years	26 (27.7)
		Between18-35 years	65 (69.1)
		More than 35 years	3 (3.2)
4.	Level of Tournament	National	76 (80.9)
		International	18 (19.1)
5.	Intensity of Workout before Lockdown	Light	10 (10.6)
		Moderate	45 (47.9)
		Vigorous	39 (41.5)
6.	Intensity of Workout during Lockdown	Light	47 (50.0)
		Moderate	38 (40.4)
		Vigorous	9 (9.6)
7.	Total Time duration of Workout before Lockdown	Less than 2 hour	39 (41.5)
		Between 2-4 hours	39 (41.5)
		More than 4 hours	16 (17)
8.	Total Time duration of Workout during Lockdown	Less than 2 hour	84 (89.4)
		Between 2-4 hours	07 (7.4)
		More than 4 hours	03 (3.2)
9.	Daily food intake	Decreased	33 (35.1)
		Increased	26 (27.7)
		Unchanged	35 (37.2)
10.	Will lockdown have impact on your future sports performance?	Yes	56 (59.6)
		No	38 (40.4)
11.	Do you feel motivated to improve your game?	Yes	71(75.5)
		No	23 (24.5)
12.	Do you feel Anxiety/Tension/ Stress about your future	Yes	42 (44.7)
	sports performance?	No	52 (55.3)
13.	Are you suffering from any financial loss?	Yes	26 (27.7)
		No	68 (72.3)
14.	How you are feeling about not being able to play?	Irritated	37 (39.4)
		Aggressive	9 (9.6)
		Depressed	20 (21.3)
		None of the above	28 (29.8)

Continue Table

15.	How is your Mood nowadays?	Relaxed Stressed	59 (62.8) 35 (37.2)
16.	Do you have any thoughts about quitting your game?	Yes No	10 (10.6) 84 (89.4)
17.	Are you practicing Meditation/ yoga or other relaxation techniques daily?	Yes No	51 (54.3) 43 (45.7)
18.	Are you enjoying this time?	Yes No	81 (86.2) 13 (13.8)

Table 3. Influence of the Lockdown measures on athlete training by demographic variables (n=94)

Training Parameters	Gender		Characteristics n (%) Age in years			Level of Participation	
	Male 73(78)	Female 21(22)	<18 y 26(28)	18-35 y 65(69)	>35 y 3(3)	National 76(81)	Inter-national 18(19)
Training Intensity							
Mild							
Before lockdown	7(10)	3(14)	2 (8)	8(12)	0(0)	10(13)	0(0)
During lockdown	40 (55)	7(33)*	13(50)	34(52)	0(0)	42(55)	5(28)
Moderate							
before lockdown	36(49)	9(43)	12(46)	31(48)	2(67)	39(51)	6(33)
During lockdown	27 (37)	11(52)*	8 (31)	27(42)	3(100)	28(37)	10(56)
Vigorous							
before lockdown	30(41)	9 (43)	12(46)	26(40)	1(33)	27(36)	12(67)
During lockdown	6 (8)**	3 (14)*	5(19)	4(6)	0(0)	6(8)	3(17)
Training duration each day <less than 2 h							
before lockdown	32(44)	7 (33)	8 (31)	29 (45)	1(33)	33 (43)	6 (33)
during lockdown	66 (90)*	18 (86)*	23 (88)	58 (89)	1(33)	69(91)**	15(83)
2-4 hours							
before lockdown	28 (38)	11 (52)	13 (50)	25 (38)	1(33)	30 (39)	9 (50)
during lockdown	5 (7)*	2 (10)*	2 (8)	5 (8)	1(33)	5 (7)**	2 (11)
More than 4 h before							
lockdown	13 (18)	3 (14)	5 (19)	11 (17)	1(33)	13 (17)	3 (17)
during lockdown	2 (3)*	1 (5)*	1 (4)	2 (3)	1(33)	2 (3)**	1(6)

* p value<0.05, ** p value <0.01

Most of the participants reverted within 10 minutes to one day with their completed forms. In case of non-response, reminders through the same social media platform were sent every 48 hours. Online surveying was preferred as it is easily accessible, less expensive and time saving. Google® form automatically analyzed the data. Responses of each participant were entered in excel sheets and the data was analyzed. For the data analysis, the statistical software, IBM® SPSS version 20.0 was used. At a confidence interval of 95%, data analysis was represented with a descriptive statistics at 0.05 levels of significance.-Due to submission of incomplete forms, 6 responses were excluded, with the data analyzed for 94 participants. The response rate to survey was 100 (91%) and completion rate was 94(94%).The normality of data was established using Kolmogorov-Smirnov test. The frequency and percentage n (%) of ordinal data of participant responses were calculated. Independent t-test was used to compare participant responses by demographic variables.

RESULTS AND DISCUSSION

The lockdown due to the ongoing pandemic is thought to have major consequences on the sports fraternity. Sudden cessation of all sports activities, lost opportunities as well as uncertain financial and sporting futures could have their significant impact on general well being of athletes and their safe return to sport. Although the process of unlocking has started in many parts of the world, but an athlete's return to sports as well as regular practice sessions have not been resumed in most of

them. The present study was conducted to observe the impact of COVID-19 Pandemic in India on the physical, nutritional and psychological aspects of elite athletes. The lockdown has caused an unexpected stop not only to various sporting events and competitions, but the routine practice sessions of elite athletes have also hampered to a great extent. In the present study, out of a total of 94 athletes, 40% were professional cricketers followed by 10% badminton and 10% table tennis players and rest 40% belong to various other sports. 76% of total athletes played their sport at the national level (Table 2) (Chang et al., 2009).

Physical Health and Nutrition: It is a well-known fact that detraining leads to reduced maximal oxygen consumption (VO2max), decline in endurance capacity and a marked reduction in flexibility, muscle strength, power and volume (Madsen et al, 1993). Normally, the average time span of athletes ceasing or reducing their training parameters should last only up to two weeks, to a maximum of four weeks. As the duration of lockdown in India, at the time of sample collection, had extended to nearly 3 months, athletes were asked about modifications in their daily training regimens. Before the COVID-19 Pandemic induced lockdown began in India, a total of 10% male athletes were practicing with mild, 49% with moderate and 42% with vigorous intensity.

With the implementation of lockdown, these numbers changed, with now 55% practicing with mild, 37% with moderate and just 10% practicing with vigorous intensity (Table 3). The lubrication and nutrition (hyaluronic acid and lubricin) of joint cartilage is compromised due to inactivity, resulting in a possible degeneration and imbalance of the maintenance and preservation of cartilages, ligaments and the synovium. The reduced activity was observed among female athletes as well, although a higher number of them, i.e., 14% were still maintaining a vigorous intensity of training (Chang et al., 2009).

The shift of training intensity from vigorous to mild was more apparent among male national players belonging to the age group of 18 -35 years. Similarly, total duration of training sessions per day significantly reduced from 17 % to 3% among national players during the lockdown (Table 3). Hence the training routines of elite athletes have abruptly been interrupted. Retraining phase to gain similar levels of physical fitness requires a time twice of what the player spent in detraining(Paoli, 2020). 26 (28% of the total) athletes reported an increase in their daily food intake despite a reduction in the intensity of their training (Table 2). A similar study showed an unprecedented number of Achilles tendon ruptures at the beginning of pre-season of the National Football League (NFL) following a lockdown period (Myer et al., 2011; Frizziero et al., 2016).

Unfortunately, such injuries can be career-altering or even career-ending. Elite athletes require a high level of regular physical training, balanced nutrition as well as mental wellbeing to maintain their peak physical fitness levels, irrespective of their specific sport(Lorenz, 2013). Detraining also shows its effects on tendon structure and properties causing an alteration in structural organization and mechanical properties of the tissues which, in turn, impair normal tendon reaction to load application (Frizziero et al., 2016). An increased caloric intake, coupled with a phase of inactivity, induces an alteration in body composition, which includes, but is not limited to, an addition in body fat levels, which has been associated negatively with physical performance. Some drastic measures need to be taken by the athlete to improve body composition before competition, which could increase the risk of injury once a player return to sport (Mcmanus, Murray and Parry, 2017). Training regime schedules of elite athletes generally follow periodization patterns, where long periods of passive rest are avoided. A sudden phase of detraining among elite athletes would impact their future sports performance, as well as increase their chances of injuries (Haugen et al, 2019).

Mental Health: Along with physical, the impact of COVID-19 lockdown on psychological aspects of athletes is bound to be inevitable. Surprisingly, a majority of athletes were relatively stress-free, with 86% reportedly enjoying this increased time of stay at home with family. However, 60% of the athletes did believe that the lockdown could have an impact on their future sports performances, with 48% feeling anxious about the same. 76% of total athletes were self motivated to improve their performance (Table 2), and 11% thinking about quitting their game. A positive attitude, self-motivation, mental imagery, self-talk are some of the key mental skills all athletes should practice during these testing times (Peluso et al., 2005).

Psychological factors significantly impact an athlete's focus and preparation of game, with a negative thought or foul mood profoundly influencing his performance(Serrano et al., 2013). At the same time, to avoid any physical, technical, and psychological damage, players can also use this time to invigorate and improve their fitness with basic exercise regimens like core exercises, aerobics, resistance exercises, yoga, and meditation etc. A cross sectional study has also been conducted in South Africa to analyze impact of coronavirus crisis on elite and semi elite athletes (Pedlar et al., 2020), showing similar results. Efforts should be put to maintain both physical health and mental wellbeing at home, and the players needing to maintain a conditioning routine during the lockdown. The limitations of this study include that the sample size was not estimated since there are few previous studies done on similar aspects.

CONCLUSION

A well-planned restart of the training phase and "return to play" strategy is the need of the hour, all around the world, to overcome the risks involved for athletes. Too rapid resumption of events by sports federations need to be avoided at every cost. The results of this study could

help the government sporting federations and sports science professionals to formulate strategies to support athletes develop and implement guidelines to minimize the potential risk to a sportsperson's career caused by this global pandemic.

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Options of Dental Students on Learning Methods in Riyadh Elm University: A Questionnaire-Based Study

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ABSTRACT

The most important challenge experienced by dental educators presently is enhancing the learning environment, and improve student satisfaction with the curriculum. The recent dental students reflect a variety of cultures, backgrounds, attitudes, and learning styles. Therefore, a questionnaire-based study was implemented in the dental students. The aim of this research is to describe and compare male and female dental students' preferences on various aspects of teaching, demonstration and assignment of various dental colleges in the Riyadh city. This study has been designed as a questionnaire-based one and the questionnaire was categorized into three parts. A total of 150 students from couple of dental colleges from different institutes such as King Saud University and Riyadh Elm University, participated. The questionnaire was distributed to male and female undergraduate dental students and a stratified random sampling method was applied to collect the targeted samples. In this study, 58% were males and 42% of them were females. The 52.7% of majority of the students have opted the visual teaching method with 67.3% as multiple-choice questions. However, overall 14% of the students opted the all the choices. Among the innovative teaching methods, 58% of them opted the short quizzes and 55.3% of them opted the small group discussions. Among the combines teaching methods, 50.7% of the students have opted the exhaustive text book content and recent update from the journals. Additionally, 55.3% of the students were interrupted with the lecture timings. The present study concludes that the majority of respondents favored 45 minutes of lecture classes in the morning hours. The most favored teaching aid was the visual method, while the most preferred method for assessment was multiple choice questions and assignment. Nevertheless, respondents were of the belief that novel approaches should be adopted to promote the process of learning. The key factors for disinterest in a class were the pacing of the lecture and the length of the class. In terms of the average percentage of the lecture material they were able to understand, a substantial gap between male and female research subjects was noted.

KEY WORDS: LEARNING STYLES, LEARNING METHODS, TEACHING TYPES, INNOVATIVE METHODS AND DENTAL STUDENTS.

INTRODUCTION

Deep learning systems have been implemented in recent years, one of the artificial intelligence tools for different clinical tasks. Communicative skills are an important component of medical and dental education and contribute positively to many facets of health care, including the performing of a thorough evaluation, the proper diagnosis and the creation of a detailed plan (Nourein et al., 2021,

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Yuzbasioglu, 2021). Professionalism and ethics are required for the academic programs of accredited dental schools in the American Dental Education Association (AlHamdan et al., 2016). Professionalism in education is fundamental to dental education and necessary for life-long education and good dental practice. In general, the word "learning style" defines the preferred way to collect, process, interpret, organize and evaluate knowledge by a person. In accordance with sensory methods involved in the taking of information, the VARK model developed by Fleming et al., (1992) provides students profile of their learning styles. VARK is an acronym for sensory modalities such as Visual (V), Auditory (A), Read/Write (R) and Kinesthetic (K). If they can see it the visual students better process knowledge. Audience students enjoy hearing knowledge. The students of read-writing tend to see the written words. The students enjoy the know-how and experience of kinesthetics (Kharb et al., 2013, Al-Khalifa et al., 2020).

Existing teaching trends have shown that increased enrollment in online courses and programs provides learners with the ability to gain credit towards graduation from secondary education remote learning. These online services can be hosted in different ways: a combined learning center in remote schools supervised by a school or agency without a computer in a student's home or alternate settings such as residential treatments, hospitals and home health centers (Kenrick et al., 2020). There are many types, systems and patterns of learning mentioned in the book; 71 schemes have been identified in one review. The most widely used models are VARK and Kolb. Learning models. The inventory of Kolb's learning styles (LSI), probably one of the most common and most commonly used surveys, uses Kolb's learning styles to help students recognize their style of learning.

It also offers information on how educators can better support students through this information and potential methods for integrating various types of learning. The successful learning system relies on four different modes of learning: concrete experience (CE), retrospective reflection (RO) and abstract conceptualization (AC). Students need to be able to completely, freely and without prejudice to active experiences (CE). The students need to reflect and observe these experiences from a wide range of viewpoints. They need to construct ideas that incorporate insights into logically-sonic hypotheses (Hernandez et al., 2020).

Universities face new difficulties which put growing pressure on learning environments to be created. Many such challenges concern the use of modern pedagogical methods, the quick evolution of education technologies, the diversification of the population of non-traditional students in need of flexible courses, and the increasing demands about the skills required for today's and future working lives. These changes often align themselves with the major transition that Barr et al., (1995) defines as a shift from an instructional model to a learning paradigm, or from transmitting information to students in the construction of knowledge.

In other words, more student-centered teaching and learning activities have been shifted. Although these references are relatively old, they still apply to current development goals. Based on the Horizon annual reports, physical environments require improvements to best fulfill the requirements of the pedagogical activities of today, which underline the active role of students. The conventional university lecture halls make it possible to change learning environments to meet the needs of neither contemporary pedagogy nor the efficient use of modern technology (Valtonen et al., 2020).

Teaching to speak is important if someone only learns English for academic purposes and is not able to speak English, which is very uncommon. Strong command on speech skills gives learners a real sense of progression and reinforces their confidence. Written communication is a valuable lesson, since it is a fundamental skill in life. Students can need notes, forms, letters, papers, stories, etc. Many need to complete comprehensive health, education and job questionnaires.

Adequate writing ability gives one the faith and characterizes one's language knowledge (Hossain et al., 2015). The downside of lecturing is that the audience has little to no interaction. The principal goal of education at all levels of education is to transform the learner fundamentally and strengthen the process of transmission of knowledge (Reymus et al., 2020; Szabo et al., 2020). Limited studies have been implicated in the Saudi population and current study aimed to perform a questionnaire-based study in the dental students. Therefore, the aim of this study is to describe and compare male and female dental students' preferences on various aspects of teaching, demonstration and assignment of various dental colleges in the Riyadh city.

MATERIAL AND METHODS

Study design: For this study, ethical approval from an IRB Research Center in Riyadh Elm (REU) was granted. The informed consent document has been signed by all participants before the data from participants was collected. This was a sectional survey, performed by the general population of Saudi Arabia through an online survey. The survey was required both for men and women under the age of 18 who were eligible to participate in the report. In the social media, 200 students were contacted. An online questionnaire with questions regarding personal and demographic details was prepared using Google forms based on awareness and questions relevant

to preferences. The e-questionnaire is categorized into three phases and Phase-I consisted of the three parts: Gender,

Student Level and University: Phase-II, covered details about the length and timing of lectures, schedule notes, participation, material preferences before the session, interactive sessions and clinical demonstration and handouts; and Phase-III, covering preferences. Preferences A 3-point Likert scale (1 = accept, 2 = neutral and 3 = disagree) was used to answer most questions. The questionnaire was circulated to students of both the KSU and the REU. The selection of a sample of 200 participants representing dental students in the couple of schools included a stratified random sampling process. In order to assess the acceptability and ensure that the questionnaire is true and clear, a pilot study was conducted on 20 students. Minor modifications were made prior to the delivery of the questionnaire, based on the responses (Tulbah et al., 2019).

Validity and reliability of the instrument: In order to determine the validity by Chronbach's alpha coefficient, a pilot study was performed with 20 parents and the data inserted in SPSS Version 22. The reliability of the survey was checked by passing it to professional REU researchers and their suggestions and comments will allow improvements.

Statistical analysis: The data was analyzed both descriptively and inferentially with the SPSS version 22. Comparisons have been made between groups and the importance value was held below 0.05 (Khan et al., 2019).

RESULTS AND DISCUSSION

The study comprises a survey of 150 graduate students, interns and graduate dentists. The study participants had an average age of 18 to 27 years. There were 58% of male and 42% of female respondents were involved in this questionnaire-based study. Most of the participants chose classes for lectures over afternoon (20%) or evening (11.3%) during morning hours (62%), and time for 6.7% participants didn't matter. Although, 82.7% of students preferred to take lecturers for at least 30 to 45 minutes (34 and 90 respectively), while, 13.3% preferred lectures for 60 minutes and for 4% of subjects no time was important. The preferred teaching method by category was visual (52.7%), while general estimates were the least preferred teaching method.

Fifty percent of respondents were interested in audio vision and 42% were interested in audiovisual vision, 18.7% in blackboard, 20% in LCD projection, and 6.7% in OHP projections. The remaining 14.7% of learners were involved in oration lectures and 17.3% chose assignments. In Table-1, the full details have been listed. The students were mainly selected for 49.3% of assignments, 67.3% for MCQs, 20% for periodic examinations, and 8.7% for Viva Voce. Nevertheless, 14% of students selected all the teaching strategies. In Table 2, the complete information have been documented.

Table 1. List of availability of teaching methods involved in this study

S. No	Types of Teaching methods	Number	Percentages
1	Audio	75	50%
2	Visual	79	52.7%
3	Audiovisual	63	42%
4	Blackboard	28	18.7%
5	LCD Production	30	20%
6	OHP sheets	10	6.7%
7	Oration lectures	22	14.7%
8	Assignments	26	17.3%

Table 2. List of availability of teaching option methodologies

S. No	Additional options	Number	Percentages
1	Assignments	74	49.3%
2	Multiple choice questions (MCQs)	101	67.3%
3	Periodic Tests	30	20%
4	Viva Voce	13	8.7%
5	Combination of all	21	14%

Table 3. List of innovative methods applied in teaching

S. No	Innovative Teaching methods	Number	Percentages
1	Video clips	63	42%
2	Small group discussions	83	55.3%
3	Short quizzes	87	58%
4	Handouts of study material	27	18%
5	Problem solving sessions	39	26%

The students have opted 58% as short quizzes for innovative methods along with 55.3% of small group discussions, 42% for video clips, 18% for handouts of study material. Finally, 26% of the students have opted for problem solving sessions. The respondents thought that the content of the ideal theory class should include a mixture of content from a detailed textbook, recent

newspaper updates and free use of audiovisual materials. The complete details have been listed in Table 3.

The participants believed that the content of an ideal theory class would include 8.7% of Exhaustive textbook content, 8.7% of recent update from journals and 50.7% opted the combined combination of exhaustive textbook content cum recent update from journals. Only, 6.7% of the students have opted the liberal use of audiovisual aids and 25.2% of students requested for all the above options. The complete details have been shown in Table 4. The timing of the lectures (55.3%) and duration of classes (45.3%) were the key reasons for student disinterest in a lecture class and the timing of boring lectures (37.3%) and Unimpressive presentation by the lectures (23.3%). The complete details have been documented in Table 5.

Table 4. List of combined additional teaching methods

S. No	Combined Teaching methods	Number	Percentages
1	Exhaustive textbook content	13	8.7%
2	Recent update from journals	13	8.7%
3	Combination of S. No 1 and 2	76	50.7%
4	Liberal use of audiovisual aids	10	6.7%
5	All of the above	38	25.2%

Table 5. List of combined additional teaching methods

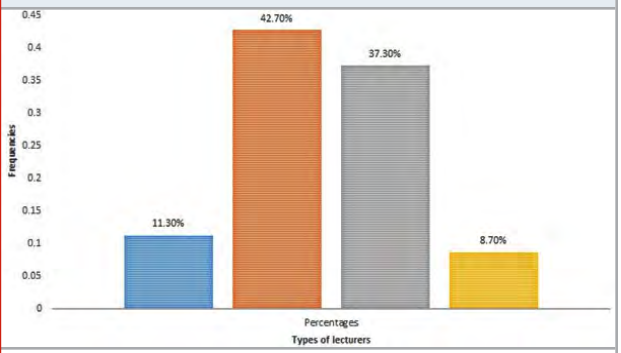
S. No	Unimpressive Teaching methods	Number	Percentages
1	Duration of classes	68	45.3%
2	Timing of Lecture	83	55.3%
3	Boring lecture content	56	37.3%
4	Unimpressive presentation of the lecture	35	23.3%

The categorization and frequencies of lecturers are listed in Figure-1 and 42.7% of students reported that 50% of lecturers could only understand and 37.3% of students reported that 75 % of lecturers could understand for different reasons. Nonetheless, 11.3% and 8.7% of the students indicated that 25% of the lecturers and entire lectures were followed without confusion. Table 6 has been categorized as per the gender wise criteria and 38.6% of males and 61.4% of females were involved. The male students have been given high priority for understanding 25% and 50% of appreciated lecturers for 12.1% and 44.8% respectively. For the remaining 75% and 100% of appreciated lecturers, 38.1% and 9.8% of female students were given high priority and the complete details were recorded in Table 6.

Table 6. Gender variation frequencies about understanding of lecturers

S. No	Frequency of lecturer understanding	Males (n=58)	Females (n=92)
1	25% of appreciated lecturer	07 (12.1%)	10 (10.8%)
2	50% of appreciated lecturer	26 (44.8%)	38 (41.3%)
3	75% of appreciated lecturer	21 (36.2%)	35 (38.1%)
4	100% of appreciated lecturer	04 (6.9%)	09 (9.8%)

Figure 1: prevalence and frequencies of understanding the lectures



Lane 1 represents the 25%, lane 2-50%, lane 3-75% and lane 4 with 100%

The aim of this study is to identify and compare the preferences of male and female dental students on various aspects of dental school teaching, demonstration and assignment in Riyadh. This study was carried out in couple of dental colleges in the capital city of the kingdom i.e., dental colleges at KSU and REU. In the present study, the majority of study participants chose morning hour classes, which may be attributed to the opportunity to better understand things during that time span. This result is in line with the findings of the

previous studies which have been linked to improved morning focus, (Parolia et al.,2012; Thilakumara et al., 2018; Tulbah et al., 2019; Faust, 2020; Al-Khalifa et al., 2020).

As this community forms the future of dentistry, dental graduates are the subjects of several research studies. There is a need to review the current teaching framework and put about the requisite improvements in order to improve the learning process of dental students. While the most widely used form of teaching is lecturing, previous studies have highlighted the importance of incorporating other active approaches to promote learning. The majority of respondents in the current study favored a 30-minute lecture class followed by 45 minutes, which is consistent with the findings of the prior studies (Parolia et al., 2012; Stuart and Rutherford, 1978).

One of the previous studies by Stuart and Rutherford (1978) stated that the student concentration was maximal for the initial 10 to 15 minutes and subsequently declined. A total of 1,760 lecture hours for undergraduate dental education is recommended by the Dental Council of India in its 2007 guidelines. It may not be feasible to introduce 30 to 45-minute lecture classes if these recommendations are to be achieved. Therefore, 60 minutes of lectures using different novel teaching techniques are suggested to combat students' limited attention span.

Allers (2010) carried out a study among dental students and stated that strong visual modalities such as video/TV, posters/charts, models, and simulations were preferred by them. A previous study by Parolia et al., (2012) found that the most favored teaching modes were the PowerPoint display, chalkboard, and clinical demonstrations. In the present research, respondents favored various teaching aids with no clear option, such as audiovisual, blackboard, PowerPoint presentations. Similarly, respondents were of the opinion that for the evaluation of students, different assessment modalities should be used without any clear preference for a single modality.

The current subjects of the study claimed that exhaustive textbooks, latest updates from journals with liberal use of audiovisual aids should be included in the contents of the ideal class. Such results can be due to the dynamic nature of dental education, which involves stimulation of the dental students' different senses to grasp and assess the composite dental curriculum. Lecturing is the most widely done modality of teaching, which has a significant downside of losing contact between the lecturer and the students. When asked about the level of comprehension of the substance of the lecture,

In our study, only 13 study subjects indicated that they were able to comprehend the entire content of the lecture, while the remaining participants ranged from 25 to 75% in their comprehension of the lecture content. These findings are consistent with the previous studies (Amini et al., 2010; Keefe, 1978), who found that each person's learning styles are different and improved learning occurs if effective learning methods are used. It is important to implement other active teaching methods, such as handouts, workshops, problem-based learning, discussions, tutorials, etc in order to promote learning among all students. The primary reasons for being disinterested in a lecture class were the timing of a lecture and class length.

By incorporating more imaginative and active learning methods that encourage a healthy relationship between the students and the lecturer, lecturers should avoid becoming passive orators and facilitate learning among the students. We analyzed the variations in learning styles based on gender in the current research. In understanding the content of the lecture, a significant difference was observed between male and female subjects, although no significant difference was observed between other questionnaire objects. While designing teaching plans, these findings can be significant. One of the prior studies by Khan et al., (2017) have contributed to a paradigm change from in-class lectures and discussion to mobile learning. WhatsApp M-learning can be an alternative, imaginative and interactive method for achieving the necessary objectives in medical education.

Although the study findings for a more precise and validated finding have to be checked in a larger sample size. In addition, questionnaire-based studies are vulnerable to prejudices that need to be considered when evaluating their findings. The following aspects of teaching are illustrated in the present study: While lecturing is the most common form of teaching, it is associated with major disadvantages, especially the lack of interaction between the lecturer and the audience. The current research has policy implications for improvements in the patterns of teaching conducted at present times. In order to address the disadvantage of lack of engagement and also to promote learning for all learners, more constructive learning initiatives need to be implemented (Lone et al., 2019; Jum'ah et al., 2020).

CONCLUSION

In conclusion, the maximum participants preferred 45 minutes of lecture classes in the morning hours. The most favored teaching aid was the visual method, while the most preferred method for assessment was multiple choice questions and assignment. Nevertheless, respondents were of the belief that novel approaches should be adopted to promote the process of learning. The key factors for disinterest in a class were the pacing of the lecture and the length of the class. In terms of

the average percentage of the lecture material they were able to understand, a substantial gap between male and female research subjects was noted. Future studies should be implemented with the large sample size.

Conflict of Interest: None

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In-Silico Analysis of Nonsynonymous Single Nucleotide Polymorphism in Human PCSK1 Gene

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ABSTRACT

The proprotein convertases (PCs) are involved in variety of cellular precursors in the secretory pathway. Polymorphisms in proprotein convertase subtilisin/kexin type 1 (PCSK1) have been associated with adult and childhood obesity. In this work non synonymous SNPs of the PCSK1 gene were retrieved from the dbSNP database. In order to predict the damaging or deleterious nsSNPs, multiple consensus tools were employed by using online tool VEP. Further we also employed SNP-GO tools to predict pathogenic nonsynonymous SNPs. Mutants like D176Y, E345A, G228V, G308E, G310R, G440E, G442R, R110C, S382L, W130S and W404R have shown deleterious and highest pathogenicity. These predicted deleterious and pathogenic nsSNPs are expected to have impending functional influence and may contribute in understanding the functional roles of PCSK1 gene associated with obesity.

KEY WORDS: NSSNP, PROPROTEIN CONVERTASE SUBTILISIN/KEXIN TYPE 1, NEUROENDOCRINE CONVERTASE 1, IN SILICO ANALYSIS, PCSK1.

INTRODUCTION

An obesity increasing worldwide and polymorphisms in proprotein convertase subtilisin/kexin type 1 (PCSK1) gene have been associated with adult and childhood obesity. Body mass index variation (risk of common obesity) is associated with more than 60 single-nucleotide polymorphisms (SNPs), identified by genome-wide association studies (Philippe 2015). The proprotein convertases (PCs) are involved in variety of cellular precursors in the secretory pathway and due to homology of their catalytic domains to bacterial subtilisin and yeast kexin, the genes are known as subtilisin and kexin-like proprotein convertases (PCSKs) (Stijnen, 2016 Loffler , 2016). Human PCSK1 gene consists of 14 exons located on chromosome 5 (Ramos-Molina, 2016), and its promoter contains transcriptional elements CRE-1 and CRE-2 which can be transactivated by CREB-1 and ATF1 transcription factors (Espinosa, 2008; Stijnen,2016). Analysis of human tissues and cells revealed the presence of a dominant transcript and the major sites of expression being endocrine pancreas, pituitary and brain (Stijnen, 2016). 71% of PCSK1 variant were located in coding region of the catalytic domain and 21% are located on the P domain (Akinci 2019).

Many studies show a strong evidence about rs6232 and rs6235 involving with obesity (Jackson ,2003). association with three variants are found in PCSK1 gene rs6232 encoding by N221D substitution involve in reduce the activity of PC1/3 while rs234 encodes by Q665E compatible with rs6235 that encodes by S690T are essential to form a linkage between PC1/3 and its sorting in secretory granules (Stijnen, 2016; Frank 2013), these

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variants have a significantly role in reducing the level of plasma glucose rapidly and increase serum insulin levels causing a hyperglycemia as type 2 diabetes (T2D) by increasing in glocuse production, insulin resistance and a dysfunction in β cell that found in pancreatic cells (Gjesing, 2011), along with the effect of blood pressure and energy ratio, causing a hypertension in the blood vessels which lead to a cardiovascular (Heni, 2010 Pepin et al 2019).

The R405X mutation cause a deletion of P and C-terminal tail domain (Bandsma, 2013). Identified N309K a deleterious in PCSK1 gene which make C-terminal domain incapable of cleave in intermolecular interaction (Wilschanski, 2014). K26E is located before the signal peptide cleavage site, M125I, T175M, N180S, Y181H, G226R and S325N are located in the catalytic domain and the T558A is located in the middle domain. These mutations have an impact on PC1/3 folding and its stability. also, G209R and G593R mutation might affect on the PC1/3 misfolding due to their enzymatic activation (Blanco, 2015).In addition, T175M was defined as induce the inhibition in N-glycosylation site which is responsible for cellular signal and altering the protein maturation (Creemers, 2012 Pepin et al 2019).

MATERIAL AND METHODS

Datasets: The SNPs of the PCSK1 gene were retrieved from the dbSNP database (Sherry, 2001). Keyword "Human PCSK1" used as our search term. Furthermore, it is filtered by selecting variation class as SNV, function class as missense. The protein sequences (P29120) were retrieved from the UniProt (https://www.uniprot.org).

Predicting deleterious and damaging nsSNPs: In order to predict the damaging or deleterious nsSNPs, multiple consensus tools were employed by using online tool VEP (http://www.ensembl.org/Tools/VEP). VEP advantages include: it uses latest human genome assembly GRCh38. p10, and can predict thousands of SNPs from multiple tools including SIFT, PROVEAN, Condel, and PolyPhen-2, at a time. nsSNP rs-ids were uploaded to VEP tool to get the prediction results

SIFT: The algorithm predicted that the tolerant and intolerant coding base substitution based upon properties of amino acids and homology of sequence (Choi Y, 2015). The tool considered that vital positions in the protein sequence have been conserved throughout evolution and therefore substitutions at conserved alignment position is expected to be less tolerated and affect protein function than those at diverse positions., SIFT predicted substituted amino acid as damaging at default threshold score <0.05, while score 0.05 is predicted as tolerated.

PolyPhen-2: This tool is predicting the structural and functional consequences of a particular amino acid substitution in human protein (Adzhubei, 2010). Prediction of PolyPhen-2 is based on a number of features including information of structural and sequence comparison. The PolyPhen-2 score varies between 0.0 (benign) to 10.0 (damaging). The PolyPhen-2 prediction output categorizes the SNPs into three basic categories, benign (score < 0.2), possibly damaging, (score between 0.2 and0.96), or probably damaging (score >0.96).

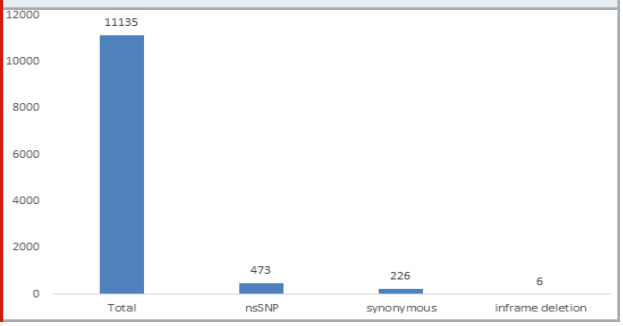
PROVEAN: This tool (http://provean.jcvi.org/) uses an alignment-based scoring method for predicting the functional consequences of single and multiple amino acid substitutions, and in-frame deletions and insertions (Choi, 2015). The tool has a default threshold score, i.e. -2.5, below which a protein variant is predicted as deleterious, and above that threshold, a protein variant is neutral.

CONDEL (CONsensus DELeteriousness): This tool evaluates the probability of missense single nucleotide variants (SNVs) deleterious. it computes a weighted average of the scores of SIFT, PolyPhen2, MutationAssessor and FatHMM (Hecht , 2015).

Predicting disease associated nsSNPs

SNPs&GO: A web server predicting whether an amino acid substitution is associated to a disease or not (http://snps.biofold.org/snps-and-go) (). It is a SVM (Support Vector Machine) based tool which takes features of protein sequence, evolutionary information, and functional annotation according to Gene Ontology terms. We input isoform 1 of Swiss-Prot Code of LSP1 (P33241) and provided the list of amino acid mutations. The results predicted the probability for the polymorphisms of helicase whether being disease- associated or not by three methods: (a) SNPs&GO, (b) PhD-SNP, and (c) PANTHER. Probability score >0.5 is predicted as disease associated variation (Calabrese , 2015).

Figure 1: Number of SNPs in different function class of PCSK1 gene of human from dbSNP database



RESULTS AND DISCUSSION

473 nsSNP ids of human PCSK1 gene was downloaded from dbSNP database of NCBI (Supplementary Table 1), after filtering variation class SNV, function class missense, there were 473 SNP mapped to missense, 226 SNPs mapped to synonymous and 6 SNPs mapped to inframe deletion, while 11135 mapped to total SNPs of different variation class (Figure 1).

Figure 1: Frequency of aggressive behavior of patients/attendants faced by study subjects.

SNP-ids	AA-change	SIFT (score)	PolyPhen (score)	Condel (score)	PROVEAN (score)	PANTHER Prediction	RI
rs759379849	D193G	*(0)	#(0.999)	*(0.935)	* (0.92173)	Disease	8
rs1561374455	D195G	*(0)	#(0.96)	*(0.848)	*(0.91956)	Disease	8
rs752416942	D272G	*(0)	#(1)	*(0.945)	*(0.93175)	Disease	8
rs749888385	T353I	*(0.02)	#(0.967)	*(0.792)	*(0.78636)	Disease	8
rs762403860	A213V	*(0)	#(0.998)	*(0.919)	*(0.71639)	Disease	9
rs1475050973	C212R	*(0)	#(1)	*(0.945)	*(0.99425)	Disease	9
rs552958813	D176N	*(0)	#(1)	*(0.945)	*(0.80172)	Disease	9
rs752416942	D272V	*(0)	#(1)	*(0.945)	*(0.9783)	Disease	9
rs1363728113	G155D	*(0)	#(0.98)	*(0.869)	*(0.90023)	Disease	9
rs1382566997	G155S	*(0)	#(0.986)	*(0.879)	*(0.83899)	Disease	9
rs1490377137	G158A	*(0)	#(0.999)	*(0.935)	*(0.873)	Disease	9
rs768031892	G209R	*(0)	#(1)	*(0.945)	*(0.95336)	Disease	9
rs142673134	G279A	*(0.04)	#(0.959)	*(0.752)	*(0.86296)	Disease	9
rs1312543959	G298A	*(0)	#(0.999)	*(0.935)	*(0.87223)	Disease	9
rs778681269	G311R	*(0)	#(1)	*(0.945)	*(0.95276)	Disease	9
rs567641208	G390S	*(0)	#(0.999)	*(0.935)	*(0.88839)	Disease	9
rs1389330621	N180K	*(0)	#(0.999)	*(0.935)	*(0.85994)	Disease	9
rs1269967613	N429K	*(0)	#(0.994)	*(0.897)	*(0.87835)	Disease	9
rs1246203022	P280S	*(0)	#(1)	*(0.945)	*(0.96058)	Disease	9
rs775618000	P341L	*(0)	#(1)	*(0.945)	*(0.98437)	Disease	9
rs775136858	P386L	*(0)	#(0.998)	*(0.919)	*(0.98692)	Disease	9
rs1332430207	Q408R	*(0)	#(1)	*(0.945)	*(0.73267)	Disease	9
rs748072514	R110H	*(0)	#(0.999)	*(0.935)	*(0.76822)	Disease	9
rs768934109	R296I	*(0)	#(1)	*(0.945)	*(0.95246)	Disease	9
rs1421014042	S186N	*(0)	#(0.996)	*(0.906)	*(0.59873)	Disease	9
rs137852824	S307L	*(0)	#(0.999)	*(0.935)	*(0.86222)	Disease	9
rs1166018774	T210S	*(0)	#(0.999)	*(0.935)	*(0.71762)	Disease	9
rs1303515025	T276I	*(0)	#(0.996)	*(0.906)	*(0.84742)	Disease	9
rs766414747	T375K	*(0)	#(0.998)	*(0.919)	*(0.88839)	Disease	9
rs766414747	T375M	*(0)	#(0.993)	*(0.895)	*(0.88839)	Disease	9
rs1346360455	T381I	*(0)	#(1)	*(0.945)	*(0.88839)	Disease	9
rs1434467255	W130L	*(0)	#(1)	*(0.945)	*(0.99433)	Disease	9
rs868424536	W152L	*(0)	#(0.985)	*(0.877)	*(0.99023)	Disease	9
rs1245583638	W342G	*(0)	#(0.998)	*(0.919)	*(0.99704)	Disease	9
rs1246742230	W98R	*(0.02)	#(0.994)	*(0.835)	*(0.99587)	Disease	9
rs552958813	D176Y	*(0)	#(1)	*(0.945)	*(0.97364)	Disease	10
rs864309557	E345A	*(0)	#(1)	*(0.945)	*(0.873)	Disease	10
rs747169606	G228V	*(0)	#(1)	*(0.945)	*(0.97617)	Disease	10
rs990328651	G308E	*(0)	#(1)	*(0.945)	*(0.95246)	Disease	10
rs748808191	G310R	*(0)	#(1)	*(0.945)	*(0.95276)	Disease	10
rs865777271	G440E	*(0)	#(1)	*(0.945)	*(0.95665)	Disease	10
rs761336991	G442R	*(0)	#(0.999)	*(0.935)	*(0.95665)	Disease	10
rs774036542	R110C	*(0)	#(1)	*(0.945)	*(0.93582)	Disease	10
rs1561368007	S382L	*(0)	#(0.998)	*(0.919)	*(0.88839)	Disease	10
rs1434467255	W130S	*(0)	#(1)	*(0.945)	*(0.99699)	Disease	10
rs1180593976	W404R	*(0)	#(0.998)	*(0.919)	*(0.99969)	Disease	10

(*Deleterious, #Probably Damaging)

Predicting deleterious and damaging pathogenic nsSNPs: In order to predict the damaging or deleterious nsSNPs multiple consensus tools were employed. Initially, online tool VEP was used (McLaren, 2016). VEP advantages include: it uses latest human genome assembly GRCh38.p10, and can predict thousands of SNPs from multiple tools including SIFT, Condel, and PolyPhen-2, at a time. 473 nsSNP rsids were uploaded to VEP tool and the prediction results were taken on default scores of consensus tools based on sequence and structure homology methods: (a) SIFT (score <-0.5) (b) Polyphen (score >0.96) (c) PROVEAN (score< 2.5) and Condel (score >0.522).

In order to get a very high confident nsSNPs impacting structure and function of PCSK1 gene, 46 nsSNPs out of 473 nsSNP (Table 1) were found to be deleterious by all four tools and predicted disease by panther tools, and these eleven nsSNPs rs552958813 of mutation D176Y, rs864309557, of mutation E345A, rs747169606 of mutation G228V, rs990328651 of mutation G308E, rs748808191 of mutation G310R, rs865777271 of mutation G440E, rs761336991 of mutation G442R, rs774036542 of mutation R110C, rs1561368007 of mutation S382L, rs1434467255 of mutation W130S and rs1180593976 of mutation W404R were predicted highly pathogenic with more than 9 RI score (Table-1).

Studies show a strong evidence about variants are found in PCSK1 gene involving with obesity, association with variants N221D, S690T and Q665E substitutions found in PCSK1 gene involve in reduce the activity of PC1/3, linkage between PC1/3 and its sorting in secretory granules (Jackson 2003, Stijnen 2016; Frank 2013), Identified N309K a deleterious in PCSK1 gene which make C-terminal domain incapable of cleave in intermolecular interaction (Wilschanski, 2014). K26E is located before the signal peptide cleavage site, M125I, T175M, N180S, Y181H, G226R and S325N are located in the catalytic domain and the T558A is located in the middle domain.

These mutations have an impact on PC1/3 folding and its stability. also, G209R and G593R mutation might affect on the PC1/3 misfolding due to their enzymatic activation (Blanco EH, 2015). In addition, T175M was defined as induce the inhibition in N-glycosylation site which is responsible for cellular signal and altering the protein maturation (Creemers, 2012). Pickett had proposed that R80Q have the most influence part in PC1/3 maturation and its activity (Pickett, 2013). In another report, the S357G mutant that low the calcium dependence and highly resistance the peptide inhibitors (Blanco, 2015).

CONCLUSION

Our investigation shows mutants D176Y, E345A, G228V, G308E, G310R, G440E, G442R, R110C, S382L, W130S and W404R with deleterious and highest pathogenicity, and may offer valuable information in selecting SNPs that are expected to have impending functional influence and pathogenicity also eventually may contribute in understanding the functional roles of PCSK1 gene associated with obesity.

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Prevalence and Microbiological Pattern of Blood Stream Infection Caused by Multi Drug Resistance Gram Negative Bacteria in Western Saudi Arabia

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ABSTRACT

Bloodstream infection (BSI) is one of the primary causes of morbidity and mortality worldwide. The management of nosocomial BSI is challenging. BSI may be associated with Multidrug-resistant Gram-Negative Bacteria (MDR-GNB), which are difficult to treat with conventional and available antimicrobial drugs. Globally, the increased prevalence of MDR -GNB has led to a significant change in the spectrum of microorganisms isolated from patients with BSI. The aim of this study to investigate the prevalence , epidemiological aspects and Microbiological pattern of BSI caused by MDR-GNB at King Abdulaziz University Hospital in Jeddah, Saudi Arabia, to facilitate the development of Multidrug-Resistant Organisms (MDROs) Prevention and Control policy and to support proper selection of antimicrobial treatment and management of MDR-GNB infection . Method: a retrospective analysis conducted in patients with GNB BSI, which included all hospital departments, using the data from the Clinical and Molecular Microbiology Laboratory database. All positive blood culture results from June 2017 to June 2020 were reviewed. Result: a total of 302 patients with positive blood culture were identified. The major risk factors for acquiring BSI were immunocompromised conditions, such as cancer (25%) and kidney disease (24.5 %,). The emergency room was the department with the most isolated cases (39.4%). *Escherichia coli* (43%) was the principal Gram Negative Bacilli responsible for BSI, and *Acinetobacter baumannii* was the most extensively drug-resistant GNB (84%). In conclusion, this study illustrates the importance and value of continuous surveillance of MDROs. Clinical microbiology laboratories should monitor MDR , XDR and Pan drug-resistance (PDR) bacterial strains to reduce the incidence of antimicrobial resistance and to help in the formulation of effective antimicrobial stewardship programmes in healthcare facilities.

KEY WORDS: BLOODSTREAM INFECTION, BLOOD CULTURE, MULTIDRUG-RESISTANT GRAM-NEGATIVE BACTERIA , ANTIMICROBIAL RESISTANCE.

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INTRODUCTION

Blood stream infection (BSI) and bacterial sepsis are public health threats. Recently World Health Organization listed BSI as a global health priority (Leal et al., 2019). The management and treatment of BSI have become challenging during the last decade due to the emergence of Multidrug-resistant organisms (MDROs) that are difficult to treat using conventional antimicrobial drugs (Gudiol et al., 2011). The increased prevalence of Multidrug Resistance Gram Negative Bacteria (MDR-GNB) has led to a significant change in the spectrum of microorganisms isolated from patients with BSI (Breijyeh et al., 2020). Understanding the definition and the mechanisms of antibiotic resistance and how these mechanisms can evolve and spread is essential for surveillance and tracking the spread of drug resistance Bacteria (Iredell et al., 2016).

MDROs is defined as non-susceptible or resistance of a microorganism to the antimicrobial agents in spite of previously susceptible to it (Tanwar et al., 2014). Basak et al. (2016) defined extensively drug-resistant (XDR) as bacteria non-susceptible to at least one drug in all but two or fewer antimicrobial categories (i.e., bacterial isolates sensitive to only one or two antimicrobial types); and pan drug-resistance (PDR) as no susceptibility to all agents in all antimicrobial categories. Infections due to MDR-GNB are an increasing threat to human health and are associated with excessive morbidity, mortality, and healthcare costs (Morris and Cerceo, 2020). It has become more challenging to control the spread of MDROs due to their growing antibiotic resistance (IDSA, 2011). A rising attention about the clinical and economic impact of MDROs has led to a major focus on antibiotic stewardship to reduce inappropriate antimicrobial prescribing (Thatrimontrichai, et al., 2020).

MATERIAL AND METHODS

2.1 Study design: A retrospective study for all patients with Gram Negative Bacteria (GNB) BSI was conducted at King Abdulaziz University Hospital (KAUH) in Jeddah, Saudi Arabia. All departments of KAUH (ER, ICU/CCU, MMW, MICU, FMW, NICU,PW & SICU) were included in our study. The sample population included all age groups.

All positive blood culture results from June 2017 to June 2020 were reviewed using study data obtained from the Clinical and Molecular Microbiology Laboratory (CMML) database. We only considered blood cultures and did not include other types of microbiology culture. If a patient had multiple admissions for GNB, they were included in the study as different episodes. However, if a patient developed recurrence of BSI during the same admission, it was considered as a single-patient episode of BSI. There were no other exclusion criteria.

Patients' electronic medical records were reviewed. Data collection included the following clinical variables: (a) age and gender (b) comorbid conditions; (c) use of antibiotics in the last 30 days; (d) source of infection; (e) use of a central venous catheter ≥48 h before the onset of GNB (f) antimicrobial resistance patterns in GNB blood culture isolates; and (g) mortality within 30 days. Ethical approval for all patients was obtained from the KAUH Research Ethics Committee (reference no.: 543-20 Oct.29.2020). The requirement of patient consent was waived due to the retrospective nature of the study.

2.2 Study Definitions

The following definitions were used: MDROs were defined according to the US Centres for Disease Control and Prevention definitions. MDR-GNB were defined as ESBL-producing *Enterobacteriaceae* and any GNB (e.g., *Acinetobacter* spp., *Enterobacteriaceae*, and *Pseudomonas* spp.) resistant to three or more of the following drug classes: piperacillin/tazobactam, Cephalosporins (Cefazolin, Ceftriaxone, Ceftazidime, and Cefepime), Carbapenems (Imipenem), Monobactams (Aztreonam), Aminoglycosides (Gentamicin, Tobramycin, and Amikacin), and Fluoroquinolones (Ciprofloxacin and Levofloxacin). Recurrence of BSI was defined as a positive blood culture with same GNB after ≥1 negative blood culture and after an interval of ≥7 days. Mortality was defined as death by any cause within 30 days of the onset of BSI.

2.3 Identification and characterisation of the bacterial isolates: Blood culture bottles were incubated at CMML using the BacT/Alert VIRTUO Microbial Detection System (bioMérieux, Durham, NC, USA), which is fully automated and yields real-time results. The blood culture bottles were incubated until a signal-positive alarm was sounded or for a maximum of 5 days. Samples from the positive blood culture bottles were processed using Gram staining, the results were entered in the system and the department was verbally informed. Then, following the CMML's blood culture manual, all positive blood culture bottles were sub-cultured on 5% sheep blood agar, chocolate agar and MacConkey agar (Saudi Prepared Media Laboratories). The MacConkey agar plates were incubated at 35–37 °C for 18–24 h in an ordinary incubator (Forma Scientific Incubator, Germany). The blood agar and chocolate agar plates were incubated at 35–37°C in 5–10% CO2 (Sanyo CO2 Incubator, Japan).

Antibiotic sensitivity was assessed using a manual technique (the disc diffusion method). Mueller-Hinton

plates (Saudi Prepared Media Laboratories, Riyadh, Saudi Arabia) were inoculated with blood samples taken directly from the positive blood culture bottles. The plates were incubated at 35–37°C for 18–24 hours in an ordinary incubator (Forma Scientific Incubator). Antibiotic discs were selected according to the guidelines provided by the Clinical and Laboratory Standard Institute (CLSI).

After 24 h of incubation, gram-negative bacilli colonies were identified using a VITEK 2 system (bioMérieux, Marcy-L'Étoile, France) according to the manufacturer's instructions. This automated system uses a turbid metric method with VITEK 2 GN ID (BioMérieux), namely Gram-negative identification cards including members of the family *Enterobacteriaceae* as well as non-enteric bacilli. The suspension was prepared from a pure sub-culture plate by mixing the colony with 3.0 mL of 0.45% sterile saline, which was aseptically added to the plastic test tube. Density was measured by a VITEK 2 DensiCheck System (bioMérieux), and results equivalent to 0.5–0.63 of McFarland standards were used. The suspension tube was placed in a cassette and followed by an empty tube. The VITEK 2 ID Card was inserted in the suspension tube. Less than 30 min elapsed between the preparation of the suspension and the card filling. The cassettes were then loaded into the VITEK 2 system. When the process was completed, on board software and automation moved the cards to the discard area after analysing the data. Finally, the results were collected from the VITEK 2 system after 10–18 h. When the sample cycle was finished, the used cards were discarded in a biohazard bag.

2.4 Antimicrobial susceptibility testing: The VITEK 2 system was used for antibiotic susceptibility testing. AST-GN susceptibility cards (panels N91 and N92) were used according to the manufacturer's instructions. The VITEK 2 system controlled the cards automatically, including their filling, sealing, and transfer to the incubator (35°C). Each AST-gram-negative susceptibility card was placed next to a VITEK 2 card in an empty tube. The results were collected from the VITEK 2 system after 10–18 h. When the sample cycle was finished, the VITEK 2 cassette and tube were discarded in a biohazard bag. The results from the VITEK 2 system were compared to the Gram-negative bacteria identification databank. CMML's antibiotic susceptibility reporting criteria for interpreting resistance, sensitivity and intermediate resistance were based on the updated guidelines of the CLSI. A renewal of that guideline is made with the issuance of each new annual edition by CLSI.

2.5 Data analysis: All data were analysed using SPSS version 22 statistical software (IBM Corp., Armonk, NY, USA). Numerical data were reported as mean ± standard deviation, and categorical data were reported using frequencies and percentages. Chi-square test was used to assess the significance of associations between the study variables and the pathogen types. P-values < 0.05 were considered significant.

Table 1. Epidemiological and clinical characteristics of (302) patients diagnosed as BSI associated with MDR-GNB strain in a period from June 2017 to June 2020 in KAUH.

Demographic Characteristics	No (%) n= 302
Age (years)	46.9 ± 28.2 (54)
Age groups (years)	
0–2	48 (15.9%)
2–18	19 (6.3%)
18–50	65 (21.5%)
>50	170 (56.3%)
Sex	
Male	153 (50.7%)
Female	149 (49.3%)
KAUH Department	
ER	119 (39.4%)
ICU/CCU	22 (7.3%)
MMW	20 (6.6%)
MICU	51 (16.9%)
FMW	15 (5%)
NICU	12 (4%)
PW	25 (8.3%)
SICU	12 (4%)
(immunocompromised patients)	
Cancer	76 (25%)
Heart disease	38 (12.6%)
Pulmonary disease	40 (13.2%)
Kidney disease	74 (24.5%)
Sepsis and meningitis	10 (3.3%)
Liver diseases (cirrhosis)	7 (2.3%)
Diabetes mellitus	14 (4.6%)
Infection Route	
Exovascular	197 (65.2%)
Endovascular	100 (33.1%)
Not determined	5 (1.7%)
Number of Deaths	160 (53%)

All numerical data are presented as mean ± standard deviation (median). All categorical data are presented in n (%). Abbreviations: CCU, coronary care unit; ER, emergency room; FMW, female medical ward; ICU, intensive care unit; MICU, medical intensive care unit; MMW, male medical ward; NICU, neonatal intensive care unit; SICU, surgical intensive care unit; PW,Peadiatric ward.

RESULTS

3.1 Demographic and clinical characteristics: A total of (302) patients were included in the analysis. As shown in Table (1), the numbers of males and females were similar. The majority of the patients (n=170, 56%) were

aged >50-years. Most of the BSI cases were obtained from the emergency room (ER). Within the sample population, the groups with highest number of BSI were patients diagnosed with immunocompromised conditions such as cancer (25%), or kidney disease (24.5%). Most of the BSIs (65%) had an exovascular infection route as secondary infections. The overall mortality rate of the study population was considerably high (n=160, 53%).

3.2 Microbial spectrum and susceptibility patterns of pathogens causing bloodstream infections: From figure (1) *E.coli* was the most prevalence GNB organisms causing BSI (n=130, 43%) & the second most common organisms causing BSI was *K. pneumoniae* (n=94, 31%) . Nearly 97% of the *E. coli* were ESBL producers (Table 2), and 77% were resistant to Ciprofloxacin (Table 3). Moreover, 90% of the *K. pneumoniae* were ESBL producers, while only 5% were CRE (Table 2). *P. aeruginosa* occurred less frequently than GNB BSIs due to the other major organisms (about 9%, p<0.001). The prevalence of MDR was highest among *P.* aeruginosa (66%, p<0.001) (Table 2). Furthermore, the susceptibility pattern of *P. aeruginosa* showed a higher prevalence of Imipenem resistance (63%) (Table 3), resulting in 33% of the isolates being reported as carbapenem-resistant *P. aeruginosa*. A further 51 cases (17%) were caused by *A. baumannii* (Figure 1), of which 84% of the isolates were extensively drug resistant (XDR) (p<0.001) (Table 2).

Table 2. Distribution of MDR-GNB causing BSI. Data collect during a period from June 2017 to June 2020 in KAUH.

Pathogen	Total	Percentage	ESBL	MDR	XDR	CRP	CRE	P-value
E. coli	130	43.0%	126 (96.9%)	1 (0.8%)	0	0	3 (2.3%)	<0.001
K. pneumoniae	94	31.1%	85 (90.4%)	4 (4.3%)	0	0	5 (5.3%)	
P. aeruginosa	27	8.9%	0	18 (66%)	0	9 (33%)	0	
A. baumannii	51	16.9%	0	6 (12%)	43 (84%)	0	0	

The P-value was calculated using the chi-square test. Values <0.05 are statistically significant.
Abbreviations: *A. baumannii, Acinetobacter baumannii*; CRE, carbapenem-resistant Enterobacteriaceae; CRP, carbapenem-resistant *P. aeruginosa; E. coli, Escherichia coli*; ESBL, extended-spectrum beta-lactamase producers; *K. pneumoniae, Klebsiella pneumoniae*; MDR, multidrug-resistant; *P. Aeruginosa, Pseudomonas aeruginosa*; XDR, extensively drug-resistance

Table 3. Susceptibility patterns of multidrug-resistant Gram-Negative Bacteria (GNB) causing BSI.

GNB	TZP	CAZ	CRO	IMP	MEM	CIP	GM	AK	CO
E. coli n=130 (%)	129 (99)	130 (100)	130 (100)	3 (2.3)	3 (2.3)	100 (77)	44 (34)	1 (0.8)	0
K. pneumoniae n=94 (%)	94 (100)	94 (100)	94 (100)	3 (3.2)	3 (3.2)	60 (64)	38 (40)	10 (11)	0
P. aeruginosa n=27 (%)	21 (78)	19 (70.4)	0	17 (63)	17 (63)	15 (56)	6 (22.2)	5 (18.5)	0
A. baumannii n=51 (%)	0	50 (98)	0	50 (98)	50 (98)	50 (98)	40 (78)	39 (76)	2 (4)

Abbreviations: *A. baumannii, Acinetobacter baumannii*; AK, Amikacin; CAZ, Ceftazidime; CIP, Ciprofloxacin; CO, Colistin;CRO, Ceftriaxone; *E. coli, Escherichia coli*; IMP. Imipenem; GM, Gentamicin; GNB ,Gram Negative Bacteria ; MEM, Meropenem; *K. pneumoniae, Klebsiella pneumoniae; P. aeruginosa, Pseudomonas aeruginosa*; TZP,Pipracillin _ tazobactam.

3.3 Major risk factors for BSI: The three most common risk factors leading to BSI were an impaired immune system, an underlying chronic disease, and older age (Table 4). In addition, 26% of the patients had indwelling devices.

DISCUSSION

BSI are a significant cause of morbidity and mortality worldwide. Over the last decades, there has been a significant increase in the number of pathogen isolated from BSI cases that are resistant to antimicrobial drugs

(Leal et al., 2019). Worldwide, numerous MDROs are the leading causes of nosocomial infections (Exner et al., 2017). On other hand, the incidence of community-acquired MDR-GNB infection has also been increasing (Tseng et al., 2017). In our study, the hospital department with the highest number of MDR-GNB infections was the Emergency Room whereas; all the patients arrived to this department were from different segments of the community.

Figure 1: The microbial spectrum of multidrug-resistant gram-negative bacteria causing BSI a period from June 2017 to June 2020 in KAUH. Abbreviations: *A. baumannii, Acinetobacter baumannii; E.coli, Escherichia coli; K. pneumoniae, Klebsiella pneumoniae; & P. aeruginosa, Pseudomonas aeruginosa*

Recognising the risk factors in the development of MDR-GNB in BSI could significantly influence patient management. (Bassetti et al. 2017) reported that the factors that have contributed to the spread of MDR-GNB include the overuse of existing antimicrobial drugs, which has promoted the development of adaptive resistance mechanisms by bacteria. BSI is a life-threatening condition, especially for vulnerable individuals, such as those who are immunocompromised, older adults and individuals with underlying diseases (Exner et al., 2017). Similarly, our study found that underlying chronic diseases and impaired immune systems were major predisposing factors in the development of MDR-GNB and that the groups with the highest frequency of BSIs were immunocompromised patient cases, such as cancer (25%) and kidney diseases (24.5%). MDR-GNB is common among residents in long-term care facilities, particularly those residents with indwelling devices, and these facilities are an important source of such strains among patients admitted to healthcare facilities (Kaye and Pogue, 2015). In our study, we found that over one-quarter of the patients with MDR-GNB infections had a history of an indwelling device used.According to Kuntaman et al. (2018), most patients with MDR-GNB are seriously ill and have a poor prognosis with a high mortality rate. As it shown as evident in the results of our study, the mortality was increased in BSI associated with MDR-GNB (53 %).

Table 4. Major risk factors for BSI among patients in KAUH

Pathogen	Immunocompromised Patients	Underlying Chronic Diseases	Aged >65 Years	Indwelling Devices
E. coli n=130 (%)	100 (77%)	110 (85%)	79 (60.8%)	42 (32.3%)
K. pneumoniae n= 94 (%)	62 (66%)	78 (83%)	47 (50%)	21 (22.3%)
P. aeruginosa n=27 (%)	21 (78%)	24 (89%)	12 (44.4%)	6 (22.2%)
A. baumannii n=51 (%)	38 (74.5%)	41 (80.4%)	32 (62.7%)	14(27.5%)

In MDR-GNB counting, the non-fermenter GNB have a lower frequency of isolation than *Enterobacteriaceae* such as *Escherichia coli* (*E.coli*) and *Klebsiella pneumonia (K.pneumonia)*, while the primary non-fermenter GNB that cause human infections are *Acinetobacter baumannii (A.baumannii)* and *Pseudomonas aeruginosa* (*P. aeruginosa*) (Oliveira and Reygaert, 2019). The prevalence of MDR *E. coli* strains is rising worldwide (Allocati et al., 2013) .The most common MDR-GNB in our study were *E. coli*, followed by *K. pneumoniae, A. baumannii*, and *P. aeruginosa.*

Ruppé et al. (2015) determined that Enterobacteriaceae, were the most important MDR-GNB and that dramatic increase drug-resistance trend in most of the anti-gram-negative agents (β-lactams, Fluoroquinolones, and Aminoglycosides) was the most important resistance issue. Rawat and Nair (2010) determined that extended-spectrum β-lactamases (ESBLs) were a mechanism by which the GNB developed antibiotic resistance in the face of introduction of new antimicrobial agents. ESBLs efficiently hydrolyse extended-spectrum β-lactams, such as Cefotaxime, Ceftriaxone, Ceftazidime, and Aztreonam. *E. coli* and *K. pneumoniae* are the most prevalent members of the *Enterobacteriaceae* group and are responsible for widespread ESBL production such as: SHV-1, TEM-1, and TEM-2 (Al-Otaibi et al., 2016). In our study too, the *E. coli* ESBL producers were the predominant isolates among the GNB-causing BSI Carbapenems, such as Imipenem and Meropenem, which are classes of β-lactam, are the most effective treatments for infections caused by ESBL-producing

bacteria (Breijyeh et al., 2020).Carbapenem-resistant *Enterobacteriaceae* (CRE) or carbapenemase-producing *Enterobacteriaceae*, *Acinetobacter baumannii* (CRAB), and *Pseudomonas aeruginosa* (CRPsA) are earnest cause of nosocomial infections (Tomczyk et al., 2019).

A. baumanniiand P. aeruginosa are increasingly acquiring carbapenem resistance which, given their intrinsic antibiotic resistance, can cause difficult-to-treat infections (Gniadek et al., 2016). Zhang et al. (2016) reported that *P. aeruginosa* can cause severe infections, such as BSI, with a high prevalence of Carbapenem resistance. In our study, the resistance to Imipenem and Meropenems was low for *E. coli* and *K. pneumonia* but high for *A. baumannii* and *P. aeruginosa*. Due to a variable resistance mechanism, such as altering the target position (penicillin-binding proteins), the development of β-lactamase, the narrowing of membrane permeability, and efflux pump, *A. baumannii* MDR infections are difficult to treat, owing to the extremely limited armamentarium (Lee et al., 2017). This is evident from the results of our study on this type of GNB, wherein most of the *A. baumannii* isolates were of XDR strains.

Limitations of our study include the following: (1) it was a single-centre study; (2) it was based on the retrospective analysis of clinical data and (3) the time to source control, which can impact the mortality rate, was not assessed.

CONCLUSION

This study found a rise in the prevalence of MDR- GNB highlighting the importance of continuous surveillance for this type of drug-resistancet bacteria. It is vital to identify the GNB-MDR responsible for the infection and their antimicrobial susceptibility profiles. We recommend that all clinical microbiology laboratories implement early detection and close monitoring of MDR, XDR and PDR bacterial strains to reduce the problem of antimicrobial resistance, manage and cure hospitalised patients appropriately and help in the formulation of effective antimicrobial stewardship programmes in healthcare facilities.

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A Web-Based Survey of COVID-19 Pandemic and its Impact on Physical, Recreational, Mental Health and Socio-Economic Factors of General Population of India

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ABSTRACT

Long duration of quarantine has shown to significantly influence lifestyles of the entire population. The study was taken to determine the impact of the COVID-19 Pandemic era on physical, mental, recreational and socio-economic factors of the general population of India. Four hundred and forty healthy volunteers were enrolled from different zones of the country using chain-referral sampling in a web-based E-survey on Google form platforms. A structured and validated questionnaire consisting of participants' demographic details, physical, mental, recreational and socio-economic changes during the COVID-19 Pandemic was sent via social networking sites (WhatsApp, Facebook, and Messenger). The association between demographic characteristics and self-reported physical, mental, recreational and socio-economic changes by participants during COVID-19 crisis was analyzed using chi square and spearman rho test. The response rate to survey was (sent to 500 individuals; 440 reverted back) 88%. The demographic characteristics were significantly associated with physical, mental, recreational and socioeconomic changes observed during the ongoing COVID-19 crisis among the general population ($p < 0.05$). Strict compliance was observed among (n=239) 54.31% participants who were staying indoors all the time during the Lockdown phase. (n= 238) 54.18% reported they were regularly performing moderate-intensity activities (50%-70% Max. HR), (n=282) 64% were indulging in recreational activities and (n=322) 73.18% participants were doing regular household chores. (n=269) 61.13% self-reported being happy. Financial loss was perceived by (n=230) 52.27% of participants. Amid all this external state of crisis significantly large proportion of participants were observed being in a happy relaxed state of mind and also utilized this time to gain health benefits and pursue their hobbies. Overall Participants self-reported an enhanced sense of wellbeing.

KEY WORDS: HEALTH, PANDEMIC, POPULATION, PSYCHOLOGICAL, SOCIAL.

INTRODUCTION

Within a span of a few months a situation of disconsolation and confusion has been created by a rapidly evolving novel form of coronavirus (COVID-19) (Gupta et al. 2020). In many countries and territories, the situation is being compared to "the end of the world", raising a concern about the scarcity of basic facilities and health services for all human races. The WHO declared it as a pandemic

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and also a major disaster source of the 21th century (Amawi et al. 2020; Preskorn, 2020).

Social distancing is widely practiced all over the world to prevent the transmission of this life-threatening viral infection. Being the second most populous country with insufficient medical resources and enormous demands, India is at a high risk of facing irremediable damage. Keeping in frame the rising critical situation, on 24th March, 2020, the Prime Minister of India announced the 21 days lockdown as reported in Hindustan times, March 23, 2020.The duration has further been extended thrice; first till 3rd May then 17th May 2020 and finally till 31st May 2020 respectively. Unlock phase started from 1st June 2020 and has seen lesser shift in current lifestyle of most of the population in country. Quarantine, Isolation and social distancing are being practiced either voluntarily or mandatory to check further spread of COVID-19 (Khanna et al. 2020). Previous outbreaks witnessing quarantine imposition reported of emotional disturbances and generated substantial anger (Brooks et al. 2020).

Also being homebound increases the rate of sedentary lifestyle. However, flipping the coins on the other side, a positive approach can enhance mental and general wellbeing of the Individuals by utilizing this time with family and practicing recreational activities. Both physical and mental health is considered vital for overall wellbeing of an individual in long run. Global humanitarian crisis of the COVID-19 pandemic, mental health issues have been reported from all over the world (Roy et al. 2020). During the early stages of the pandemic in India, this study was focused mainly to assess its physical, mental, recreational and socio-economic factors. Lockdown and concern about the disease's future effects and transmission had a huge impact on people's lives. Because of the high death tolls and global spread of COVID -19, people are becoming increasingly worried. This could assist policymakers in designing systematic interventions. The whole situation impacts physical, psychological, social and economic domains of society and may have a long-lasting impact on public health (Varshney et al. 2020).

The objective of our study was to determine the impact of ongoing COVID-19 Pandemic on physical, mental, recreational and socio-economic factors of the general population of India and will also be helpful to frame better strategies to cope with current situation (Varshney et al. 2020). This study can have potential limitations. A post pandemic survey also needs to be done which can later on explain the impact on physical, recreation, mental health and socio-economic factors once the pandemic is over.

MATERIAL AND METHODS

The study protocol was approved by the Institutional Ethics Committee (IEC) of Maharishi Markandeshwar (Deemed to be University), Mullana, Ambala district, Haryana (IEC-114F) and is in accordance with the National ethical guidelines for Biomedical and Health Research involving human subjects-ICMR guidelines (Revised 2017) and guidelines of Helsinki declaration 2013. Participation and return of completed survey were implied as Consent by the participant. At 95 % of Confidence level, the Minimum required Sample Size for this online Cross-sectional Survey was estimated to be 384 with 5% margin of error (Sakpal, 2010).

Anticipating 10 % of online forms being incomplete, the target sample size was set at 427. However a total of 440 complete responses were obtained for the present study and were used for statistical analysis. The individual data was collected from all the participants in the month of April 2020 and May 2020 using chain referral sampling method. The General population belonging to all the age groups and gender; who were able to understand English language and had access to social networking sites was included from various regions of the country (Pourhoseingholi et al. 2013).

A self-structured and validated questionnaire was used to collect comprehensive information about impact of ongoing COVID-19 lockdown on the general population of India. It consists of 30 questions which included Demographic details, daily activities routine modification if any; amid lockdown period, perceived stress or anxiety levels, Physical and sedentary activity during lockdown, Diet and weight fluctuations, Mood swings, social and family interaction, recreational activities and perception about the financial loss during lockdown period. Objective questions were formulated to access all the items in the questionnaire. The questionnaire was validated using face validity and pilot testing of Questionnaire on 50 Individuals. The likert questions included in the questionnaire had test value >0.6 using Cronbach's Alpha (CA) indicating a higher internal consistency (Pourhoseingholi et al. 2013).

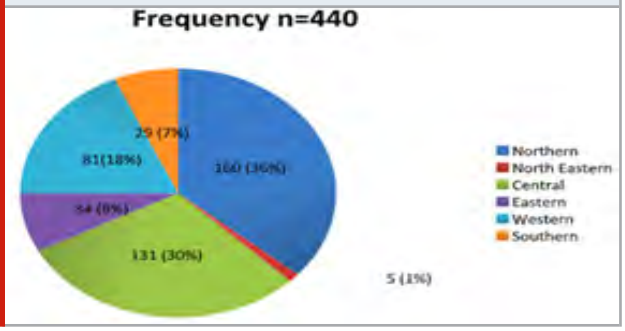
The questionnaire was included in the Google form Link: https://docs.google.com/forms/d/e/1FAIpQLSdsxRd4UEjvJDABNSrCF7OYP_FyaRC_cn-_M_NjtBElvdajrg/viewform and was circulated on various social media sites (WhatsApp, Facebook, and Messenger). The statistical software, SPSS version 20 was used at 95% confidence interval. Response rate of survey was calculated. Categorical data was represented with total number of participants and frequency as percentages n (%). Chi square and Spearman Rho tests were used to find difference and associations between demographic variables and participants' responses at 0.05 levels of significance.

RESULTS AND DISCUSSION

The survey link was forwarded to 500 individuals and complete forms were obtained from 440 participants. Majority of participants belonged to Northern, Western and Central zones of country (Figure 1) and were in the age group of 20-40 years (n= 383; 87.05%); majority were residing in the cities (n= 342; 77.3%). Henceforth the results of our study are ought to be generalized to

above mentioned characteristics of participants. The participants' response to questionnaire items in terms of number of sample (n) and frequency in brackets (%) is tabulated in Table 1. In Table 2, the association between demographic characteristics and participant response to physical, mental, recreational and socio-economical changes during the Pandemic has been represented (Singh and Misra 2009).

Figure 1: Frequency and percentage (in brackets) n (%) of country (India) zone wise distribution of participants (n=440)



COVID-19 is the third human epidemic that has occurred in the last two decades, triggering clinical manifestations of infectious, digestive and systemic disorders, manifested mainly by pneumonia. Amid lack of specific antiviral drugs or vaccines; quarantine is the only best preventive measure. India had been in a lockdown phase from 24th of March to 31st May 2020. Unlock phase has also seen lesser shift in current lifestyle of most of the population in country. In this cross-sectional survey, we provided an insight to the ongoing COVID-19 Pandemic on physical, mental, recreational and socio-economic dimensions of the general population of India. The response rate to survey was calculated to be 88% (Singh and Misra 2009).

Table 1. Frequency and percentages (in brackets) of participants' response n (%) to physical, mental, recreational and socio-economic changes during COVID-19 lockdown phase (n= 440)

S. No.	VARIABLE		Frequency (Percentage)
1.	Physical and Sedentary Activities during the COVID-19 lockdown phase		
a.	Stepping out of House	Not at all	239 (54.31)
		Sometimes	157 (35.68)
		Often	19 (4.32)
		Most of the time	17 (3.86)
		all the time	8 (1.82)
b.	Daily Physical activity	Decreased	259 (58.86)
		Increased	110 (25.0)
		Unchanged	71 (16.14)
c.	Doing any Household chores	Yes	322 (73.18)
		No	118 (26.82)
d.	Moderate intensity Exercises regularly (50%-70% Max. HR)	Yes	238 (54.09)
		No	202 (45.91)
e.	Meditation regularly	Yes	126 (28.64)
		No	314 (71.36)
f.	Total duration of time spent on physical activity in a day	NIL	67 (15.23)
		Up to 30 minutes	132 (30.0)
		30-60 minutes	126 (28.64)
		>60 minutes	115 (26.14)
g.	Daily Sleep duration	Decreased	67 (15.23)
		Increased	216 (49.09)
		Unchanged	157 (35.68)
h.	Time spent on Mobile, Laptop, Television, other gadgets per day	Decreased	20 (4.54)
		Increased	346 (78.64)
		Unchanged	74 (16.82)
i.	Workload in a day	Decreased	179 (40.68)
		Increased	143 (32.5)
		Unchanged	118 (26.82)

Table 1 Continue

j.	Routine Appetite	Decreased Increased Unchanged	78 (17.73) 186 (42.27) 176 (40.0)
k.	Body weight	Decreased Increased Unchanged	54 (12.27) 151 (34.32) 235 (53.41)
l.	Changes in body weight	0 <2 kg 2-4 kg >4 kg	1 (0.23) 239 (54.32) 189 (42.95) 11 (2.5)
2.	Self-reported mental Health of participants during the COVID-19 lockdown phase		
m.	Present State of mind Happy	Sad 269 (61.13)	171 (38.86)
n.	Do you Feel	Lazy Active	269 (61.14) 171 (38.86)
o.	Mood nowadays	Relaxed Stressed	294 (66.82) 146 (33.18)
p.	Worry or tension about daily routine	Not at all Sometimes Often Most of the time All the time	97 (22.05) 206 (46.82) 52 (11.82) 60 (13.64) 25 (5.68)
q.	Worry or tension about being infected with COVID-19	Not at all Sometimes Often Most of the time all the time	93 (21.14) 194 (44.09) 46 (10.45) 71 (16.14) 36 (8.18)
r.	Worry or tension about your loved ones being infected with COVID-19	Not at all Sometimes Often Most of the time All the time	40 (9.09) 153 (34.77) 66 (15.0) 101 (22.95) 80 (18.18)
3.	Recreational activities during the COVID-19 lockdown phase		
s.	Pursuing Hobbies, recreational activities	Yes No	282 (64) 158 (35.9)
4.	Socio-economic factors during the COVID-19 lockdown phase		
t.	Enjoying family time	Yes No	381 (86.59) 59 (13.4)
u.	Communication and relation with family members has improved	Yes No	371 (84.31) 69 (15.68)
v.	Feeling about the lockdown	Necessary Unnecessary	421 (95.68) 19 (4.32)
w.	Should Government increase the lockdown Period	Yes No	424 (96.36) 16 (3.64)
x.	Financial loss	Yes No	230 (52.27) 210 (47.72)

COVID-19 Pandemic Impact on Physical health: Previous Studies have proved that confinement, lack of daily routine and decreased social and physical interaction with others have often resulted in boredom, dissatisfaction and a sense of alienation from the rest of the world that was distressing to the participants (Singh and Misra 2009). However, in our study (n= 171) 38.86% of total participants felt active during these days. (n=238) 54.18% reported they were regularly doing moderate intensity exercises at 50%- 70% of maximum heart

rate and 312 (73.18%) participants were doing various household chores regularly. Only (n=11) 2.5 % of total participants felt an increase beyond 4 kg in their body weight (Table 1).

In contrast to age old beliefs a good percentage of males 136 (66.4%) agreed to doing various household chores. A higher percentage of females reported increase in Daily Physical activity and time spent on doing various household chores as well as other physical activities (Table 2). (n=126) 28.64 % of total participants were having daily physical activity between 30-60 minutes equivalent to 100-200 MET minutes/day and (n= 115) 26 (Singh and Misra 2009).

Table 2. An association between demographic characteristics and physical, mental, recreational and socio-economic changes in participants during COVID-19 lockdown phase (n=440).

Independent variable	Category of Independent variable	Dependent Variables				
Age (years)	1. <20 years 25 (5.7%) 2. 20-40 years 383 (87%) 3. 40-60 years 25 (5.7%) 4. >60 years 7 (1.6%)	1. Stepping out of House				
		Not at all	Sometimes	Often	Most of the time	All the time
		1. 19 (76)	05 (20)	0(0)	01(4)	0 (0)
		2. 206 (53.8)	140 (36.6)	19(4.3)	13 (3.4)	05 (1.3)
		3. 12 (48)	08 (32)	0(0)	03(12)	02(8)
		4. 02 (28.6)	04(57.1)	0(0)	0(0)	01(14.3)
		Spearman Rho p= 0.008, Correlation Coefficient= .12				
		2. Present State of mind				
		Sad		Happy		
		1. 14 (56)		11 (44)		
		2. 149 (38.9)		234 (61.1)		
		3. 6 (24)		19 (76)		
		4. 2 (28.6)		5 (71.4)		
		χ^2 value= 4.83, df= 1, p-value= .028				
Gender	1. Male 208 (47.27%) 2. Female 232 (52.73%)	1. Stepping out of House				
		Not at all	Sometimes	Often	Most of the time	All the time
		1. 81 (38.9)	101 (48.6)	8 (3.8)	13 (6.2)	5 (2.4)
		2. 158 (68.1)	56 (24.1)	11 (4.7)	4 (1.7)	3(1.3)
		χ^2 value= 23.5, df= 1, p-value= <.0001				
		3. Daily Physical activity				
		Decreased		Increased		Unchanged
		1. 137 (66.9)		49 (23.6)		22 (10.6)
		2. 122 (52.6)		61 (26.3)		49(21.1)
		χ^2 value= 10.94, df= 1, p-value= .001				
		4. Doing any Household chores				
		Yes		No		
		1. 136 (66.4)		70 (34.6)		
		2. 186 (80.2)		46(19.8)		
		χ^2 value= 12.22, df= 1, p-value= < .0001				
		5. Total duration (time spent) of Household chores/ Exercises/ Meditation/day				

Table 2 Continue

		NIL	Up to 30 minutes	30-60 minutes	>60 minutes
		1. 35(16.8)	73(35.1)	61 (29.3)	39(18.8)
		2. 32(13.8)	59(25.4)	65(28)	76(32.8)
		χ^2 value= 9.20, df= 1, p-value= .002			
		6. Pursuing Hobbies, recreational activities			
		Yes	No		
		1. 121(58.5)	86(41.5)		
		2. 160(69)	72(31)		
		χ^2 value= 5.25, df= 1, p-value= .022			
Education	1. Up to 10th 7 (1.59%)	1. Do you Feel			
		Active	Lazy		
		1. 5(71.4)	2(28.57)		
	2. Up to 12th 20 (4.55)	2. 7 (35)	13(65)		
		3. 83(36)	146(63.8)		
		4. 73(40.8)	106(59.2)		
	3. Graduation 229 (52.04)	5. 3(60)	2(40)		
		χ^2 value= 11.14, df= 1, p-value= .049			
	4. Post-Graduation 179 (40.68%)	2. Doing any Household chores			
		Yes	No		
		1. 1(14.29)	6(85.7)		
	5. Ph.D. 5 (1.14%)	2. 10(50)	10(50)		
		3. 166(72.5)	63(27.5)		
		4. 140(78.2)	39(21.8)		
		5. 5(100)	0(0)		
		χ^2 value= 16.90, df= 1, p-value= <.0001			
		3. Communication and relation with family members has improved			
		Yes	No		
		1. 5(71.4)	2(28.6)		
		2. 16 (80)	4(20)		
		3. 183(79.9)	46(20.1)		
		4. 162(90.5)	17(9.5)		
		5. 5(100)	0(0)		
		χ^2 value= 10.69, df= 1, p-value= .001			
		1. Stepping out of House			
		Not at all	Sometimes	Often	Most of the time	All the time

Table 2 Continue

Occupation	1. Student 182 (41.36%) 2. Unemployed 36 (8.18%) 3. Employed 214 (48.63%) 4. Retired 8 (1.82%) 9	1. 116(63.7) 2. 18(50) 3. 101(47.2) 4. 4(50)	58(31.9) 15(41.7) 80(37.4) 4(50)	3(1.6) 0(0) 16(7.5) 0(0)	3(1.6) 2(5.6) 12(5.6) 0(0)	2(1.1) 1(2.8) 5(2.3) 0(0)
		Spearman Rho p= 0.000, Correlation Coefficient= .174				
		2. State of Mind				
		Sad	Happy			
		1. 84(46.2) 2. 13(36.1) 3. 71(33.2) 4. 3(37.5)	98(53.8) 23(63.9) 143(66.8) 5(62.5)			
		χ^2 value= 6.54, df= 1, p-value= .011				
	1. Village 36 (8.18%) 2. Town	1. Physical Activity				
		Decreased	Increased	Unchanged		
		1. 16 (44.4) 2. 30 (48.4) 3. 213 (62.3)	15 (41.7) 20 (32.3) 75 (21.9)	5 (13.9) 12 (19.4) 54 (15.8)		
		Spearman Rho p= 0.021, Correlation Coefficient= .110				
Residence	62 (14.09%) 3. City 342 (77.73%)	2. Doing any Household chores				
		Yes	No			
		1. 23 (63.9) 2. 37 (59.7) 3. 262 (76.6)	13(36.1) 25 (40.3) 80 (23.4)			
		χ^2 value= 6.98, df= 1, p-value= .008				

* Frequency of participants and Percentages (in brackets) are represented as n (%).

14% were spending>60 minutes in a day equivalent to >200 MET minutes/day of energy expenditure hence fulfilling the guidelines of American Heart association for moderate intensity activity to maintain cardiovascular health as well as adult Physical activity per day recommendations (Fuzeki and Banzer 2018). Thus, it can be assumed that the majority of participants utilized COVID-19 lockdown time to gain health benefits (Ruegseggar and Booth 2018). Education was also found to be positively associated with doing household chores (p value < .0001) (Table 2). A high percentage of city population 262 (76.6%) denoted an increase in doing household chores (p value .021) (Table 2). As the city households rely more on maids for their daily household chores, this lockdown phase has caused them to be more self-dependent on these aspects of life (Fuzeki and Banzer 2018).

COVID-19 Pandemic Impact on Mental Health: Studies have recommended that open wellbeing crises can have numerous mental impacts on the overall population, which can be communicated as anxiety, fear, stress, and apprehension. The developing mental wellbeing issues related to this world-wide occasion may advance into long-lasting wellbeing issues, segregation and stigma (Roy et al, 2020). But surprisingly, the results from our study reported a different scenario where (n= 294) 66.8% of study sample reported that their mood is relaxed now days and (n= 269) 61.13 % stated of being in a Happy State of Mind (Table 1). A Positive association between Age and State of Mind of Participants revealed that as the age of participants increased, they were reportedly in a Happier State of Mind (p Value 0.28). The percentage was also found highest among the employed section of Participants (n=143; 66.8%) (p value .011); Table 2.

The results indicated that the lockdown period provided a break from routine life and the aged employed section of society found this time as relaxing and henceforth were in a happy State of Mind. Also, physical and mental health has an intriguing direct relationship. Duration of quarantine, fear of infection, frustration and boredom are the major stressors during quarantine (Gupta et al, 2005). It is worth noting that, due to lockdown, 206 (46.82%) of the study sample reported sometimes feeling worried or tensed about their everyday routine while

only 25 (5.68%) were worried all the time. Along with it 36 (8.18%) were having fear all the time of getting themselves infected with the coronavirus.

While comparatively a relatively higher percentage of 80 (18.18%) participants feared of their loved ones being infected (Table 1). The tendency to feel more concerned towards near ones had been reported earlier as well among Indians in the week, May 11 2020. It can be exposed to more barriers in accessing timely health services, because of discrimination associated with mental ill-health in health-care settings mental health disorder co morbidities to COVID-19 will make the treatment potentially less effective and more challenging. People with mental health conditions could be more substantially influenced by the emotional responses brought on by the COVID-19 pandemic, resulting in relapses or worsening of an already existing mental health condition because of high susceptibility to stress compared with the general population (Talevi et al. 2020).

COVID-19 Pandemic Impact on Recreational dimensions: This lockdown period has been taken as an opportunity by people to indulge themselves in extracurricular activities like painting, dancing, gardening etc. 282 (64%) participants were pursuing their hobbies or other recreational activities (Table 1). Pursuing one's hobbies has shown carryover effects later in the day. It helps one to engage time in something they enjoy doing and also improves both Physical and mental health (Takeda, 2015). Since a higher percentage of individuals were spending time on their hobbies and other recreational activities this could be one of the reasons that the majority of participants reported being relaxed and happy in our study (Takeda, 2015).

A Positive association was found between a higher percentage of female participants being engaged in their hobbies and other recreational activities (p value .001) (Table 2). Also, a remarkable increase in the usage of laptop, mobile and television has been reported by 78.8% of the study sample (n=346) (Table 1). Having a working versatile phone is presently a need, not a luxury, and those venturing off a long flight to enter isolate will likely welcome charger or connector more than anything else (Takeda, 2015).

COVID-19 Pandemic Impact on Socio- Economic dimensions: As shown in Table 1, 239 (54.31%) reported that they were never stepping out of their houses. Interestingly, (n=381) 86.6% of study samples reported that they are enjoying their family time at home and 84.3% of study samples (n=371) felt that their communication and relation has been improved with their family members during the lockdown period, which is truly a positive impact on the general population. A positive association (p value.001) was found between higher the Education level more improved were communication and relation with family members of participants (Table 2).

Participants residing reported an improved relation and better communication with family members. This study reported 95.7% of sample size feels that this lockdown was a necessary step to be taken by the government. Also (n= 424) 96.4% of participants think that the government should increase the lockdown period if cases of coronavirus increase in India, which reflects that this whole lockdown period is being taken in a positive manner by the general population of India (Bashir et al. 2020).

At the cost of Economy, the Government of India prioritizes saving as many lives as possible. Increased workload per day had been reported by (n= 143) 32.5% of total participants. Still the Indian Economy is estimated to lose more than $4.5 billion each day during this lockdown phase (Businessline, 2020). So, it is quite obvious to have a fear of financial loss in the general population during the lockdown period. This is supported by our findings which suggest that 52.27% of study samples have faced a financial loss during the lockdown period. Age has found to be positively associated (p value 0.028) with financial loss reported by participants (Table 2). The study outcomes will provide practical guidance on strategies and will help to design a better protocol for lockdown period in future.

COVID-19 could be a wakeup call for greater global empathic solidarity, great logical education, trust between individuals and public authorities, and better international participation; all due to new crises and untrue divisions rolling on the skyline (Bashir et al. 2020). Recent COVID- 19 researches indicate that residential areas with lower mean income are likely to be at a greater danger of getting infected than areas with higher income as a research project about New York City has shown that poor residential areas have a much higher infection rate than other areas of the city. As a result, we can confidently assume that socioeconomic demographics are at the core of the COVID-19 pandemic, which explains why heavily populated areas have higher infection and mortality rates (Bashir et al. 2020).

CONCLUSION

This research survey sought to document a variety of quarantined people's perspectives in order to better understand their needs and concerns. Amid COVID-19 spread in country along with an active lifestyle, social distancing, basic precautionary measures, maintenance of personal hygiene and special attention to high-risk population is necessary to tackle this situation.

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Analysis on in-Silico Identification of Novel Activator of Pyruvate Kinase M2

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ABSTRACT

Pyruvate kinase M2 isoform (PKM2) in a less active state (dimer form) regulates the rate-limiting step of glycolysis that switches the glucose metabolism to aerobic glycolysis in tumor cells and thus promotes cell proliferation. Allosteric regulated PKM2 switches low to high activity state and prevent growth of cancer. Activators of PKM2 promote tetramer formation and suppress tumorigenesis. We present a structure based virtual screening of a diverse chemical compound collection (Diverse-lib) to identify novel activators of Pyruvate kinase M2 (PDB ID: 4G1N) from *Homo sapiens*. In order to rank potential small molecule hits, two separate docking algorithms were used to produce a consensus score. Four compounds out of 99,288 leads having lowest binding affinity even lower than control NZT compound were identified as activators of PKM2 using MTiOpenScreen and MTiAutoDock servers. Further, these best predicted compounds were subjected for physicochemical, pharmacokinetic and toxicological investigation using preADMET tool and cross verified by SwissADME tool. Compound PubChem SID 17517397was satisfied all the ADME/Tox parameters out of four activators. In the AutoDock Vina and AutoDock programmes, the binding energy of compound SID 17517397 was -10 kcal / mol and -11.11 kcal/mol with PKM2.Compound SID 17517397 had human intestinal absorption, Caco2 cell permeability, Plasma Protein Binding and Blood-Brain Barrier penetration values as 94.18%, 23.86, 89.50%, 3.30, respectively, which indicates that it is in the range of well absorbed and active compound range. Compound had negative carcinogenicity value in mouse and rat. Therefore, it is concluded that compound could be promising novel activator for PKM2 as drug target but it must be verified by experimental studies.

KEY WORDS: ADMET/TOX, CANCER, MTIAUTODOCK, MTIOPENSCREEN, PYRUVATE KINASE M2

INTRODUCTION

In cancer cells, the metabolism differs significantly from that of healthy cells (Kim and Dang, 2006; Vander Heiden et al., 2009; Zhao et al., 2013). In normal cell rely on glycolysis to produce energy but in tumor cells it switches the glucose metabolism to aerobic glycolysis and this mechanism is called Warburg effect (Warburg, 1956; DeBerardinis et al., 2008). Tumor's glycolysis interventions are a novel approach for targeted anti-cancer therapies (Chen et al., 2007; Gatenby and Gillies, 2007; Porporato et al., 2011). In cancer cell metabolism, the regulation of Pyruvate kinase M2 isoform (PKM2) plays a key role. The last rate-limiting enzyme in the glycolytic pathway is pyruvate kinase (PK), which catalyses the transfer of a phosphate group from phosphoenolpyruvate to ADP to obtain pyruvate and ATP. There are four distinct subtypes of Pyruvate kinase. PKL isoforms exist predominantly

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in the liver, kidney and red blood cells, whereas PKR is mostly present in red blood cells (Gupta and Bamezai, 2010; Israelsen et al., 2013, Wong et al., 2013; Yang and Lu, 2013; Otto et al., 2016).

In myocardium, skeletal muscle and brain tissue, PKM1 is distributed, and in tissues such as the brain and liver, PKM2 is distributed (Israelsen et al., 2013). For cancer metabolism and tumour development, PKM2 is essential, yet tetramer and dimer of PKM2 consist of the same monomer (Ashizaw et al., 1991; Yang and Lu, 2015). There are substantially different biological effects between the tetramer and dimer form (Muñoz-Colmenero et al., 2015). In the sense of glucose metabolism, the tetramer primarily plays the role of pyruvate kinase and controls glycolysis and dimer PKM2 as a switch for energy metabolism and material synthesis (Dombrauckas et al., 2005). In the dimer state, PKM2 can enter the nuclear to regulate gene expression, epithelial–mesenchymal transition (EMT), invasion and metastasis and cell proliferation. Zhang et al. (2019) first used the switching effect of PKM2 in glucose metabolism to expand and enrich the Warburg effect (Zhang et al., 2019).

Endogenous and exogenous activators allosterically regulate the change between PKM2 dimers and tetramers. PKM2 dimers are tetramerized using activators that allow PKM2 to behave like PKM1 induces reversal of the Warburg effect in cancer cells. Phosphorylation or acetylation of native tetrameric PKM2 in cancer cells causes a transition to a dimeric/monomeric form, which translocate into the nucleus and causes oncogene transcription. However, it is unclear how these post-translational modifications (PTMs) cause PKM2 to lose its oligomeric state. Nandi et al. (2020) performed crystallographic and biophysical studies of PKM2 mutants containing residues that mimic phosphorylation and acetylation Nandi et al. (2020).

They discovered that PTMs cause a significant structural reorganization of the binding site for fructose 1, 6-bisphosphate (FBP), an allosteric activator, affecting the interaction with FBP and causing oligomerization disruption (Nandi et al., 2020). In the current research, we have implemented a virtual screening approach to search out the novel activator for the treatment of cancer targeting the PKM2 protein. In-silico physicochemical, pharmacokinetic and toxicological properties of activators were analysed by preADMET and cross-checked with the SwissADME web tool (Lee et al., 2003; Daina et al., 2017).

MATERIAL AND METHODS

The 3D crystal structure of Pyruvate kinase isoform M2 in complex with an activator was retrieved from the Protein Data Bank with PDB ID: 4G1N (Kung et al., 2012). All the water molecules, Oxalate, Magnesium ions and N-(4-{[4-(pyrazin-2-yl) piperazin-1-yl] carbonyl} phenyl) quinoline-8-sulfonamide (NZT) were removed and polar hydrogen added to PKM2 protein for structure based virtual screening as well as molecular docking processes. Small-molecule activator NZT bind PKM2 at the subunit interaction interface, a site that is distinct from fructose-1,6-bisphosphate (FBP) was used as activator binding site in molecular docking (Kung et al., 2012). The virtual screening was performed using the diverse chemical compound library (Diverse-lib) database. The compound library consisted of 99,288 diverse drug-like PubChem compounds from in house Diverse-lib database of RPBS Web portal. The compound library was filtered by using the criteria such as molecular weight <500 Dalton; hydrogen bond donor <5, hydrogen bond acceptor <10, octanol–water partition coefficient logP <5; Number of rotatable bonds <8, polar surface area <140 Å (Labbé et al., 2015).

Docking was carried out on the MTiOpenScreen server with AutoDock Vina and with AutoDock on MTiAutoDock server (Labbé et al., 2015). The rankings of AutoDock Vina and AutoDock were combined to construct a consensus list of compounds with both techniques that scored well. Validation and Optimization process of best predicted compounds had been processed by PreADMET tool and cross check by Swiss ADME tool, which are web-based application for predicting absorption, distribution; metabolism, elimination and toxicity (Lee et al., 2003; Daina et al., 2017). Using Python Molecular Viewer software, docking findings were visualized to display the 3D structure and position of activator binding to the protein (Sanner, 1999; Daina et al., 2017).

RESULTS AND DISCUSSION

Activator binding site analysis: Small molecule activator PubChem SID 17517397 bind PKM2 at the subunit interaction interface, a site different from that of the endogenous activator fructose-1, 6-bisphosphate (FBP). The activator binding sites of PKM2 comprises 8 residues on chain A such as TYR390, ASP354, GLN393, ILE389, PHE26, GLU397, LEU353, LEU394 and 7 residues on chain B as MET30, PHE26, TYR390, LYS311, LEU394, LEU353, ASP354. Binding of activator to PKM2 promoted a constitutively active enzyme state (tetramer form).

Structure based virtual screening: 1500 small molecules were screened after applying filter criteria in database. A gradient-based conformational search approach was employed by AutoDock Vina. The grid box parameters were set to values of 3.743 Å,-12.72, and 48.977 Å for the grid box center and 34 Å ×28 Å ×32 Å for the box dimensions. We use a total of 10 binding modes and 8 for exhaustiveness. The scoring of the docking poses produced and the ranking of the ligands were based on the empirical scoring function of Vina approximating the affinity of binding in kcal/mol. Top 100 compounds were selected based on their lowest binding energy and further docked with activator binding site of PKM2 using AutoDock. Docking results of both were shown in table 1.

Four compounds with PubMed SID 17517397, 26649876, 49737693, 4247715 were selected based on binding energy of compound with PKM2 protein having >-9.5kcal/mol in

AutoDock Vina and >-11kcal/mol in AutoDock, which is even lower than control NZT compound (binding energy: -7.95kcal/mol with PKM2 protein).

ADME/Tox properties of best predicted compounds: The properties of human intestinal absorption were critical for the production of drugs that purport to be orally administered (Zhao et al., 2001; Postigo et al., 2010). Human intestinal absorption (%HIA) values of four best predicted compounds with PubMed SID 17517397, 26649876, 49737693 and 4247715 were shown in table 2. These compounds have been identified in the category of well absorbed compounds (HIA: 70 ~ 100 %) (Yee, 1997). *In vitro* cell permeability Caco-2 is an important test that measures drug intestinal absorption (Yazdanian et al., 1998). In the MDCK method, the cell permeability *in vitro* used canine kidney cells and has a shorter growth rate than the Caco-2 cells were used as a tool for the rapid analysis of permeability (Irvine et al., 1999). In the pharmaceutical industry, skin permeability is used to measure the toxicity of chemical products in the event of unintended skin touch (Singh and Singh, 1993). Moreover, the blood-brain barrier (BBB) was essential for drug pharmacology. PCaco-2, MDCK, Skin Permeability, PPB and BBB of four compounds were shown in table 2. Compound SID 26649876 had a BBB value greater than 2.0 and was graded in the central nervous system with high absorption (Ma et al., 2005).

Table 1. Docking results of Autodock Vina and Autodock programmes with physicochemical properties of compounds.

Sl. No	PubChem SID	BE (AutoDock Vina)	BE (AutoDock)	nRot	HBA	HBD	LogP	MW	TPSA
1	17517397	-10	-11.11	6	4	2	3.98	374.9043	58.2
2	26649876	-9.9	-11.34	6	6	1	3.29	374.4357	72.95
3	24838666	-9.6	-9.25	4	7	2	3.11	400.8587	99.94
4	49737693	-9.5	-11.28	8	7	1	3.65	413.8542	86.48
5	24339460	-9.5	-7.35	2	7	2	0.18	193.1628	96.51
6	4247715	-9.5	-11.71	6	7	1	3.77	398.4108	118.38
7	24798741	-9.4	-9.38	7	7	0	1.98	340.3764	85.26
8	4256388	-9.4	-9.34	1	4	0	3.1	218.2981	43.6
9	17477384	-9.4	-9.47	4	5	3	2.95	352.4069	97.64
10	26664864	-9.2	-8.57	1	5	0	1.63	204.1821	57.38
11	49736489	-9.2	-8.92	2	4	0	3.98	338.7876	46.34
12	17469113	-9.2	-8.24	3	4	1	2.75	260.1661	52.6
13	24779480	-9.2	-10.05	6	7	2	2.68	360.4108	153.7
14	17479262	-9.1	-9.95	5	6	0	3.88	295.2927	84.74
15	56317084	-9.1	-9.31	4	6	0	2.22	256.2136	78.36
16	26534815	-9.1	-9.85	2	5	0	3.39	317.386	103.68
17	7971621	-9.1	-8.93	3	7	1	1.52	331.3464	107.75
18	22413776	-9	-9.75	2	5	0	3.62	253.256	63.12
19	49716534	-8.9	-9.62	6	7	1	2.23	392.4477	113.63
20	49817894	-8.9	-8.26	6	6	1	2.41	356.2965	73.59
21	26537356	-8.9	-8.70	3	4	2	3.97	337.8028	57.78
22	17449251	-8.9	-10.08	6	7	1	3.88	369.3944	128.51
23	89852923	-8.9	-8.00	2	6	1	0.97	190.1588	87.39
24	862504	-8.9	-8.81	4	6	0	3.29	330.3401	73.93
25	849520	-8.9	-10.84	8	7	2	2.36	386.4216	106.02
26	4242876	-8.9	-8.57	2	3	1	3.49	275.3013	42.24
27	22402150	-8.8	-9.88	4	7	1	3.01	352.3672	121.87
28	26620089	-8.8	-11.71	6	6	1	3.18	414.5178	84.09
29	7967102	-8.8	-9.46	4	6	0	3.44	349.1363	78.36
30	57263884	-8.8	-10.18	10	7	2	2.99	372.415	97.64
31	24784913	-8.8	-9.11	6	6	0	3.24	376.4516	62.47
32	26650965	-8.8	-11.12	5	7	0	3.04	394.399	80.82
33	49674809	-8.8	-6.59	7	6	2	3.5	376.4052	95.15
34	24296145	-8.8	-8.14	2	7	1	3.1	323.3293	123.89
35	26725965	-8.8	-10.68	5	7	2	3.51	420.3899	102.68
36	17447282	-8.8	-8.45	2	4	1	3.59	305.7362	84.61

Table 1 Continue

37	24831749	-8.7	-9.88	6	5	0	3.53	335.3533	65.49
38	26649874	-8.7	-9.13	6	6	2	3.03	368.4263	76.66
39	7964748	-8.7	-10.28	5	7	1	3.03	375.442	111.78
40	17456133	-8.7	-8.24	4	6	3	2.55	329.3736	103.01
41	26647975	-8.7	-8.81	3	5	0	3.79	378.4641	43.21
42	26648535	-8.7	-7.27	5	5	1	3.47	353.4116	56.79
43	24809269	-8.7	-8.75	6	6	1	3.45	300.3092	84.15
44	17465009	-8.7	-6.39	1	4	2	3.69	329.3751	104.53
45	26533701	-8.7	-7.74	2	4	0	2.64	280.2021	48.03
46	26727266	-8.7	-9.89	4	6	0	3.29	327.3313	81.35
47	14730768	-8.7	-9.50	5	5	0	3.3	336.338	73.58
48	49737701	-8.7	-9.69	6	7	1	1.75	346.3777	82.82
49	24364860	-8.7	-6.77	3	4	2	2.98	296.3636	66.13
50	93577670	-8.7	-10.07	7	7	1	2.03	351.356	94.31
51	49645683	-8.6	-8.94	4	6	1	2.96	296.2775	88.28
52	17470642	-8.6	-9.50	5	5	2	3.37	361.3906	67.79
53	4246955	-8.6	-8.87	4	7	2	3.63	338.4069	84.73
54	17512468	-8.6	-7.58	1	5	4	3.06	284.3363	112.04
55	14744922	-8.6	-9.57	7	6	1	2.89	387.4494	97.92
56	26648015	-8.6	-7.70	7	7	2	2.02	390.4814	88
57	860027	-8.6	-7.02	2	4	0	3.48	362.4448	67.2
58	11534585	-8.6	-9.51	3	5	0	3.1	261.2813	67.39
59	851712	-8.6	-9.26	4	6	0	1.56	274.2984	103.14
60	16952534	-8.6	-8.96	4	5	1	3.96	297.3053	72.56
61	17402129	-8.5	-7.61	2	4	1	3.43	319.3324	58.11
62	24811377	-8.5	-11.15	7	7	0	3.64	350.3249	98.15
63	124949736	-8.5	-8.04	6	5	2	3.57	406.3982	67.43
64	24334833	-8.5	-9.20	3	5	1	3.97	292.2888	78.94
65	24293703	-8.5	-10.63	3	7	1	3.02	289.2667	129.19
66	24366734	-8.5	-10.40	4	4	1	3.98	327.7617	59.31
67	49640535	-8.5	-7.27	6	7	2	2.78	384.3793	97.28
68	57259063	-8.5	-7.65	5	6	1	2.76	327.3313	73.86
69	24807319	-8.5	-6.83	5	7	1	1.42	300.2662	86.75
70	24412722	-8.5	-5.59	7	7	1	2.08	434.5108	119.25
71	26729749	-8.5	-7.86	3	5	1	1.68	210.6204	63.5
72	144203702	-8.5	-5.71	9	5	2	3.85	414.3427	64.17
73	4247822	-8.5	-7.67	4	6	2	3.42	400.473	71.33
74	49718523	-8.5	-8.99	5	6	0	3.47	383.484	60.01
75	89855761	-8.5	-7.13	1	5	1	1.08	195.2184	68.01
76	3714881	-8.5	-10.69	6	6	0	3.84	365.4057	99.48
77	14720958	-8.5	-9.47	8	5	0	3.82	404.5014	57.69
78	17445644	-8.5	-9.34	5	5	1	3.9	336.2653	74.92
79	26652683	-8.4	-8.94	3	4	1	3.13	336.3612	93.48
80	24787424	-8.4	-9.58	4	7	1	1.19	234.2114	92.96
81	17452005	-8.4	-8.47	5	7	2	3.42	359.3813	96.02
82	4247583	-8.4	-9.82	6	7	2	1.22	388.4639	137.67
83	7977979	-8.4	-10.98	8	6	1	3.76	383.464	102.55
84	49733540	-8.4	-10.37	5	6	2	2.97	385.3905	108.86
85	24834152	-8.4	-8.08	6	6	3	2.43	387.4958	100.88
86	24348650	-8.4	-10.17	6	7	1	3.94	383.7852	97.04
87	24801340	-8.4	-7.24	2	4	0	2.74	277.2741	52.21
88	24365159	-8.4	-9.17	3	5	0	2.3	246.2619	67.41
89	847046	-8.4	-8.00	5	6	0	2.26	252.6571	69.9
90	104223077	-8.4	-9.72	8	6	2	1.78	354.3997	88.4
91	14722665	-8.4	-9.49	4	7	1	1.67	280.2566	122.81
92	17506536	-8.4	-9.37	4	6	0	2.82	292.3303	75.36

Table 1 Continue

93	24824418	-8.4	-10.57	1	6	2	1.69	293.2768	84.08
94	24320463	-8.4	-9.80	4	7	1	2.78	357.2846	98.39
95	24798468	-8.4	-10.25	6	6	2	2.65	338.4036	83.81
96	85269299	-8.4	-10.17	8	7	1	3	332.3511	101.22
97	26618413	-8.4	-8.21	4	7	2	1.03	383.4673	112.7
98	17515744	-8.4	-11.00	5	6	1	3.55	339.3684	116.05
99	24782536	-8.4	-10.26	3	7	2	1.94	272.2594	99.84
100	17453955	-8.3	-9.17	1	6	2	1.1	242.2334	83.54

BE: binding energy (kcal/mol), nRot:number of rotatable bonds, HBA: hydrogen bond donor Count, HBD: hydrogen bond acceptor Count, LogP: partition coefficient of a molecule between an aqueous and lipophilic phase, MW: molecular weight, TPSA:topological polar surface area.

Carcinogenicity is the toxicity in the body that causes cancer. Compounds SID 26649876 had positive carcinogenicity value in rat. Compounds SID 26649876, 49737693 and 4247715 had a BBB value less than 2.0 and was graded in the central nervous system with low absorption (Ma e al., 2005). Further, ADME properties of compounds PubChem SID17517397, 26649876, 49737693 and 4247715 were analysed by SwissADME tool (Daina et al., 2017). All the compounds were qualified five different rule-based filters such as Lipinski filter implemented rule-of-five, Ghose, Veber, Egan and Muegge rules shown in table 3 (Ghose et al., 1999; Egan et al., 2000; Muegge et al., 2001; Veber et al., 2002; Lipinski, 2004). The result provided in table 3 shows that all the compounds investigated have high gastrointestinal absorption and good skin permeation.

Table 2. ADMET properties of best predicted activators.

Pubchem SID	P_{Caco}-2 (nm/sec)	MDCK (nm/sec)	Skin Permeability	%HIA	%PPB	BBB	Carcinogenicity		
							Ames test	Mouse	Rat
17517397	23.86	0.11	-3.91	94.18	89.50	3.30	mutagen	-ve	-ve
26649876	31.32	0.13	-3.25	96.35	99.32	0.19	mutagen	-ve	+ve
49737693	26.33	1.55	-3.80	96.77	87.55	0.02	mutagen	-ve	-ve
4247715	19.85	0.84	-4.13	97.98	92.05	0.18	mutagen	-ve	-ve

PPB:Plasma Protein Binding, BBB: blood brain barrier.

Table 3. The pharmacokinetics and drug likeliness prediction of four best predicted compounds

Pubchem SID	GI	BBB	Log Kp (skin permeation Coefficient) (cm/s)	Bioavalia-bility Score	Lipinski	Ghose	Veber	Egan	Muegge
17517397	High	Yes	-5.76	0.55	Yes	Yes	Yes	Yes	Yes
26649876	High	Yes	-6.25	0.55	Yes	Yes	Yes	Yes	Yes
49737693	High	No	-6.23	0.55	Yes	Yes	Yes	Yes	Yes
4247715	High	No	-6.05	0.55	Yes	Yes	Yes	Yes	Yes

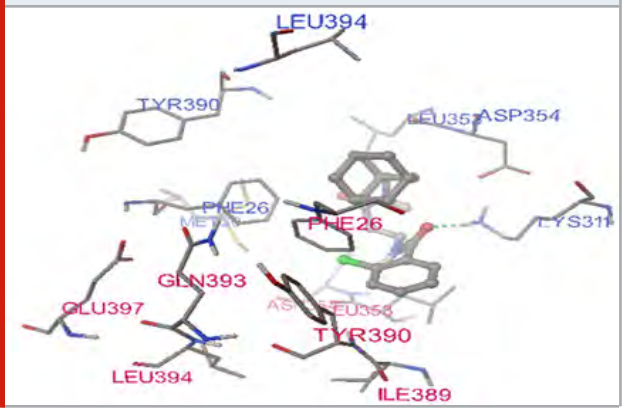
GI: Gastrointestinal absorption, BBB: Blood Brain Barrier penetration.

Compounds SID 49737693 and 4247715 indicate negative results in blood-brain barrier (BBB) permeation. The SwissADME estimates for passive human gastrointestinal absorption (GI) and permeation to the blood-brain barrier (BBB) consists of the BOILED-Egg model reading (Butina et al., 2002). Therefore, only one compound PubChem SID 17517397 qualifies all parameters of ADME /Tox on analysis of four best predicted compounds using PreADMET and SwissADME tools (Butina et al., 2002). Li et al. (2018) used structure-based virtual screening to find successful activators targeting PKM2 in the ZINC database. ZINC08383544, a screened compound, promotes the development of PKM2 tetramers, effectively blocks PKM2 nuclear translocation, and inhibits tumour growth, suggesting that it may be a novel PKM2 activator (Li et al., 2018).

Visualization of protein-ligand interaction: Best docked complex was analyzed through Python Molecular Viewer

for their interaction study shown in figure 1. It is evident from this analysis that compound SID 1751739 was located at the subunit interaction interface of protein and was stabilized by hydrogen bonding.

Figure 1: Docking pose of compound SID 17517397 on PKM2 protein structure. One H-bond was formed between amino acid LYS311 of protein Chain B with compound. Chain A and B binding residues of PKM2 protein were colored with pink and royal blue, respectively.



CONCLUSION

The current research utilizes structure based virtual screening to identify human Pyruvate kinase M2 isoform (PKM2) protein activator that is required for cancer treatment. From thousands of chemical structures and a sequence of steps of rational refinement, including virtual screening, molecular docking and ADME/Tox studies, we identified compound PubChem SID 17517397 as novel activator of pyruvate kinase M2 protein as drug target for further experimental testing.

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Conflict of Interests: Authors declare no conflict of interests among themselves.

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Screening and Characterization of Endophytic Bacteria from Heliotropium pterocarpum found in Hot Springs

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ABSTRACT

Under harsh environmental stresses, some plants can survive due to their association with microorganisms. These microorganisms residing within the specific host plant, form unique group of endophytes that can extend diverse sorts of positive impacts on plant. Endophytes related to their functions to host plant and factors affecting their association are poorly studied. Thus, this study concentrated on isolation, identification, and characterization of endophytic bacteria from *Heliotropium pterocarpum* growing at Hot spring, Gomyqah, Saudi Arabia, for their biological impacts as plant growth promoting (PGP) traits. Seven endophytic bacteria were isolated from root, stem, and leave of *H. pterocarpum* plant. These bacterial isolates were molecular identified based on 16S rDNA, as *Serratia* sp., *Exigobacterium indicum, Kocuria sediminis, Variovorax paradoxus, Staphylococcus epidermis, Siphingobium yanoikuyae* and *Serratia rubidea*. In addition, 42% of these bacterial isolates have efficacy of phosphate solubilizing with clear zones ranging from 5.2 to 6.6 mm, and siderophore producing ranging from 14 to 16.3 mm. Moreover, most of these bacterial isolates have ability to produce different enzyme activities. Furthermore, all the selected bacterial isolates were able to produce Indole acetic acid (IAA) and Gibberellic acid (GA3) in broth media, ranging from 0.002 to 0.056 mg/ml, and from 0.006 to 0.144 mg/ml, respectively. Considering all these activities of bacterial isolates,endophytes could be exploited as effective resource for promoting plant growth and nutrients uptake without chemical effect on the environment. Thus, endophytic bacteria could be used as biological productin in agriculture fields.

KEY WORDS: HELIOTROPIUM PTEROCARPUM; ENDOPHYTIC, BACTERIA; 16S RDNA, EXTRACELLULAR ENZYMES.

INTRODUCTION

Under extreme environmental stresses, plants can survive by developing symbiotic association with any components of their ecosystem to survive and development in their natural environments. Microorganisms are one of the most vital components that create useful associations with plants (Santoyo et al., 2016). Symbiotic bacteria or "endophytes" can associate internally with a wide-range of plants species (Guo et al., 2008). Endophytes defined as a group of microorganisms that colonizing internally different parts of host plant, without causing symptomatic impacts to the plant (Wilson, 1995). Endophytes distribution within plants depends on their ability to colonize and obtain plant resources. Endophytic metabolic pathways played major roles in endophytic diversity (Conrath et al., 2006; Singh et al., 2009) which were contained mainly four

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phyla: Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes, with dominant genera of *Pseudomonas, Bacillus, Burkholderia, Stenotrophomonas, Micrococcus, Pantoea* and *Microbacterium* (Goryluk-Salmonowicz et al., 2018).

Researchers are supposed that presence of these endophytes improve plant ecology by increasing the capacity of host plants to grow under environmental and biological stresses (Miliute et al., 2015). It is believed that endophytes live in close association with plants and can extend different kinds of positive effects on plant growth and health. These include increased nutrients availability through the fixation of nitrogen, solubilization of phosphate, and production of siderophores. Furthermore, endophytes could improve plant through production of plant growth regulators phytohormones, such as indole-3-acetic acid (IAA), gibberellic acid (GA3), and cytokinins in addition to their ability to tolerate stress, drought, salinity, and metal toxicity (Nogema et al., 2013).

Endophytes also could provide resistance to diseases and potential pathogens through production of secondary metabolites and extracellular enzymes (Nair and Padmavathy, 2014). Moreover, the mechanisms of endophytes to deal with natural ecology may give superior survival preferences to host plants. Bacteria of endophytic plants were first studied in temperate regions, but recently the studies were extended to plants in tropical regions as well. However, a little knowledge is available on diversity, and interaction of endophytes communities in plants struggling for existence in extreme environment. Thus, focusing on isolating and screening of endophytes with diverse properties from plants at Hot spring are necessary and urgent. These endophytes could have many roles to manipulate plant growth (Patten and Glick 2002, Fadiji and Babalola, 2020).

Using these interesting of endophytes with many roles are useful especially in poor soil and desert regions. *Heliotropium pterocarpum* is widely spread at Hot spring, Gomyqah (Saudi Arabia). However; the biological properties of this plant related to their endophytic biology have not yet been well studied. Hence, this study was focused on isolation, identification, and characterizing of endophytic bacteria from the previous plant and screening these endophytes for production of plant growth regulators and other promoting materials to understand their functional roles.

MATERIAL AND METHODS

Plant collection from the study area: This study was carried out at Hot spring area (N 40˚28'11.4E), located 17 Km North-East of Gomyqah city, and 25 km East of Al-Leeth city, Saudi Arabia. Sterile technique was used during the collection of plant specimens. The plant was collected, labeled, and immediately transferred in sterile polyethylene bags to the Microbiological laboratory at faculty of Science, and placed on a dry cool place to avoid moisture accumulation or excessive drying (stored at 4°C). The plants were collected from 2 meters distance of a stream bank at Hot spring area, during February 2019 at 32°C for the isolation of endophytes.

Identification of collected plants: The plant sample was identified by Dr. Amal Aldhebiani (Botany/Biology Department, King Abdulaziz University, Jeddah, SA).The plant sample was characterized, identified and checked with some books and online checklist of the Flora of KSA (Alfarhan and Thomas,1994;Chaudhary, 2001), as well as with Thomas (2011). Then, nomenclature and family of the plant was identified and checked by follow Catalogue of Life website.

Surface sterilization and isolation of bacterial endophytes: To remove dust and debris, the plant parts were firstly washed with water, then surface sterilized by following sterilization- culturing dependent method (Hallmann et al. 1997; Zinniel et al. 2003), using 70% ethanol and aqueous solution of 5% sodium hypochlorite, rinsed with sterile distilled water and dried using sterile filter paper. Sterilized surgical blade was used to cut the plant into small pieces (1-3 mm long) and each parts of plant were put on Nutrient Agar plate (Hi Media, Mumbai, India). To confirm the disinfection process, aliquots of the steriledistilled water that used in the final plant rinse was plated onto the previous medium. All plates were incubated at 28˚C for 1-2 weeks to allow growth of endophytes and examined for bacterial endophytic growth from the used plant segments. Bacteria growing out of the plant segments were isolated, purified, and identified based on phenotypic and genotypic characteristics.

Molecular identification of endophytes bacterial isolates: The bacterial isolates were identified by extracting the genomic 16S rDNA (Govindarajan et al., 2007) from the bacterial colonies, using a commercial kit for bacterial DNA extraction (MQ Bacterial DNA Isolation Kit, MOLEQULE-ON Company, Auckland, New Zealand). Then, 16SrDNA was amplified in using the genomic DNA as template and bacterial universal primers, 27 F (5'GAGTTTGATCCTGGCTCA-3'), and reverse primer 1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR product was visualized, sequenced at Macrogen Online Sequencing Company, Korea and then, checked by BLAST analysis in the NCBI database for microbial identification. The 16S rDNA sequence of the strains was used to search the GenBank database and determine phylogenetic relative strains.

Screening of endophytic bacterial isolates for biological impacts: Solubilization of phosphate: The bacterial isolates were screened for phosphate solubilization using Pikovskaya's medium (pH 7.0) which composed of (g/l): glucose 10; tri-calcium phosphate 5; ammonium sulphate 0.5; sodium chloride 0.2; magnesium sulphate heptahydrate 0.1; potassium chloride 0.2; ferrous sulfate heptahydrate 0.002; yeast extract 0.5; manganese (II) sulfate dehydrate 0.002; bromo phenol 0.025g, Bacto agar (Difco) 15. The medium was inoculated by spotting 10 µl of overnight shaken bacterial broth cultures on the surface of Pikovskya agar and the plates were incubated

at 28°C for 3-5 days. Formation of a clear halo zone around the colony was due to the utilization of tricalcium phosphate present in the medium (Lavakush and Verma, 2012).

Production of siderophores: Siderophores produced by the endophytic isolates were determined using qualitative assay as described by Schwyn and Neilands (1985) using chrome azurol S (CAS), and hexadecyltrimethylammonium bromide (HDTMA) as indicators. The CAS/ HDTMA react with ferric iron to produce a blue color. Removing a siderophore (iron chelator) from the dye complex changes the color from blue to orange. On each plate of CAS medium, 10 µl of 48 hours old cultures of endophytic bacterial filtrate were spotted and all plates were incubated at 28°C for 2 days. A color change of the CAS medium around the colony from blue to yellow was considered positive result (Louden et al., 2011).

Exoenzymes activity: The extracellular hydrolytic enzymes activity was detected by growing the bacterial isolates on different indicator media, including amylase activity medium (Glucose Yeast Extract Peptone Agar (GYP) medium containing 2.0% (w/v) starch and 1.5% agar (w/v)(Claus 1988), lipas eactivity medium (NB medium containing 1.0% Tween 80 (v/v) and 1.5% agar (w/v) (Rajanet.al, 2011), protease activity medium(NB medium containing 1.0% (w/v) skim milk and1.5% agar (w/v) (Tennalli et al, 2012), pectinase activity medium (NB medium containing 0.5% poly galaturonate (v/v) and 1.5% agar (w/v) (Cotty et al. 1990), cellulase activity medium (carboxy methyl cellulose (CMC) medium containing 0.5% (w/v) carboxyl methyl cellulose and 1.5% agar (w/v) (Zaghloul et al. 2016). All the isolates were spotted inoculated on respective enzymes screening media and incubated at 28°C for 48-72 hours. Clearing zones in the medium indicated positive enzyme activity.

Production of Indole Acetic Acid and Gibberellic Acid: Estimation of IAA was recorded using a colorimetric spectrophotometric method (Patten and Glick, 2002). Each endophytic bacterial isolate was grown in250 ml flasks Erlenmeyer flask containing 50 ml of nutrient broth containing 0.2 % of L-tryptophan (v/v). After incubation in darkness for 7days at 30 °C and 120 rpm,the culture filtrate was centrifuged at 10,000 rpm for 15 min., and then 2 ml of each culture supernatant was mixed with 2 drops of concentrated orthophosphoric acid, followed by 4 ml of Salkowski reagent. The mixture was incubated in darkness at room temperature (25°C) for 25 min, and the presence of pink color indicated IAA production. The absorbance was read at 530 nm using a spectrophotometer (SpectroSC™ Spectrophotometer, LaboMed.inc).

A standard curve of known concentrations of IAA was prepared to determine the quantities of IAA in each filtrate. Similarly, the amount of GA3 produced by the endophyte's isolates was estimated by the method of Holbrook et al., (1961). Two ml of zinc acetate solution was added to 50 ml of the bacterial culture filtrate and after two minutes of incubation, two ml of potassium ferrocyanide solution was added, the reaction volume was centrifuged at 8000 rpm for 10 minutes. Five ml of supernatant was added to five ml of 30 % HCl and the mixture was incubated at 28°C for 75 min. Five ml of the supernatant with five ml of 5% HCl was used as blank. The absorbance of the sample and blank were measured at 254 nm. A standard curve was prepared by using gibberellic acid to calculate the GA3 quantities in each bacterial filtrate (Holbrook et al., 1961).

RESULTS AND DISCUSSION

Hot spring at Gomyqah, Al-Leeth city, Kingdom of Saudi Arabia (Figure 1- A) was visited and a plant from the normal flora was collected from two meters distance far away from the stream bank of Hot spring (Figure 1-B). This collected plant was identified as *Heliotropium pterocarpum* (A. DC.) Hochst. & Steud. ex Bunge (Figure 1-C), which belong to Borage family (Boraginaceae) (Table 1). The plant sample was mounted on sheet bearing a label and saved at King Abdulaziz University herbarium.

Figure 1: A: Google map of the Hot spring area at Gomyqah village at Al-Leeth city, B: The study area, C: The collected *H. pterocarpum* at natural habitat

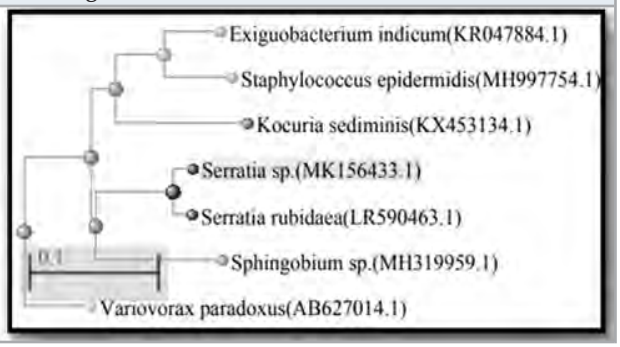
Table 1. The scientific name and classification of *Heliotropium pterocarpum* plant according to catalog of life website.

Accepted scientific name	*Heliotropium pterocarpum* (A. DC.) Hochst. & Steud. ex Bunge (accepted name).
Basionym	*Heliophytum pterocarpum* A. DC. in DC. (1845, p. 552).
Taxonomic synonym	*Bourjotia pterocarpa* (DC.) Pomel, *Heliophytum pterocarpum* DC. & A. DC, and *Heliotropium kassasii* Täckh. & Boulos.

A total of 7 endophytic bacterial isolates were obtained from *H. pterocarpum*. The bacterial isolates namely P1M4, P1M6, P1M7, P1M8, P1M9 were isolated from the leaves, and P1SM10 and P1SM11 were isolated from the stem segments of *H. pterocarpum*. These isolates were morphologically characterized by Gram staining test, 57% were Gram negative, rods shape, while 43% were Gram positive, bacilli and cocci in shape. The DNA sequences were analyzed by BLAST analysis for alignment,the results were compared with NCBI database and phylogenic tree

were obtained. The sequence analysis of 16S rDNA of P1M4, P1M6, P1M7, P1M8, P1M9, P1SM10, and P1SM11 showed the maximum identity of 84 % to Serratia sp. (MK156433.1), 92 % to *Exiguobacterium indicum* (KR047884.1), 99 % to *Kocuria sediminis* (KX453134.1), 94% to *Variovorax paradoxus* (AB627014.1), 95% to *Staphylococcus epidermidis* (MH997754.1), 99% to *Sphingobium yanoikuyae* (MH319959.1), and 99% to *Serratia rubidaea* (LR590463.1), respectively. Results of their closest relatives are shown in phylogenetic tree (Figure 2).

Figure 2: Phylogenetic tree of the identified endophytic bacterial isolates based on the 16S rDNA sequences. The GenBank accession number is given in parentheses for each organism.



Out of 7 bacterial isolates, four isolates (*Serratia* sp., *Variovorax paradoxus, Staphylococcus epidermis*, and *Serratia rubidaea*) showed their ability to solubilize complex calcium phosphate and developed clear zones, ranging from 5 to 6.6 mm on Pikovskya's agar plates (Figure 3). The same isolates also were able to chelate the iron and form yellow zone around the colony in CAS plate (Figure 4), while three isolates (*E. indicum, K. sediminis*, and *S. yanoikuyae*) had negative results (Table 2).

Figure 3: Production of extracellular enzymes by endophytic bacterial isolates from *H. pterocarpum*

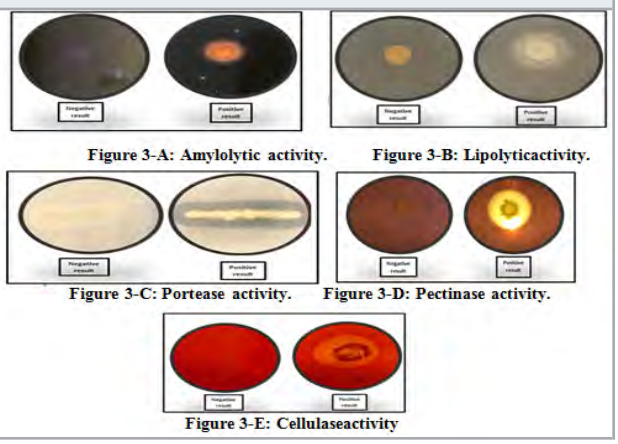


Table 2. Some activities of endophytic bacterial isolated from *H. pterocarpum*.

Plate code	Bacterial isolates	Biological activities	
		Phosphate solubilization (mm)	Siderphore production(mm)
P1 M4	*Serratia* sp.	+ (6.0)	++ (16.0)
P1 M6	*Exiguobacterium indicum*	-	-
P1 M7	*Kocuria sediminis*	-	-
P1 M8	*Variovorax paradoxus*	+ (5.0)	++ (16.0)
P1 M9	*Staphylococcus epidermis*	+ (6.6)	++ (16.0)
P1 SM10	*Sphingobium yanoikuyae*	-	-
P1 SM11	*Serratia rubidaea*	+ (6.0)	++ (16.3)

Note: (+): Positive result, (-): Negative result (no clearing zone).

The enzymatic activity of the bacterial isolates revealed that all theseisolates produced at least one or other extracellular enzymes; however, none of the isolates were able to produce all the five tested enzymes (Table 3). The result showed the maximum a mylolytic activity for *Serratia* sp., *E. indicum*, and *S. yanoikuyae* (Figure5-A) while lipase activity was prominent in *S. epidermis* followed by *Serratia* sp., *V. paradoxs* and *S. rubidaea* (Figure5-B). Additionally, all the bacterial isolates had ability to produce protease enzyme (Figure 5-C). Pectinase activity was observed in most of the isolates, *S. yanoikuyae, Serratia* sp., and *E. indicum, K. sediminis* and *S. rubidaea* (Figure5-D).The maximum cellulase activity was observed in *S. yanoikuyae*, followed by *E. Indicum* (Figure 5-E).

The quantity of phytohormones varied between the bacterial isolates obtained from *H. pterocarpum*. The result showed that all the seven isolates producedI AA andGA3, the quantity ranged from 0.002 to 0.056 mg/ml and from 0.006 to 0.144 mg/ml, respectively (Table 4).The IAA data showed that, *S. rubidaea* produced the maximum amount (0.056 mg/ml), followed by *V. Paradoxus* and *E. indicum* with IAA production ranged from 0.12 to 0.13mg/ml. The lowestamounts of IAA (0.002 to 0.007 mg/ml) were obtained for *Serratia sp.*,

S. yanoikuyae, K. sediminis and *S. epidermis*. On the other hand, *K. sediminis* showed the maximum amount of GA3 (0.144mg/ml), followed by *Serratia* sp. (0.104mg/ml) while the minimum amounts of GA3were ranged from 0.006 to 0.099 mg/ml.

The collected plant was studied and identified as *Heliotropium pterocarpum* (A. DC.) Hochst. & Steud. ex Bunge. Based on investigation, the beneficial effects of endophytes on plant at extreme environment, diverse bacterial endophytes were isolated from various tissues of *H. pterocarpum*. It is a very remarkable plant in Saudi Arabia. It is a genus of the flowering plant in the family Boraginaceae, commonly known as heliotropes and has diverse bioactive metabolites including pyrrolizidine alkaloids (Kakar et al., 2010, Radwan and El-shabasy (2020).

Table 3. Extracellular hydrolytic enzymes activity of endophytic bacteria isolated from *H. pterocarpum*.

Plate code	Bacterial strains	Amylase	Lipase	Protease	Pectinase	Cellulase
P1 M4	*Serratia* sp.	+	++	+++	+++	-
P1 M6	*Exiguobacterium indicum*	+	-	++++	+++	+
P1 M7	*Kocuria sediminis*	-	-	++++	++	-
P1 M8	*Variovorax paradoxus*	-	++	+++	-	-
P1 M9	*Staphylococcus epidermis*	-	+++	+++	-	-
P1 SM10	*Sphingobium yanoikuyae*	+	-	+++	++++	+++
P1 SM11	*Serratia rubidaea*	-	++	+++	+	-

Note: (-) no clearing zone, (+) weak clearing zone ≤ 5 mm; (++) moderate clearing zone > 5-10 mm; (+++) strong clearing zone ≥ 10-15 mm; and (++++) very strong clearing zone > 15 mm.

Table 4. Production of Indole acetic acid (IAA) and Gibberellic acid (GA3) from endophytic bacteria isolated from *H. pterocarpum*.

Plate code	Bacterial isolates	Concentration of IAA (mg/ml)	Concentration of GA3(mg/ml)
P1 M4	*Serratia* sp.	0.002±0.01	0.104±0.05
P1 M6	*Exiguobacterium indicum*	0.013±0.01	0.006 ±0.01
P1 M7	*Kocuria sediminis*	0.006±0.001	0.144 ±0.1
P1 M8	*Variovorax paradoxus*	0.012±0.01	0.099 ±0.05
P1 M9	*Staphylococcus epidermis*	0.007±0.01	0.047±0.4
P1 SM10	*Sphingobium yanoikuyae*	0.002±0.002	0.031±0.04
P1 SM11	*Serratia rubidaea*	0.056±0.01	0.056±0.01

Endophytic bacteria inhabitant tissues of *H. pterocarpum* is relatively unstudied to their endophytic biology and being considered as potential source of novel natural products to be used in industry or agriculture fields. In this study, leaves of *H. pterocarpum* harbored more endophytic bacteria (5 isolates) compared to stems (2 isolates) and roots (no isolate). This is confirmed with previous studies that have shown leaves of *Arabidopsis thaliana* harbored more endophytes than roots (Bodenhausen et al., 2013). As result ofhigh photosynthetic metabolism occurs in the leaf, these products could be utilized by the endophytes. In addition, the most important technique to obtain endophytes from plant is surface sterilization to remove epiphytes on tissues. Hence, a variety of chemical disinfectants have been selected forisolation of bacterial endophytes, however sequential immersion of the plant segments in 70% ethanol and sodium hypochlorite ensured the removal of surface microbial flora (Bacon and Hinton, 1996; Coombs and Franco, 2003).

The confirmation of proper surface sterilization of tissues carried out by inoculating last water wash on nutrient agar plate. Absence of any growth after three days incubation indicated the proper surface sterilization of the plant tissues. The taxonomic status at phylogenetic level of the endophytic bacterial isolates was defined by16S rDNA. The identification of bacterial isolates was recorded and they belonged to Protobacteria (57%), as: (*Serratia sp., V. paradoxus, S. yanoikuyae* and *S. rubidea*), *Firmicutes* (28%) as: (*E. indicum* and *S. epidermis*), and Actinobacteria (14%), as: (*K. sediminis*). In the same regard, endophytic bacteria have been isolated from a number of plant species, as Proteobacteria

which is the most predominant phylum, frequently isolated from plants (Afzal et al., 2019). Also, members of Actinobacteria and Firmicutes are the most commonly found as endophytes (Reinhold-Hurek and Hurek, 2011, Fadiji and Babalola, 2020).

The importance of endophytic bacteria is known since long time and they play specific roles in promoting plant growth and protecting the host plants against pathogens and diseases (Muzzamal et al., 2012). Hence, the biological impact of these bacterial strains as plant growth promoting activities were screened for P-solubilization, siderophore production, extracellular enzyme, IAA and GA3 production. In particular, 57% of endophytes isolates were able to solubilize phosphor and produce of siderophor. Similar research has been documented by Rodríguez et al. (2006) bacterial strains belonging to the genera of *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aerobacter, Flavobacterium*, and *Erwinia* have the ability to solubilize inorganic phosphate (tri-calcium phosphate), (di-calcium phosphate), and rock phosphate.

The endophytes isolate which have ability to solubilize phosphorwere able to produce siderophore, *in vitro*. This can occur by lowering pH by endophytes producing low molecular weight organic acids, in which hydroxyl and carboxyl groups can chelate cations bound to phosphate (Kpomblekoua and Tabatabai, 1994, Fadiji and Babalola, 2020).The prominence of siderophore for promoting plant growth through attracting the available iron, in the rhizosphere was reported. As a result, as, iron be available to plants, but unavailable to phyto-pathogens which could contribute to protect the plant (Pashapour et al., 2016). Malfanova (2013) suggested that endophytes including strains of *Pseudomonas fluorescens* produce siderophores and can act as biocontrol agents that antagonize growth of some fungal pathogens.

Most of the isolated bacteria in this study were able to produce extracellular enzymes such as amylases, lipases, proteases, pectinase, and cellulase. It can be summarized that extracellular enzymes may play a significant part in mechanism of endophytes colonization into host plant. Also, these enzymes could be included within the attack of plant pathogens in host plant, as reported for *Azoarcus* sp. (Hurek et al., 1997). Therefore, these bacterial isolates could be used as potential sources of commercial enzyme production for exploitation in medicine, agriculture, and industry (Guo et al. 2008, Fadiji and Babalola, 2020). Additionally, the isolates produced varied amounts of IAA, and GA3 hormones. These hormones enhance the growth of various plants. The amount of IAA produced by the isolate was increased by the addition of precursor tryptophan in the medium (Uma-Maheswari et al., 2013).

The result exhibited the efficiently isolate *Serratia rubidaea* produced the highest IAA (0.056 mg/ ml). Similarly, Kamilova et al., (2005) reported that *Pseudomonas fluorescens*- WCS365, stimulated growth of radish root through production of IAA in the presence of tryptophan. Thus, IAA has many different effectsin stimulating elongation and division of plant cell which posterior to growth and development of plant (Phetcharat and Duangpaeng, 2012, Fadiji and Babalola, 2020). In addition, the bacterial isolates were produced GA3,ranged from 0.006 to 0.144 mg/ml.The GA3 has a role in plant growth, promotes primary and lateral root elongation, and increases yield (Bottini et al., 2004). Report study indicated that *B. pumilus* isolated from the rhizosphere had the growth promoting effect of red pepper and this effect originated from GA production (Joo et al., 2004). Briefly, beneficial activities by bacterial endophytes for promoting plant growth are vital factor that could affect plant development in extreme environment.

CONCLUSION

This is probably the first study that demonstrates the diversity of endophytic bacteria in *H. pterocarpum*, which was collected from Hot spring, Gomyqah, Saudi Arabia. Characterization of these bacterial endophytes includes P-solubilization, siderophore production, extracellular enzymatic activity, phytohormones production was performed in terms of their plant growth-promoting abilities. The successful traits of these bacterial endophyte suggest that they can be utilized in future applications, as biological product through increasing and promoting of plant growth, and protecting plant against pathogens, which help to eliminate or minimize using of commercial fertilizers, and pesticides. Putting all these in consideration, endophytes have a positive impact on plant, environment, and agriculture field.

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The Protective Effects of Melatonin on the Monocrotophos and Quinalphos Induced Oxidative DNA Damage in Rats

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ABSTRACT

Organophosphate pesticides are widely used in agriculture and household for pest control and consequently have been a major cause of toxicity in farmers and others especially in India. The present study was carried out to evaluate the ability of two pesticides: monocrotophos (MCP) and quinalphos (QNP) to generate oxidative stress and to explore the possible protective effects of melatonin in combating the caused stress. The purpose of the study was to find a suitable agent which could reduce the toxic symptoms generated due to acute as well as chronic exposure of monocrotophos and quinalphos. We studied the potential of MCP and QNP to generate oxidative stress and subsequent oxidative damage to DNA in the rat tissues and lymphocytes. Oxidative stress was measured by quantitating the levels of reactive oxygen species (ROS), total antioxidant capacity, accumulation of lipid peroxidation end products while DNA oxidation was measured by the modified comet assay using the bacterial repair enzymes, formamidopyrimidine glycosylase (Fpg) and endonuclease III (Endo III) in the liver, brain and lymphocytes of rats given for two days and subchronic exposure of MCP and QNP, both separately and in combination. The results showed that both acute and subchronic pesticide exposure, separately and in combination, lead to the generation of oxidative stress. Extensive oxidative damage of both purine and pyrimidine bases was observed in liver, brain and lymphocytes of rats given exposure with MCP or QNP, separately or in combination. MCP was found to be more toxic than QNP as highest DNA damage was observed in this group of rats. The combined exposure of MCP and QNP does not potentiate each other's action. However, co-treatment of melatonin, a well established antioxidant, decreased the oxidative stress and damage caused to the DNA.

KEY WORDS: ORGANOPHOSPHATE PESTICIDES, OXIDATIVE STRESS, DNA DAMAGE, MELATONIN.

INTRODUCTION

Pesticides are the chemicals that are extensively applied in agriculture to fulfill the increasing food demands of steadily rising population and for the eradication of numerous vector borne diseases. The ubiquitous dispersion of these substances contaminated the food as well as surface, ground and drinking water. In almost all parts of the world, low level poisoning of human beings poses a risk of chronic illness and adverse health effects. (Sabarwal et al., 2018). Organophosphates (OP) compounds are some of the most common, and most toxic insecticides used today, adversely affecting the human nervous system even at low levels of exposure by irreversibly inhibiting the enzyme acetylcholinesterase (AChE). Besides being potent anticholinesterase compounds, many studies suggest that both acute and chronic exposure of OP pesticides cause disturbances in cells and tissues of test animals and in human beings also (Costa, 2018; Laksmidevi et al., 2020).

All the important biomolecules like proteins, lipids and nucleic acids are susceptible to oxidative DNA damage. Both, in acute or chronic OP exposure, induction of oxidative stress has been reported as the main mechanism of their toxicity (Farkondeh et al., 2020). DNA oxidation is

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known to be one of the most common kinds of damage. OP pesticides induced DNA damage has been reported by many workers. OP compounds have been shown to be genotoxic in vitro and cause extensive damage to DNA (Greeshma et al., 2019; Ali, 2020).

The primary oxidant responsible for DNA damage is OH•, 2017). A variety of oxidized bases have been identified in nuclear DNA but 8-oxo-7,8-dihydroguanine (8-oxoGua) is one of the most abundant and readily formed modified base which if not repaired prior to replication cause mis-incorporation of adenine leading to transversion mutation. It has been suggested that this kind of lesion plays important role in the initiation, promotion and progression of tumors (Poetsch, 2020). The modified comet assay has been applied to wide range to cell types and nowadays, is a well established and widely used genotoxicity test for estimation of DNA damage at the individual cell level in both *in vivo* and *in vitro* studies (Collins et al., 2020).

Melatonin, a hormone produced by pineal glands, is a ubiquitous molecule which is known to possess antioxidant properties and shown ability to detoxify H_2O_2, OH•, peroxynitrite anion, singlet oxygen, $O_2^{\cdot-}$ and peroxyl radicals. (Hacısevki and Baba, 2018). Monocrotophos [dimethyl-(E)-1-methyl-2-(methyl carbamoyl) vinyl phosphate, MCP], is an extremely toxic, systemic aliphatic OP insecticide, which is applied to kill various insects like spiders, mites which attack on cotton, sugarcane, peanuts, ornamentals and tobacco while quinalphos [O,O-diethyl-O-(2- quinoxalinyl)-phosphorothioate, QNP], another extensively used insecticide, is toxic to the unintended targets including humans and animals (Eid, 2017; Kaur and Goyal, 2019).

Since MCP and QNP are widely used OP pesticides, their overlapping application may lead to combined exposure that may potentiate the action of each other. The genotoxicity of MCP and QNP has been confirmed in our previous studies which showed highly significant extensive single and double strand breaks in DNA in tissues of rats given acute and subchronic exposure of MCP and QNP (Mishra et al., 2015). Therefore, it was considered worthwhile to evaluate the involvement of oxidative stress in damage of nitrogenous bases of DNA in tissues of rats given 2 day and 60 days oral exposure of MCP and QNP, separately and in combination. The mechanism of this DNA damage was studied by modified comet assay using bacterial repair enzymes Fpg/ Endo III, to find out whether DNA damage is caused by oxidative stress.

Table 1. Division of experimental animals and pesticide treatment

Acute		Chronic			
I (2 days pesticide exposure)		II (Total pesticide exposure = LD_{50} in 60 days)		III (Total pesticide exposure =$2LD_{50}$ in 60 days)	
Group i	Group ii	Group iii	Group iv	Group v	Group vi
Con MCP	Con + MT MCP + MT	Con MCP	Con + MT MCP+MT	Con MCP	Con + MT MCP + MT
4.5mg/Kg b.w. x 2 days	4.5 mg/Kg b.w. x 2 days	0.3 mg/Kg b.w. x 60 days	0.3 mg/Kg b.w.x 60 days	0.6 mg/Kg b.w. x 60 days	0.6 mg/Kg b.w. x 60 days
QNP 5 mg/Kg b.w. x 2 daysx 2 days	QNP + MT 5 mg/Kg b.w. b.w. x 60 days	QNP 0.33mg/Kg b.w. x 60 days	QNP + MT 0.33 mg/Kg b.w. x 60 days	QNP 0.66 mg/Kg b.w. x 60 days	QNP + MT 0.66 mg/Kg
Mix 2.25mg MCP + 2.5mg QNP/Kg b.w. x 2 days	Mix + MT 2.25 mg MCP + 2.5mg QNP/Kg b.w. x 2 days	Mix 0.15 mg MCP + 0.17mg QNP/Kg b.w. x 60 days	Mix + MT 0.15 mg MCP + 0.17 mg QNP/Kg b.w. x 60 days	Mix 0.3 mg MCP + 0.34mg QNP/ Kg b.w. x 60 days	Mix + MT 0.3 mg MCP + 0.34 mg QNP /Kg b.w. x 60 days

The levels of H_2O_2 and $O_2^{\bullet-}$ radical along with total antioxidant capacity was also estimated in pesticide exposed rats to find out any correlation with base oxidation. Since lipid peroxidation is a sensitive biomarker of oxidative stress, the accumulation of lipid peroxidation products, malondialdehyde (MDA) and 4-hydroxynonanal (4HNE) was monitored to have an idea about the extent of oxidative stress. The antioxidant effect of melatonin, if any, is also evaluated against MCP and QNP induced increase in generation of ROS and oxidative DNA damage in the present study.

MATERIAL AND METHODS

Adult male albino rats of Wistar strain (*Rattus norvegicus*) weighing about 120±10 g were used in the present study. Rats were obtained from the animal facilities of Defence Research and Development Establishment, Gwalior, India, and were maintained in a light (light-dark cycle of 12 h each) and temperature (25° ± 2°C) controlled animal room of our department on standard pellet diet (obtained from Amrut Rat & Mice Feed, New Delhi, India) and tap water *ad libitum*. Rats were acclimatized for one week

prior to the start of the experiment. The animals were handled, ethically treated, and humanly killed as per the rules and instructions of the Ethical Committee on Animal Care of Jiwaji University, Gwalior, in accordance with the Indian National Law on animal care and use. Rats were randomly divided into three groups and were given 2 days of acute pesticide exposure and 60 days of subchronic pesticide exposure. The further division of groups and treatment is as described in Table 1.

The animals were randomly divided into two groups, which were further divided into subgroups of six animals each. The rats of first group consisted of twenty four animals which were further divided into four subgroups of six animals each and were given pesticides for two consecutive days. The second group consisted of thirty animals divided into five subgroups of six animals each, received co treatment of pesticide and melatonin for two consecutive days. The rats of first sub group received MCP [4.5 mg/Kg body weight dissolved in 0.4 ml corn oil per day equivalent to 0.25 LD_{50} as the reported LD_{50} is 18 mg/Kg body weight (Gaines, 1969), orally for two consecutive days, the second sub group received QNP (5 mg/Kg body weight dissolved in 0.4 ml corn oil per day equivalent to 0.25 LD_{50} as the reported LD_{50} is 20 mg/Kg body weight, (Raizada et al., 1993).

Orally for two consecutive days), the third sub group received a mixture of both the pesticides (0.125 LD50 each, total 0.25 LD_{50} equivalent/Kg body weight, orally, for two consecutive days), while rats of the fourth sub group received 0.4 ml corn oil orally for two days and served as the control. The rats of the second group were divided into five sub groups, the first, second, third and fourth sub groups received a co treatment of melatonin (5 mg/Kg body weight intraperitoneally per day for two consecutive days(Suke et al., 2006) and MCP, QNP, their mixture or corn oil orally as in the first group. The fifth subgroup received just corn oil orally and served as control.

The blood was collected 24 h after the last treatment via ocular bleeding and used for lymphocyte (Phatak, 1978) and serum separation (Tuck et al., 2009) The cell viability was checked by trypan blue dye exclusion test (Philips 1973) The lymphocytes samples with viability >95% were used for comet assay. After the blood collection, the rats were humanly killed by cervical dislocation; liver (Martin and Neuhaus, 2007) and brain (http:// biology.mit.edu/sites/default/files/Rat Brain Dissection.pdf) tissues were excised off, washed with 0.9% NaCl and used for different estimations.

Superoxide anion release was measured by superoxide dismutase inhibitable reduction of ferricytochrome c (Cohen et al., 1980). Lymphocytes ($3X10^5$) were incubated in PBS-EDTA buffer (pH7.4) with phorbol-12,13-dibutyrate (PDBu) at 37o C for 15 min and ferricytochrome c and PDBu were added in that the final concentration should be 50 nmol/L and 100 nmol/L, respectively, in total volume of 1.0 ml. The change in absorbance was measured spectrophotometrically at 550 nm for 10 min with a double beam Shimadzu UV-160A spectrophotometer. The amount of superoxide – anion secreted into the medium was calculated using the molar extinction coefficient of reduced cyochrome c, $2.1X\ 10^4$ $M^{-1}\ cm^{-1}$, and the concentration is expressed as nmole $O_2^{\cdot-}$/ 10^6 cells/min (Pick and Keisari, 1981).

Hydrogen peroxide in lymphocytes was measured by the method of Pick (Pick, 1986). For assay of H_2O_2, 100 µl of pesticide treated lymphocytes, 100 µl of assay solution (containing 0.2 ml phenol red, 0.2 g/l and 0.2 ml of horseradish peroxidase, 20 U/ml in potassium phosphate buffer, 0.05 M, pH 7.0 and 9.6 ml of 0.9% NaCl), was taken in microwell plate and reaction was started by the addition of 10 µl of 1.0 N NaOH, and absorbance was recorded at 600 nm. Results are expressed as µmol H_2O_2 formed/ml preparation.

MDA and 4HNE were estimated by the method of Jacobson (Jacobson et al., 1999). Briefly 200 µl aliquot of tissue homogenate (10% w/v in Tris-HCl buffer, 20mM, pH 7.4) was transferred to 650 µl of 10.3 mM 1-methyl-2-phenylindole in acetonitrile and vortex mixed. To assay MDA + 4HNE, 150 µl of 15.4 M methanesulfonic acid was added, vortexed and incubated at 45 °C for 40 min. To assay MDA alone, 150 µl of 37% HCl was added instead of methanesulfonic acid, vortexed, incubated at 45oC for 60 min. After incubation, samples were kept on ice, centrifuged at 9500 g for 5 min and absorbance was measured at 586 nm. The levels of MDA and 4HNE are expressed as nmol g-1 tissue using extinction coefficient 1.1 X 105 $M^{-1}\ cm^{-1}$.

Total antioxidant capacity in serum was measured by the method described by Rice-Evans and Miller, 1994 (Rice-Evans and Miller,1994). The reaction mixture containing 8.4 µl of serum sample, 489 µl of buffer (0.1 M PBS, pH 7.4), 36 µl of 70 µM metmyoglobin, and 300 µl of 5 mM ABTS [2, 2'-azinobis-(3-ethylbanzothizoline-6-sulfonic acid) disodium salt] were taken and the reaction was started by addition of 167 µl of 450 µM H_2O_2 and the absorbance change was recorded at 734 nm for 5 min. The total antioxidant capacity was calculated using trolox (2.5 mM) as standard and values were expressed as mmol trolox equivalent L^{-1}.

Modified bases were estimated by Fpg- Endo enzyme treatment in combination with the comet assay based on Collin's protocols (Collins et al., 1993). A homogenate (25% w/v) of fresh tissues was prepared in chilled homogenizing buffer (0.075 M NaCl containing 0.024 M EDTA, pH 7.2) in a Potter Elvehjem homogenizer with a single stroke. The nuclei were obtained by centrifugation at 700 g for 10 min at 4°C and the pellet was gently resuspended in 3.0 ml of chilled homogenizing buffer. 75 µl of normal melting agarose (1% prepared in 0.1 M sodium phosphate buffer, pH 7.2, containing 0.9% NaCl) was quickly layered on end-frosted slide, covered gently with another slide, and allowed to solidify. The slides were observed at 10X magnification with a Leica Optiphase microscope equipped with an excitation filter of 515-560 nm and barrier filter of 590 nm.

A total of 100 cells were scored per tissue per animal (50 from each replicate slide). The nuclei were divided into five different categories on the basis of percentage of DNA in the tail using TriTek CometScoreTM Freeware v1.5 software. The nuclei having 0-10% of tail DNA were categorized under 0 stage, 10-25% tail DNA under stage I, 25-50% tail DNA under stage II, 50-75% tail DNA under stage III and the nuclei having tail DNA >75% were categorized under stage IV (Fig. 1). The results are expressed as DNA damage index, calculated as #0 + #1 + #2 + #3 + #4/ # of cell scored where # is the total number of nuclei counted (Figure 1).

Figure 1: Stages of DNA damage:

The nuclei were divided into four stages on the basis of percentage of DNA in tail. The nuclei having 0-10% of DNA were categorised under 0 stage, the nuclei having tail DNA from 10-25% were categorised under stage I, 25-50% tail DNA conatining nuclei were categorised under stage II, 50-75% tail DNA containing nuclei were categorised under stage III, the nuclei having tail DNA >75% were categorised under stage IV.

Stages	Criteria of classification	% of DNA in tail	Visual appearance
0	0-10%	0.001224	
I	10-25%	11.921560	
II	25-50%	48.226644	
III	50-75%	70.715544	
IV	>75%	91.362786	

Results are expressed as mean ± S.E. of six sets of observations taken on different days. Statistical analyses were performed using Sigma Stat Statistical software version 2.0. All the statistical analyses were performed using one-way analysis of variance with post hoc Bonferroni's multiple comparison test applied across the treatment groups. Significance was based on P value < 0.05.

RESULTS AND DISCUSSION

Levels of malondialdehyde and 4-hydroxynonanal (MDA and 4HNE), the two major end products of peroxidative damage of lipids, were monitored in the rat tissues following MCP and QNP exposure either singly or in combination in rat tissues. The results showed that both 2 days and subchronic exposure of MCP and QNP showed significantly high accumulation of MDA and 4HNE in the liver and brain of rats. Two days of MCP and QNP exposure caused 258% and 220% increase in the liver MDA and 225% and 192% increase in the brain MDA of rats, respectively, while the 4HNE levels were increased by 310% and 317% in the liver and 161% and 151% increase in the brain of exposed rats, (Table 2). When the rats were given combined exposure of MCP and QNP (0.125 LD50 equivalent of each pesticide per day for two consecutive days), the hepatic MDA and 4HNE levels were increased by 187% and 249%, while 132% and 128% increase was observed in the MDA and 4HNE levels, respectively in the brain, when compared with respective control.

Table 2. Effect of oral exposure of MCP (4.5 mg/Kg b.w.) and QNP (5 mg/Kg b.w.) single and in mixture on the levels of MDA and 4HNE in the liver and the brain of rats and evaluation of protective effects of intraperitoneal dose of melatonin (5mg/Kg b.w.)

	Con	Con +MT	MCP	MCP +MT	QNP	QNP + MT	Mix	Mix + MT
					MDA			
Liver	20.0 ± 0.9	18.8 ± 0.6*	71.5 ± 2.1***	53.0 ± 1.5***	64..0 ± 0.9***	55.5 ± 1.3***	57.3 ± 0.9***	41.3 ± 0.9***
Brain	19.3 ± 1.1	18.0 ± 0.4*	62.8 ± 1.8***	41.8 ± 1.3***	56.3 ± 0.9***	47.0 ± 1.2***	44.8 ± 3.4***	32.0 ± 2.2***
					4HNE			
Liver	15.0 ± 1.3	13.0 ± 1.3*	61.5 ± 4.3***	53.0 ± 1.1***	62.5 ± 2.8***	48.3 ± 1.7***	52.3 ± 1.3***	45.3 ± 1.3***
Brain	23.0 ± 0.9	21.0 ± 0.9*	60.0 ± 1.1***	48.8 ± 0.6***	57.8 ± 1.6***	57.5 ± 1.8***	52.5 ± 1.0***	46.8 ± 0.9***

Values of MDA and 4HNE are expressed as nmoles g-1 tissue.Results are expressed as mean ± SE of six set of observations take on different days.Rats were given 0.25 LD_{50} equivalent of MCP or QNP or mixture of MCP + QNP (0.125 LD_{50} equivalent of each) dissolved in 0.4 ml corn oil, orally for two consecutive days. Another group of rats was given co-treatment of melatonin (5 mg/Kg body weight intraperitoneally).*P < 0.05, **P < 0.01, ***P < 0.001and ≠P > 0.05 when compared with respective control.Abbreviations: Con, Control; MT, Melatonin, MCP, Monocrotophos; QNP, Quinalphos; Mix, Mixture; MDA, malondialdehyde; 4HNE, 4-hydroxynonenal.

The co treatment of melatonin along with pesticide showed protective effects and the accumulation of MDA and 4HNE was markedly decreased in both the tissues when compared with corresponding tissues of only pesticide treated rats. The decrease in the hepatic MDA levels were 93%, 42% and 80% while 108%, 48% and 66% decrease was observed in brain of rats given co-treatment of melatonin with MCP, QNP and Mix respectively, when compared with only MCP, QNP and Mix treated rats. The decrease in the HNE levels in the

liver and the brain ranged from 1% to 95% on melatonin co-treatment when compared with pesticide treated melatonin untreated tissues of rats (Table 2).

Subchronic exposure of MCP, QNP and their mixture also caused significantly marked accumulation of MDA and 4HNE in the liver and the brain of rats when compared with the control. The increase in MDA levels ranged from 23- 42% in the liver and 5- 28% in the brain while the increase observed in the 4HNE levels ranged from 383- 535% in the liver and 200- 404% in the brain of rat given 4.5 mg/Kg body weight of MCP, 5 mg/Kg body weigt of QNP and their mixture (2.25 mg/Kg body weight of MCP + 2.5 mg/Kg body weight of QNP orally for 60 days (Total MCP and QNP given was LD_{50} equivalent in 60 days) (Table 3).

Table 3. Effect of 60 days of oral exposure of LD50 equivalents of MCP (18 mg/Kg b.w.) and QNP (20 mg/Kg b.w.) single and in mixture on the levels of MDA and 4HNE in the liver and brain of rats and evaluation of protective effects of intraperitoneal dose of melatonin (5 mg/Kg b.w.)

	Con	Con +MT	MCP	MCP +MT	QNP	QNP + MT	Mix	Mix + MT
				MDA				
Liver	24.1 ± 1.1	22.8 ± 0.6*	34.2 ± 1.8**	31.0 ± 1.1**	30.9 ± 0.8**	31.2 ± 1.1**	29.7 ± 1.4*	28.4 ± 0.6***
Brain	22.4 ± 1.6	20.2 ± 0.2*	27.6 ± 0.5*	26.2 ± 0.4*	28.7 ± 0.7*	25.3 ± 0.3*	23.5 ± 0.5≠	23.3 ± 0.3≠
				4HNE				
Liver	20.8 ± 5.2	18.0 ± 3.2≠	100.4 ± 20.8*	82.8 ± 15.2***	118.8 ± 0.4**	66.8 ± 10.0**	132.0 ±10.0**	72.0 ± 8.0**
Brain	21.2 ± 6.8	34.4 ± 2.4*	63.2 ± 4.4***	25.2 ± 3.6≠	70.4 ± 6.8**	31.2 ± 13.6*	106.8 ± 6.8***	24.12 ± 4.4≠

Values of MDA and 4HNE are expressed as nmoles g-1 tissue. Results are expressed as mean ± SE of six set of observations take on different days. Rats were given 1/60 LD_{50} equivalent of MCP or QNP or mixture of MCP + QNP (1/120 LD_{50} equivalent of each) dissolved in 0.4 ml corn oil, orally for sixty consecutive days (total pesticide received by each animal was LD50 equivalents in 60 days). Another group of rats was given co-treatment of melatonin (5 mg/Kg body weight intraperitoneally per day for sixty consecutive days) along with 1/60 LD_{50} equivalent of MCP, QNP or their mixture.*P < 0.05, **P < 0.01,***P < 0.001and ≠P > 0.05 when compared with respective control.Abbreviations: Con, Control; MT, Melatonin, MCP, Monocrotophos; QNP, Quinalphos; Mix, Mixture MDA, malondialdehyde; 4HNE, 4-hydroxynonenal.

Table 4. Effect of 60 days of oral exposure of LD50 equivalents of MCP (36 mg/Kg b.w.) and QNP (40 mg/Kg b.w.) single and in mixture on the levels of MDA and 4HNE in the liver and brain of rats and evaluation of protective effects of intraperitoneal dose of melatonin (5 mg/Kg b.w.)

	Con	Con +MT	MCP	MCP +MT	QNP	QNP + MT	Mix	Mix + MT
				MDA				
Liver	22.5 ± 0.2	18.8 ± 1.3*	38.0 ± 1.2***	34.2 ± 1.7**	36.3 ± 1.1***	32.5 ± 0.7***	32.4 ± 0.6***	31.7 ± 1.6***
Brain	19.2 ± 1.1	16.7 ± 0.5*	30.5 ± 0.6***	29.1 ± 0.3***	29.2 ± 0.4***	27.0 ± 0.4***	27.1 ± 0.4**	24.8 ± 0.6**
				4HNE				
Liver	22.4 ± 3.2	20.8 ± 0.8*	75.0 ± 8.4**	77.6 ± 6.7**	64.3 ± 3.2***	63.8 ± 3.8***	62.3 ± 6.7**	43.2 ± 7.7**
Brain	21.6 ± 2.8	19.6 ± 1.2*	60.9 ± 6.9**	50.2 ± 6.5**	49.5 ± 6.2***	43.0 ± 1.8**	46.5 ± 5.5***	34.8 ± 2.5**

Values of MDA and 4HNE are expressed as nmoles g-1 tissue. Results are expressed as mean ± SE of six set of observations take on different days. Rats were given 1/30 LD_{50} equivalent of MCP or QNP or mixture of MCP + QNP (1/60 LD_{50} equivalent of each) dissolved in 0.4 ml corn oil, orally for sixty consecutive days (total pesticide received by each animal was 2LD_{50} equivalents in 60 days). Another group of rats was given co-treatment of melatonin (5 mg/Kg body weight intraperitoneally per day for sixty consecutive days) along with 1/60 LD_{50} equivalent of MCP, QNP or their mixture. < 0.05, **P < 0.01, ***P < 0.001and ≠P > 0.05 when compared with respective control. Abbreviations: Con, Control; MT, Melatonin, MCP, Monocrotophos; QNP, Quinalphos; Mix, Mixture, MDA, malondialdehyde; 4HNE, 4-hydroxynonenal.

When the dose of pesticides was doubled i.e. total 2 LD_{50} equivalents of MCP, QNP and their mixture was given in 60 equal doses, the accumulation of MDA and 4HNE was further increased in the liver and the brain of rats when compared with respective tissues of control rats. Melatonin co treatment showed protection against pesticide induced peroxidative damage of lipids and levels of peroxidation products, MDA and 4-HNE, were accumulated in the liver and the brain of rats when compared with melatonin untreated group. Melatonin co treatment caused 0- 15% decrease in the MDA levels in the liver and 1- 15% in the brain of rats receiving

LD_{50} equivalents of pesticides; 3-17% in the liver and 7-12% in the brain of rats receiving 2 LD_{50} equivalents of pesticides either singly or in mixture in equal doses for 60 days. Melatonin co treated rats showed 93- 289% decrease in the 4HNE levels in the liver and 39- 189% decrease in the brain of rats and 0- 85% decrease in the liver and 30-54% decrease in the brain of rats receiving 2 LD_{50} equivalent of pesticides in equal doses in 60 days, respectively, when compared with only pesticide treated group (Table 4).

Figure 2: Effect of oral acute (2 days) (A) and subchronic (60 days) exposure LD_{50} (MCP: 18 mg/Kg, QNP: 20 mg/Kg) (B), 2 LD_{50} (MCP: 36 mg/Kg, QNP: 40 mg/Kg) (C) of MCP and QNP, single and in combination on total antioxidant capacity in rat serum and evaluation of protective effect of intraperitoneal exposure of melatonin (5 mg/Kg). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$and $^{d}P > 0.05$. Abbreviations: C, Control; MT, Melatonin, MCP, Monocrotophos; QNP, Quinalphos; Mix, Mixture.

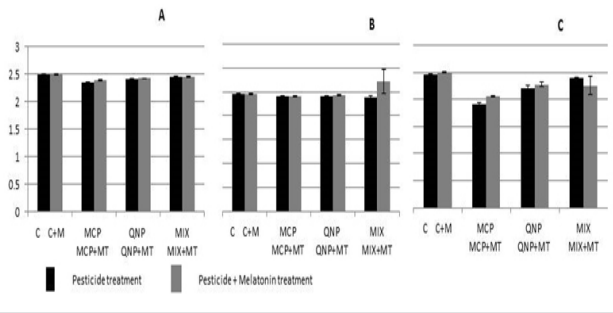
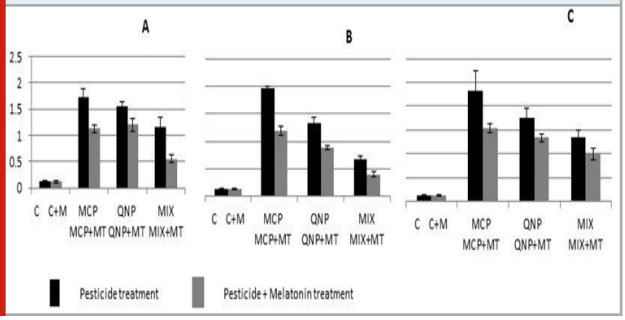


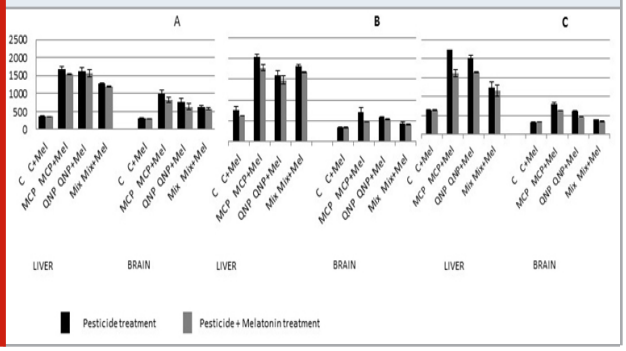
Figure 3: Effect of oral acute (2 days) (A) and subchronic (60 days) exposure LD_{50} (MCP: 18 mg/Kg, QNP: 20 mg/Kg) (B), 2 LD50 (MCP: 36 mg/Kg, QNP: 40 mg/Kg) (C) of MCP and QNP, single and in combination on superoxide anion generation in rat lymphocytes and evaluation of protective effect of intraperitonal exposure of melatonin (5 mg/Kg). The concentration of superoxide anion is expressed as nmole $O_2^{\bullet-}$/ 106 cells/min.35 a P<0.05, b P<0.01, c P<0.001, d P>0.05. Abbreviations: C, Control; MT, Melatonin, MCP, Monocrotophos; QNP, Quinalphos; Mix, Mixture.



The total antioxidant capacity (TAC) measured in serum of rats was marginally decreased on two days exposure or subchronic exposure of MCP or QNP, either alone or in combination. The decrease in TAC was 6%, 4% and 2% in the serum of rats receiving acute exposure of MCP, QNP and their mixture, respectively, when compared with control (Fig. 2A). In the group given subchronic LD50 equivalents of pesticides, TAC was reduced to 3%, 2% and 11%, while the group receiving 2 LD50 equivalents of pesticides the decrease observed was reduced to 22%, 11% and 3% in the MCP, QNP and mixture treated group, respectively, when compared with control (Fig. 2B and C). Melatonin co-treatment although tend to reduce the pesticide induced alterations in the serum TAC of rats but the effect was very marginal ranging from 0.4 - 1.3% in acute treatment and 0.4 – 7.3% in the chronic LD50 group and 3.6 – 7.3% in chronic 2LD_{50} group when compared with melatonin untreated group (Fig. 2).

The results of the present study showed that MCP and QNP exposure either singly or in mixture caused drastic increase in the rate of generation of superoxide anion ($O_2^{\bullet-}$) in the lymphocytes of rats. The increase in the level of $O_2^{\bullet-}$ was 14.3-, 13- and 9.8- folds in the rats given acute exposure of MCP, QNP and their mixture, respectively, while chronic exposure of LD_{50} equivalents caused 15.1-, 10.3- and 5.2- folds increase and exposure of 2 LD_{50} equivalents caused 17.8-, 13.5- and 10.3-folds increase in the rats receiving MCP, QNP and mixture, respectively (Fig. 3).

Figure 4: Effect of oral acute (2 days) (A) and subchronic (60 days) exposure LD_{50} (MCP: 18 mg/Kg, QNP: 20 mg/Kg) (B), 2 LD_{50} (C) of MCP and QNP, single and in combination on the levels of H_2O_2 in the liver and the brain of rats and evaluation of protective effect of intraperitoneal exposure of melatonin (5 mg/Kg). Results are expressed as µmol H_2O_2 formed/ml preparation.a P<0.05, b P<0.01,c P<0.001, d P>0.05. Abbreviations: C, Control; MT, Melatonin, MCP, Monocrotophos; QNP, Quinalphos; Mix, Mixture.



Melatonin co treatment tend to ameliorate the effect of pesticide exposure; 4.9-, 3- and 5.2- folds decrease was observed in the levels of $O_2^{\bullet-}$ in the group given two days exposure of MCP, QNP and mixture, respectively (Fig. 3A). In the group receiving melatonin co treatment along with chronic exposure of LD_{50} equivalents of MCP, QNP and mixture, the increase in the levels of $O_2^{\bullet-}$ was 5.9-, 3.5- and 2.1-folds, respectively, while 2LD50 equivalents of MCP, QNP and mixture exposure caused 6-, 3.2- and 2.6- fold decrease, respectively, when compared with only pesticide treated melatonin untreated group (Fig. 3B and 3C).

Table 5. Effect of 2 days of MCP (4.5 mg/Kg b.w.) and QNP (5 mg/Kg b.w.) single and in combination on oxidative DNA damage in rat tissues and lymphocytes and protective effects of intraperitoneal dose of melatonin (5 mg/Kg b.w.)

Tissue	Treat	Con	Con + MT	MCP	MCP + MT	QNP	QNP +MT	Mix	Mix + MT
Liver	Buffer	0.24±0.02	0.25±0.01≠	0.80±0.01***	0.64±0.01***	0.72±0.04***	0.54±0.03***	0.47±0.03***	0.37±0.03**
	Lysis	0.24±0.01	0.18±0.01***	1.48±0.02***	1.21±0.03***	1.10±0.07***	0.89±0.09***	0.88±0.06***	0.70±0.07***
	FPG	0.31±0.01	0.20≠±0.02	3.01±0.04***	2.00±0.05*	2.65±0.05***	1.70±0.02≠	2.76±0.12***	1.52±0.11≠
Brain	Endo	0.21±0.01	0.17±0.02≠	2.00±0.01***	1.13±0.14**	1.58±0.04***	1.03±0.10**	1.24±0.04***	0.89±0.07**
	Buffer	0.18±0.02	0.15±0.02≠	0.75±0.03***	0.58±0.02***	0.68±0.02***	0.60±0.03***	0.44±0.03***	0.36±0.03***
	Lysis	0.26±0.02	0.21±0.01≠	1.28±0.04***	0.96±0.04***	1.05±0.07***	0.84±0.09***	0.83±0.09***	0.64±0.07**
	FPG	0.48±0.06	0.38±0.08≠	3.21±0.07***	2.68±0.06***	2.92±0.09***	1.92±0.14***	2.37±0.10***	1.33±0.08***
	Endo	0.51±0.09	0.50±0.02≠	2.91±0.09***	1.51±0.07***	2.40±0.09***	1.38±0.07***	1.55±0.14***	0.95±0.03**
Lymp	Buffer	0.16±0.02	0.15±0.01≠	0.83±0.03***	0.57±0.01***	0.66±0.04***	0.48±0.03***	0.43±0.03***	0.34±0.03***
	Lysis	0.19±0.02	0.16±0.02≠	0.89±0.06***	0.67±0.05***	0.75±0.03***	0.56±0.03***	0.42±0.04**	0.34±0.05*
	FPG	0.48±0.06	0.39±0.07≠	3.19±0.22***	2.11±0.05***	2.47±0.09***	1.86±0.08***	1.78±0.12***	1.34±0.03***
	Endo	0.45±0.06	0.37±0.08≠	2.58±0.05***	2.07±0.01***	2.08±0.07***	1.69±0.07***	1.30±0.14**	0.73±0.08*

Values of DNA damage are expressed as damage index calculated as #0 + #1 + #2 + #3 + #4/ # of cell scored where # is the total number of nuclei counted.
Results are expressed as mean ± SE of six set of observations take on different days.
Rats were given 0.25 LD_{50} equivalent of MCP or QNP or mixture of MCP + QNP (0.125 LD50 equivalent of each) dissolved in 0.4 ml corn oil, orally for two consecutive days. Another group of rats was given co-treatment of melatonin (5 mg/Kg body weight intraperitoneally.cdcomparison of MCP + Melatonin with MCP, efcomparison of QNP +Melatonin with QNP and ghcomparison of Mix + Melatonin with Mixture treated group.
*P < 0.05, **P < 0.01,***P < 0.001and ≠P > 0.05 when compared with respective control.
Abbreviations: Con, Control; MT, Melatonin; MCP, Monocrotophos; QNP, Quinalphos; Mix, Mixture; Lymp, Lymphocytes.

The results of present study clearly showed that the levels of hydrogen peroxide were significantly increased on exposure with MCP and QNP either singly or in combination in the rat tissues when compared with control (Fig. 4). The exposure of 4.5 mg/Kg body weight of MCP for two consecutive days caused 370% increase in the liver and 229% in the brain of rats when compared with control while 5 mg/Kg body weight of QNP exposure caused 357% and 155% increase in the liver and brain, respectively, when compared with control. When the rats were exposed with the mixture of these pesticides, the increase in the levels of hydrogen peroxide were 237% and 106% in the liver and the brain, respectively, when compared with control (Fig. 4A).

Chronic exposure of LD_{50} and 2 LD_{50} equivalents of MCP and QNP in 60 days, also significantly increased the levels of H_2O_2 in the liver and the brain of rats. The increase observed in the liver was 170%, 112% and 140%; while brain showed 118%, 75% and 35% increase when exposed with LD_{50} equivalents of MCP, QNP and their mixture respectively, in 60 equal doses in 60 days (Fig. 4B). On doubling the dose, the levels of hydrogen peroxide were further increased. Recovery ranged from 4- 18% in the group given 2 days exposure and 7-89% in the group receiving subchronic exposure of pesticides when compared with melatonin untreated group (Fig. 4C).

The oxidative damage of purines and pyrimidines was studied by the modified comet assay with the use of Fpg and Endo III which remove the damaged purines and pyrimidines, respectively, and create strand breaks at abasic sites. The results showed that 2 days exposure of 0.25 LD_{50} equivalents of MCP and QNP either singly or in mixture caused extensive DNA damage and the damage index was increased 233%, 200% and 96% in the liver, 317%, 278% and 144% in the brain and 419%, 313% and 169% in the lymphocytes in buffer treated slides of rats, respectively. When the slides were treated with Fpg or Endo III significantly marked increase in damage index was observed in the liver, brain and lymphocytes of same group of rats, respectively (Table 5) when compared with control or when compared with buffer-treated slides.

The increase observed on Fpg-treatment was 870.96%, 754.83% and 790.32% in the liver, 569%, 508% and 394% in the brain and 565%, 415, and 271% in the lymphocytes of MCP, QNP and Mix treated rats, respectively, when compared with control while Endo III treatment caused 852%, 652.38% and 490.47% increase in the liver, 471%, 371% and 204% increase in the brain and 473%, 362% and 189% in the lymphocytes of the same group of animals, respectively, when compared with control. The results of the present study showed that melatonin co-treatment decreased the damaging effects of pesticides and the DNA damage index was

decreased in all the tissues tested when compared with only pesticide treated group. Melatonin co-treatment also caused significantly marked decrease in the damage index in the Fpg and Endo III treated liver, brain and lymphocytes when compared with only MCP, QNP and mix treated tissues (Table 5).

Table 6. Effect of 60 days exposure of MCP (18 mg/Kg b.w.) and QNP (20 mg/Kg b.w.) single and in combination on oxidative DNA damage in rat tissues and lymphocytes and protective effects of intraperitoneal dose of melatonin (5 mg/ Kg b.w.)

Tissues	Treatment	Con	Con + MT	MCP	MCP + MT	QNP	QNP +MT	Mix	Mix + MT
Liver	Buffer	0.36±0.03	0.31±0.03≠	1.03±0.05***	0.79±0.02***	0.95±0.04***	0.66±0.02***	0.64±0.04***	0.54±0.03**
	Lysis	0.44±0.02	0.32±0.02*	1.62±0.15***	1.18±0.05***	1.33±0.08***	0.92±0.07***	1.06±0.07***	0.75±0.04***
	FPG	0.20±0.01	0.18±0.03≠	2.62±0.04***	1.80±0.02***	2.26±0.08***	1.75±0.07***	1.80±0.10***	1.19±0.07***
	Endo	0.26±0.01	0.20±0.02≠	2.58±0.05***	1.72±0.03*	2.30±0.06***	1.39±0.03#	1.65±0.06≠	0.84±0.04**
Brain	Buffer	0.26±0.01	0.20±0.01≠	1.06±0.05***	0.80±0.03***	0.94±0.02***	0.75±0.04***	0.73±0.05***	0.55±0.02***
	Lysis	0.33±0.02	0.27±0.02≠	1.22±0.08***	0.88±0.04***	1.16±0.04***	0.92±0.05***	0.92±0.06***	0.83±0.05***
	FPG	0.30±0.02	0.21±0.02*	2.96±0.07***	2.42±0.06***	2.72±0.09***	1.74±0.04***	2.14±0.07***	1.00±0.06***
	Endo	0.37±0.02	0.28±0.02*	2.58±0.05***	1.72±0.04***	2.30±0.06***	1.39±0.03***	1.65±0.06***	0.84±0.04***
Lymp	Buffer	0.25±0.01	0.20±0.01*	0.95±0.02***	0.62±0.01***	0.77±0.02***	0.58±0.05***	0.57±0.02***	0.42±0.04**
	Lysis	0.26±0.02	0.23±0.01*	1.14±0.05***	0.86±0.05***	0.96±0.03***	0.78±0.03***	0.62±0.04***	0.44±0.05*
	FPG	0.35±0.02	0.28±0.02*	2.56±0.04***	1.89±0.04***	2.34±0.03***	1.68±0.03***	1.75±0.05***	1.26±0.05***
	Endo	0.25±0.01	0.20±0.02≠	2.60±0.05***	1.87±0.06***	2.22±0.04***	1.38±0.11***	1.46±0.10***	0.94±0.10***

Values of DNA damage are expressed as damage index calculated as #0 + #1 + #2 + #3 + #4/ # of cell scored where # is the total number of nuclei counted.

Results are expressed as mean ± SE of six set of observations take on different days.

Rats were given 1/60 LD50 equivalent of MCP or QNP or mixture of MCP + QNP (1/120 LD50 equivalent of each) dissolved in 0.4 ml corn oil, orally for sixty consecutive days (total pesticide received by each animal was LD50 equivalents in 60 days). Another group of rats was given co-treatment of melatonin (5 mg/Kg body weight intraperitoneally per day for sixty consecutive days) along with 1/60 LD50 equivalent of MCP, QNP or their mixture.

*cdcomparison of MCP + Melatonin with MCP, efcomparison of QNP +Melatonin with QNP and ghcomparison of Mix + Melatonin with Mixture treated group.

*P < 0.05, **P < 0.01, ***P < 0.001and ≠P > 0.05 when compared with respective control.

Abbreviations: Con, Control; MT, Melatonin, MCP, Monocrotophos; QNP, Quinalphos; Mix, Mixture; Lymp, Lymphocytes.

Subchronic treatment of LD_{50} and $2LD_{50}$ equivalents of MCP, QNP and their mixture also caused significantly marked increase in the DNA damage index in the liver, brain and lymphocytes of rats when compared with control. The increase in DNA damage index observed was 232%, 164% and 78% in the liver, 308%, 262% and 181% in the brain and 280%, 208% and 128% in the lymphocytes of rats equivalents of LD_{50} MCP, QNP and their mixture, respectively, when compared with control (Table 6). The group receiving 2LD50 equivalents of these pesticides, the DNA damage index was increased to 215%, 136% and 54% in the liver, 330%, 233% and 250% in the brain and 219%, 156% and 113% in the lymphocytes of MCP, QNP and their mixture treated group, respectively, when compared with control (Table 7).

The DNA damage index was further increased on treatment with Fpg or Endo in slides of all the tissues of the rats of both the groups when compared with buffer-treated slides. In the liver Fpg treated slides showed 1210%, 1030% and 795% increase, brain showed 887%, 807% and 613% increase, the lymphocytes showed 631%, 569% and 400% increase while Endo treatment showed 892%, 785% and 534.61% increase in the liver, 597%, 523% and 346% increase in the brain and 940%, 788% and 484% increase in the lymphocytes on MCP, QNP and their mixture treatment, respectively, when compared with control (Table 6). The other group of rats receiving $2LD_{50}$ equivalents of pesticide treatment showed further increase in the DNA damage index when compared with respective controls (Table 7). Melatonin co treatment showed protective effects against pesticide-induced DNA damage and caused significantly marked decrease in the DNA damage index in all the tissues when compared with only pesticide treated group in case of both the doses of subchronic exposure.

Generation of reactive oxygen species (ROS) is inevitable in aerobic organisms. The ROS including superoxide anion, hydroxyl radicals, hydrogen peroxide, and others, stem from endogenous sources through cellular metabolism and exogenous sources mediated by environmental exposure of chemicals, pollutants, radiations, cigarette smoke, pesticides and related neurotoxins etc. Nuclear and mitochondrial genomes are under continuous assault by environmentally and endogenous derived ROS, including the formation and accumulation of mutagenic, toxic and/ or genome destabilizing DNA lesions. In

most cases, DNA damage from ROS-generating agents is mediated by Fenton-chemistry giving rise to the formation of chronic and persistent damage, including nucleotide base modification, apurinic/ apyrimidic sites, single and double strand breaks, and DNA crosslinks which can be measured by variety of direct and indirect assays including the comet assay (Collins et al., 2001; Azqueta et al., 2009).

Table 7. Effect of 60 days exposure of MCP (36 mg/Kg b.w.) and QNP (40 mg/Kg b.w.) single and in combination on oxidative DNA damage in rat tissues and lymphocytes and protective effects of intraperitoneal dose of melatonin (5 mg/Kg b.w.)

Tissue	Treat	Con	Con + MT	MCP	MCP + MT	QNP	QNP +MT	Mix	Mix + MT
Liver	Buffer	0.39±0.03	0.36±0.02≠	1.23±0.04***	0.96±0.01***,cd***	0.92±0.03***	0.68±0.06**,ef*	0.60±0.02***	0.48±0.03*,gh**
	Lysis	1.16±0.03	1.02±0.06≠	1.82±0.07***	1.44±0.03*,cd**	1.39±0.09≠	1.02±0.05≠,ef**	1.09±0.07≠	0.82±0.05**,gh*
	FPG	0.17±0.03	0.12±0.02≠	2.92±0.08***	2.22±0.05***,cd***	2.51±0.06***	2.00±0.08***,ef**	2.08±0.10***	1.64±0.07***,gh*
	Endo	0.26±0.01	0.20±0.02≠	2.58±0.05***	1.72±0.03***,cd***	2.30±0.06***	1.39±0.03*,ef**	1.65±0.06*	0.84±0.04≠,gh*
Brain	Buffer	0.30±0.02	0.25±0.01≠	1.29±0.05***	1.14±0.04***,cd*	1.00±0.06***	0.83±0.05***,ef≠	1.05±0.03***	0.88±0.03***,gh**
	Lysis	0.60±0.05	0.46±0.07≠	1.29±0.05***	1.14±0.05***,cd≠	1.16±0.02***	0.93±0.04***,ef**	0.99±0.01***	0.77±0.04***,gh**
	FPG	1.26±0.08	1.02±0.08≠	3.11±0.07***	1.75±0.03***,cd***	2.33±0.08***	1.31±0.05≠,ef***	2.62±0.05***	1.52±0.05*,gh***
	Endo	0.99±0.04	0.75±0.06*	2.82±0.08***	1.63±0.02***,cd***	2.74±0.08***	1.53±0.07***,ef***	2.27±0.09***	1.35±0.08**,gh***
Lymp	Buffer	0.32±0.01	0.10±0.02***	1.02±0.03***	0.92±0.04***,cd≠	0.82±0.04***	0.68±0.04***,ef*	0.68±0.04***	0.51±0.03**,gh*
	Lysis	0.42±0.03	0.34±0.03≠	1.23±0.04***	0.98±0.08***,cd*	1.10±0.02***	0.92±0.04***,ef**	0.74±0.04***	0.55±0.03*,gh**
	FPG	0.44±0.04	0.36±0.04≠	2.56±0.04***	2.16±0.04***,cd**	2.54±0.02***	2.05±0.03***,ef***	2.12±0.04***	1.84±0.02***,gh***
	Endo	0.45±0.04	0.31±0.04≠	2.61±0.04***	1.98±0.07***,cd***	2.27±0.06***	1.62±0.04***,ef***	1.83±0.04***	1.17±0.05***,gh***

Values of DNA damage are expressed as damage index calculated as #0 + #1 + #2 + #3 + #4/ # of cell scored where # is the total number of nuclei counted.
Results are expressed as mean ± SE of six set of observations take on different days.
Rats were given 1/30 LD_{50} equivalent of MCP or QNP or mixture of MCP + QNP (1/60 LD_{50} equivalent of each) dissolved in 0.4 ml corn oil, orally for sixty consecutive days (total pesticide received by each animal was LD50 equivalents in 60 days). Another group of rats was given co-treatment of melatonin (5 mg/Kg body weight intraperitoneally per day for sixty consecutive days) along with 1/30 LD_{50} equivalent of MCP, QNP or their mixture.
cd omparison of MCP + Melatonin with MCP, efcomparison of QNP +Melatonin with QNP and ghcomparison of Mix + Melatonin with Mixture treated group.
*P < 0.05, **P < 0.01***, P < 0.001and ≠P > 0.05 when compared with respective control.
Abbreviations: Con, Control; MT, Melatonin, MCP, Monocrotophos; QNP, Quinalphos; Mix, Mixture; Lymp, Lymphocytes.

Many attempts have been made to establish the mechanism of OP pesticide-induced DNA damage but the outcome of most of the studies have been inconsistent. The present study was carried out to monitor the extent of oxidative damage of DNA bases in rat tissues and lymphocytes on exposure with MCP and QNP. The levels of ROS, total antioxidant capacity and accumulation of lipid peroxidation end products was also monitored in tissues of rats exposed with MCP and QNP in order to establish a correlation if any, between oxidative stress and DNA damage, which could give a clear idea of the mechanism involved in the OP pesticides-induced DNA damage. Previous studies from our laboratory have established that chlorpyrifos, methyl parathion

and malathion, the most commonly used OP pesticides, induce oxidative stress in rat lymphocytes and also cause oxidative DNA damage by oxidation of purines and pyrimidines (Ojha and Srivastava, 2014). Moore et al., 2010 have also demonstrated oxidative stress,

DNA damage and cytotoxicity induced by malathion in human liver carcinoma (HepG2) cells (Moore et al., 2010). In order to elucidate mechanism of selected OP pesticide induced DNA damage, the Fpg and Endo III enzymes are included in the comet assay which can measure oxidized purines and pyrimidines, respectively. The study was carried out using rats given acute as well subchronic exposure of MCP and QNP, single and in combination, and monitoring DNA damage in tissues and lymphocytes of exposed rats. The prophylactic potential of melatonin was monitored by its co treatment with pesticides followed by estimation of levels of ROS and DNA damage.

Results of the present study clearly showed that the levels of ROS are significantly increased in tissues and lymphocytes of rats on exposure with MCP and QNP either single or in combination. The results showed that the MCP exposure caused more pronounced increase in the levels of ROS than QNP exposure to the rats. When combined exposure of both the pesticides was given, the ROS levels were lower than either the MCP or QNP exposed group. The increase in the levels of $O_2^{\bullet -}$ was higher in the rats given 2 LD_{50} equivalent of pesticide in 60 days while the increase in H_2O_2 levels was more in the rats given acute exposure of MCP or QNP either single or in combination.

Co treatment of melatonin has reduced the pesticide-induced increase in the levels of ROS. The results showed that both these pesticides generated oxidative stress in tissues of exposed rats which in turn caused extensive damage to lipids and accumulation of lipid peroxidation products. Increase in lipid peroxidation in response to OP pesticide exposure has been reported by many workers (Rastogi et al., 2009; Mecdad et al., 2011). Other OP pesticides have also been reported to decrease the levels of non-enzymatic antioxidants (Ojha and Srivastava, 2012).

Brain showed higher accumulation than liver in group given chronic exposure of MCP and QNP which seems justified as the distribution of antioxidants is not uniform throughout the body. Because of the low levels of antioxidant enzymes and glutathione, high concentration of iron and readily oxidizable substances such as polyunsaturad fatty acids and catecholamines and high rate of oxidative metabolic activity, the central nervous system is particularly susceptible to damaging effects of ROS.

The decrease in levels of GSH and disturbance in glutathione homeostasis in rat liver and brain exposed with MCP and QNP has already been reported earlier (Mishra and Srivastava, 2015). Chlorpyrifos exposure has caused cortical damage in wistar albino rats, manifested due to oxidative stress as observed via increased nitric oxide production, lipid peroxidation and inducible nitric oxide synthase expression. Further it was observed that there was a decrease in glutathione content and in the activities of glutathione peroxidae, glutathione reductase, catalase and superoxide dismutase in the cortical tissue (Albasher et al., 2020).

Results of the present study showed that oral exposure of MCP and QNP either single or in mixture caused extensive DNA damage in rat liver, brain and lymphocytes, measured by single cell gel electrophoresis. DNA oxidation is known to be the most common type of DNA damage to human and other species. The role of ROS in production of DNA single and double strand breaks and oxidative damage to DNA bases is well known (Azqueta et al., 2009). The damaged purines and pyrimidines bases were identified by modified comet assay using lesion specific bacterial repair enzymes, Fpg, which acts on damaged purines and Endo III which removes damaged pyrimidines, and convert base damage to breaks. The increase in number of breaks on Fpg-Endo treatment, is directly proportional to the number of oxidized bases.

Results of the present study showed that the DNA damage index was markedly increased in Fpg-Endo treated slides of the liver, brain and lymphocytes in MCP or QNP treated group when compared with buffer treated slides of corresponding tissues. It was observed that Fpg treatment caused higher increase in DNA damage index indicating that oxidation of purines is more than oxidation of pyrimidines, by these pesticides. In the present study, the difference in DNA damage index in the presence and absence of Fpg and Endo III enzymes suggest that oxidative stress is responsible for OP pesticides induced DNA damage. The results also showed that rat tissues given acute exposure of these pesticides showed more damage than the rats given low level exposure of these pesticides for longer duration.

Melatonin co treatment showed protection against oxidative injuries and the DNA damage was lowered in these groups when compared with melatonin untreated group. There have been several investigations in *in vivo* and *in vitro* on the correlation between toxicant induced oxidative stress and DNA damage. The OP pesticides, chlorpyrifos, methyl parathion and malathion, singly and in combination cause oxidative stress and oxidation of purine and pyrimidine bases in rat lymphocytes, in vitro (Ojha and Srivastava, 2014).

Other studies reported on oxidative stress and DNA damage in response to exposure with OP pesticides include methyl parathion in spermatozoa of male mouse (Rahman et al., 2002), monocrotophos in rat tissues, malathion on human liver carcinoma cells (Moore et al., 2010), chlorpyrifos in rat tissues and lymphocytes (Rahman et al., 2002; Mehta et al., 2008), fenitrothion in aquatic organism Fingerlings, *Oreochromis niloticus* L.(Zeid and Khalil, 2014), and several other OP pesticides (Lu et al., 2016).

Malathion has been widely studied for its ability to cause oxidative stress in human subjects and subsequently cause toxicity in various organs. Antioxidants have proved to be very effective in decreasing lipid peroxidation and oxidative stress. Natural products that have effectively reduced damage in biological system include aged garlic extract, Aloe Vera, caffeic acid, grape seed extract and curcumin (Badr, 2020). Not only in mammals the organophosphate pesticides have shown to induce toxicity in *Chlorella pyrenoidosa*. The pesticides that were evaluated for their toxic effects were acephate (ACE), trichlofor (TRI) and glyphosate (GIY). The possible mechanism of toxicity in this study could be affect on photosynthesis and subsequent oxidative damage to *C.pyrenoidosa* cells (Tao et al., 2020).

Melatonin also prevents the damage caused by OP pesticides such as diazinon and reduces the levels of trace and major elements (Sarbia et al., 2009; Cemek et al., 2010). Melatonin prevented the oxidative stress in the periodontal tissue of the rats receiving radiotherapy for the treatment of periodontitis (Kose et al., 2017). Melatonin also posesses the ability to protect the biological system such as the renal tissues against the oxidative damage caused by the carcinogens such as arsenic, 2-nitropropane, carbon tetrachloride etc (Gultekin and Hicyilmaz, 2007). Melatonin has recently been evaluated for its ability to reduce oxidative stress caused by a carcinogen dimethyl benz (a) anthracene (DMBA) thereby further validating its antioxidant properties (Muqbil et al., 2020).

CONCLUSION

The present study clearly demonstrates the ability of monocrotophos and quinalphos to generate oxidative stress. This oxidative stress leads to the formation of oxidized pyrimidines and purines thereby damaging the integrity of the genome. The protective effects of melatonin against the exposure of these pesticides indicate its ability to be used as a suitable therapeutic agent. Hence the use of low doses of melatonin as a dietary supplement in agriculture and household can save the non-target organisms from the harmful effects of these pesticides.

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Statistical Optimization of Lipase Production from Bacillus Species by Submerged Fermentation

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ABSTRACT

Enzymes are an important class of proteins of biological origin that act as biochemical catalysts. Lipases (EC 3.1.1.3) are one of the important extracellular microbial enzymes. Lipases catalyze the hydrolysis of triglycerides to glycerol and free fatty acids. They are soluble in water and hydrolyze insoluble substrates to more polar lipolytic products. After performing OFAT analysis, screening of media component and process parameters was performed by PB design. Process and media optimisation of lipase are significant in view of economic production of enzymes in industries. Present study emphasises on optimising the process parameters for lipase production by bacteria through submerged fermentation using statistical tools. Bacterial strains were isolates from oil contaminated soil sources and screened for their lipase production based on the zone of clearance on Tributyrin Agar media. Eight isolates showing good ability to produce lipase were characterized for morphological features and motility. Further, one isolate was chosen and screened for media components (mineral salts) for lipase production by Plackett-Burman (PB) Design using Minitab 16 software. Totally eleven different minerals salts with 2 levels were considered for screening by PB design and 12 experimental design were taken for the study. The Pareto chart showed that except G ($ZnSO_4$) and D ($CaCO_3$) remaining all mineral salts showed significant effect on lipase production. Main effect plots for enzyme production also found that for some of salts like $CaCO_3$ and $MgCl_2$ their change in concentration from low to high level does not any significance on enzyme production. Lipase activity ranged from 23U/ml to 72.3U/ml. $NH_4H_2PO_4$ was found to be the most significant salt of the media component affecting the lipase production having P-value less than 0.05. A Full factorial design (FFD) was employed to determine the effects of media components (molasses, peptone and $NH_4H_2PO_4$). The results of FFD show that 1.5ml for molasses; 0.2grams of $NH_4H_2PO_4$ and1 gram of peptone were the optimized values. There exist significant interactions between molasses and peptone and $NH_4H_2PO_4$ and peptone. The analysis of variance (ANOVA) was performed to check the adequacy of the proposed models. The results of RSM based mathematical modelling, indicates the ability of this technique to predict proposed performance at 95% confidence interval. The coefficient of determination R-sq was 97.11%, which confirms that the model was statistically significant and good fit.

KEY WORDS: LIPASE; MINERAL SALTS; PB-DESIGN; FULL FACTORIAL DESIGN; SUBMERGED FERMENTATION.

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are enzymes that are unique in catalyzing the hydrolysis of fats into fatty acids and glycerol at the water-lipid interface, and have the ability of reversing the reaction in non-aqueous media. The recent applications of lipases are additives in food (flavour modification), fine chemicals (synthesis of esters, detergent hydrolysis of

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fats), wastewater treatment (decomposition and removal of oily substances), cosmetics (removal of lipids), pharmaceuticals (digestion of oil and fats in foods), leather processing (removal of lipids from animal skins) and biomedical assays (blood triglycerides). (Manouchehr et al., 2018). Additionally, lipases have an important application in the field of bio energy, especially in biodiesel production, which is an expanding sector, as a result of the worldwide rising demand for the use of renewable energy (Daiha et al., 2015; Manouchehr et al., 2018).

Lipases are carboxyl esterase's that catalyze the hydrolysis of acylglycerols containing fatty acid chains greater than 10 carbon atoms in length. There is a great to militate these effluents which has necessitated the development of new technology for bioremediation involves the intervention of microorganism to degrade the pollutants through these metabolites like enzymes (Daiha et al., 2015). Various microorganisms producing lipase are used in bioremediation of oil contaminated soil. Hence lipases are regarded as potential agent for commercial applications. They are being explored for both academically and industrially for their applications. Lipids are essential components of living systems and are important sources of energy. They are part of cell membranes and signalling events. Lipids are needed to catalyze these reactions (Gilham and Lehner, 2005; Mateus et al., 2009; Salihu et al., 2011; Karina et al., 2016).

The enzyme from microbial sources is currently receiving more attention because of their potential diverse applications in industries such as detergent, oleo chemical, organic. Considering the fact that a substantial part of industrial enzymes production cost is contributed by the cost of the fermentation medium, the present investigation was aimed at evaluating the effects of medium components on lipase production by formulating a suitable medium containing waste groundnut oil as a low-cost renewable substrate and molasses as an economic carbon source (Pin et al., 2015; Nelison et al., 2016). Plackett-Burman (PB) design is important statistical design used for the screening and selection of medium components in shake-flask cultures. PB design offers a good and fast screening procedure and it mathematically computes the significance of a large number of factors in one experiment. It has been applied to try and reduce the number of fermentations runs to an absolute minimum. It saves much of the time and gives useful information on each component using a minimum number of experiments compared to conventional method of screening (Parimala et al., 2006; Salihu et al., 2011; Selvam et al., 2016).

It is used only to evaluate and select the significant factors, which influence the production of lipase during fermentation. It does not describe the interactions among the factors affecting the process. Full Factorial Design (FFD) provides the most complete information, it often requires so many runs that they become impractical to carry out. Thus, optimum performance has been determined using mathematical tools such as multiple regression of a partial or full factorial to obtain a model of the production system, usually involving fitting of data to a polynomial equation, using stepwise multiple regression. Response surface methodology has also been used to investigate the optimal regions of production of useful product. Detailed analyses of the optimized region using simple designs have also been applied for optimization processes. However, several interactions of the experimental design and optimization of models are required for effective application to product formation in fermentation systems (Jaeger et al., 1997; Savitha et al., 2007; Sirisha et al., 2010; Anwar et al., 2011; Pin et al., 2015).

Gap Analysis: Lipolytic enzymes are categorized into these groups- esterases, phospholipases and lipases are known to catalyze the hydrolysis of triglycerides into and free fatty acids and glycerol. Lipases usually act carboxylic bonds of triglycerides and give simple fatty acids and glycerol as by product. The key uniqueness of lipase is substrate specific; they are known to be stable at higher of pH and temperature. Lipases are extracellular in nature and act on substrate. Lipases having variety of applications are commonly regarded as third largest enzyme being produced only after protease and amylase. The present study caters the various needs of lipase enzyme as biocatalyst for different application.

MATERIAL AND METHODS

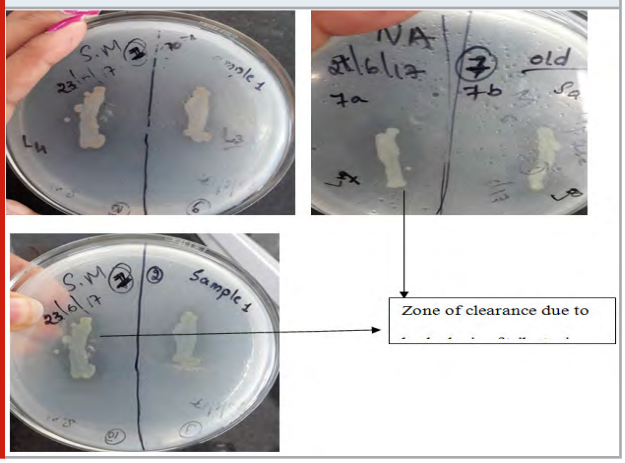
For the soil sample collection, the oil contaminated soil samples where soil was collected by digging ground up to 15-20 cm nearby Hubballi, with the help of clean spatula in sterile polythene bag. Immediately after collecting the soil it was stored in the refrigerator at 4 °C till processing (Pooja et al., 2015).

For the isolation and screening of lipase producing bacteria, 5 grams of the soil sample was added to nutrient broth (100ml.) and incubated for 24 hours. Soil sample was serially diluted (0.85% NaCl) and plated on tributyrin agar base containing 0.5% (w/v) peptone, 0.3% (w/v) beef-extract, 1% (v/v) Tributyrin and 2% agar, pH 7.0 by spread plate method. Plates were incubated at 37 °C for two days. Pure cultures of the isolates were maintained on nutrient agar slants supplemented with tributyrin. The isolated colonies were picked and maintained on nutrient agar slants as pure cultures. These were screened for lipolytic activity by Tributyrin Qualitative Plate Assay based on the zone of clearance (by incubating at 37 °C for 2 days). Isolates showing maximum zone of clearance were taken for further studies. Morphological characterization of these isolates was performed by gram staining technique and Motility test (Hanging drop technique) (Rifaat et al., 2010; Acikel et al., 2011; Veeranna et al., 2012).

For the microbial culture and inoculum preparation, one isolate having maximum lipase activity was chosen for further studies. Loop full of the culture was inoculated into the inoculum media(100ml) containing molasses(1.5ml),

waste groundnut oil (2.2ml), peptone(1g), $CaCO_3$(0.1g), $(NH_4)_2SO_4$(0.1g), $MgSO_4.7H_2O$ (0.1g), Inoculum flask was kept for incubation for 2 days at 37°C and 150rpm (Imandi et al., 2007). For the lipase production media, 1ml of inoculum in 100ml production medium containing Soya peptone (1gram), waste groundnut oil (2.2ml), KH_2PO_4 (0.5gram), $CaCO_3$ (0.1gram), $(NH_4)_2SO_4$ (0.1gram), $MgSO_4.7H_2O$ (0.1gram), Molasses (1.5gram) and submerged fermentation was carried out at 37 °C 150 rpm for 72 h (Imandi et al., 2007).

Figure 1: Tributyrin Agar media



For the fermentation medium and lipase production for screening of mineral salts, the fermentation medium was prepared by adding molasses (1.5ml), waste groundnut oil (2.2ml), peptone (1gram) and 11 combinations of mineral salts and inoculum was added to fermentation medium. For the full factorial design, in order to evaluate the factors that influence the lipase production using cheaper carbon source, a three level and three factor full factorial experiments was designed. Carbon source, nitrogen source and $NH_4H_2PO_4$ (which showed most significance by Pareto chart P (<0.005)) were chosen as the independent variables and lipase activity (U) was taken as the response of the design. The level and the range of the independent variables were examined in three levels. Thus, ranges of (0.5, 1.5 and 2.5%v/v) of molasses as carbon source, (0.5, 1 and 1.5%v/v) of peptone as nitrogen source and (0.2, 1, 1.8%v/v) of $NH_4H_2PO_4$ were considered. Practical considerations were used for selecting the three factors and the range in which they were varied (Prashant et al., 2006; Anwer et al., 2011; Ananthi et al., 2013).

RESULTS AND DISCUSSION

Isolation and Screening of bacterial strains: Many bacterial colonies were isolated from oil contaminated soil and screened for lipase production by tributyrin zone of clearance test. Totally eight isolates showing the maximum zone of clearance was chosen for morphological studies (Ajit et al., 2007; Bhavani et al., 2012).

Selection of significant mineral salts by PB Design: PB design was used to evaluate maximum lipase production as function of mineral salts. A total of eleven medium components (minerals) were studied with regard to their effects on lipase production by PB design using the Minitab16 software. The combinations of medium components (minerals) are shown in [Table 1]. Based on the design matrix selected for the screening of significant variables, the highest lipase activity realized was 72.3 U/ml at run 1 and the lowest amount was observed in run 2 (23 U/ml) (Sirisha et al., 2010; Bhavikatti et al., 2020).

Table 1. PB design matrix for screening mineral sources

S. No	$MgSO_4.7H_2O$	KH_2PO_4	$CaCl_2$	$CaCO_3$	$FeSO_4$	$MnSO_4$	$ZnSo_4$	$NH_4H_2PO_4$	KCl	$MgCl_2$	NaH_2PO_4	Enzyme Activity (U)	Lipase con(µg/ml)
1	1	1	1	1	1	1	1	1	1	1	1	72.3	0.2661
2	-1	1	-1	1	1	1	-1	-1	-1	1	-1	23	0.0946
3	-1	-1	1	-1	1	1	1	-1	-1	-1	1	24.3	0.3534
4	1	-1	-1	1	-1	1	1	1	-1	-1	-1	34	0.1311
5	-1	1	-1	-1	1	-1	1	1	1	-1	-1	53.3	0.1257
6	-1	-1	1	-1	-1	1	-1	1	1	1	-1	38	0.3364
7	-1	-1	-1	1	-1	-1	1	-1	1	1	1	25.3	0.0369
8	1	-1	-1	-1	1	-1	-1	1	-1	1	1	53	0.0851
9	1	1	-1	-1	-1	1	-1	-1	1	-1	1	44	0.1475
10	1	1	1	-1	-1	-1	1	-1	-1	1	-1	31	0.2972
11	-1	1	1	1	-1	-1	-1	1	-1	-1	1	63.3	0.2633
12	1	-1	1	1	1	-1	-1	-1	1	-1	-1	24.67	0.2828

Response Surface Methodology (RSM)/Full Factorial Design (FFD) for media components: Full factorial Design is used to determine the optimum response of lipase production. FFD for three independent variables was used to obtain the combination of values that optimizes the response. The experiments were designed using the Minitab16 software. Based on the PB design results further studies was done by performing RSM to

optimise the lipase production using significant media components. The significant independent variables of the medium components are molasses (carbon source), peptone (nitrogen source) and $NH_4H_2PO_4$.

Figure 2: Pareto graph showing variables effect on maximum lipase production, based on PB design observations

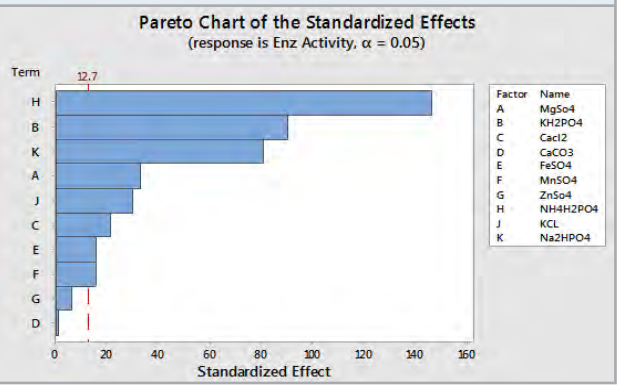


Figure 3: Main effects plot for enzyme activity

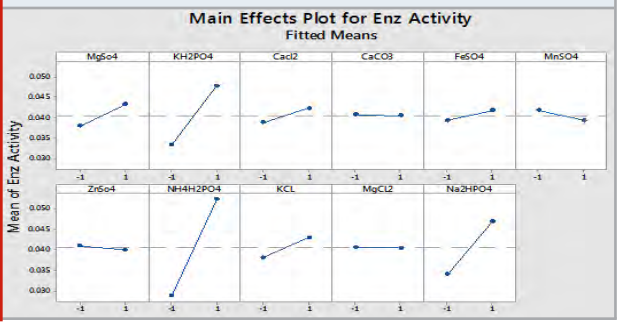


Table 2. FFD matrix showing effect of media components on lipase activity

Molasses	$NH_4H_2PO_4$	Peptone	Response-EA(U/ml)
-1	-1	-1	10.21
-1	0	-1	5.11
-1	1	-1	3.92
0	-1	-1	13.89
0	0	-1	13.59
0	1	-1	9.85
1	-1	-1	12.01
1	0	-1	9.13
1	1	-1	6.84
-1	-1	0	9.28
-1	0	0	6.69
-1	1	0	5.41
0	-1	0	18.64
0	0	0	15.90
0	1	0	12.86
1	-1	0	9.87
1	0	0	9.36
1	1	0	7.67
-1	-1	1	8.54
-1	0	1	5.14
-1	1	1	10.98
0	-1	1	13.67
0	0	1	11.24
0	1	1	11.12
1	-1	1	6.48
1	0	1	4.37
1	1	1	8.58

Analysis of Variance

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-Value	P-Value
Model	18	335.554	97.11%	335.554	18.642	14.94	0.000
Linear	6	253.536	73.38%	253.536	42.256	33.87	0.000
Molasses	2	196.996	57.01%	196.996	98.498	78.95	0.000
$NH_4H_2PO_4$	2	42.259	12.23%	42.259	21.130	16.94	0.001
Peptone	2	14.281	4.13%	14.281	7.141	5.72	0.029
2-Way Interactions	12	82.017	23.74%	82.017	6.835	5.48	0.011
Molasses*$NH_4H_2PO_4$	4	11.364	3.29%	11.364	2.841	2.28	0.150
Molasses*Peptone	4	30.972	8.96%	30.972	7.743	6.21	0.014
$NH_4H_2PO_4$*Peptone	4	39.681	11.48%	39.681	9.920	7.95	0.007
Error	8	9.981	2.89%	9.981	1.248		
Total	26	345.535	100.00%				

Regression Equation

Response-EA (U/ml) =9.643 - 2.391 Molasses_-1 + 3.776 Molasses_0 - 1.385 Molasses_1 + 1.756 $NH_4H_2PO_4$_-1 - 0.694 $NH_4H_2PO_4$_0 - 1.062 $NH_4H_2PO_4$_1 - 0.248 Peptone_-1 + 0.989 Peptone_0 - 0.740 Peptone_1

FFD experiment matrix along with response is shown in following table. The ANOVA table obtained from analyzing the response we got the value of R-Sq (pred) and R-Sq (adj) difference is less. Hence, the designed model is adequate and consistent. Analysis of variance showed a high coefficient of determination of (R^2) value of 97.11%, indicating a satisfactory fit of the model with the experimental data. All the three parameters were statistically significant at $p<0.05$ and all the parameters influence the production of lipase as shown in the Pareto chart (Kumar et al., 2006; Rao et al., 2010; Tembhurkar et al., 2012; Muhhamad et al., 2012; Bhavikatti et al., 2020).

CONCLUSION

Eleven mineral salts were screened by 12 experimental runs. The experimental plan and corresponding lipase activity were reported. The Pareto chart were used to show the effect of all the mineral salts on lipase production. The Pareto chart showed that except G ($ZnSO_4$) and D (CaCO3) remaining all mineral salts showed significant effect on lipase production. Out of these $NH_4H_2PO_4$with P-value (0.004), KH_2PO_4 with P value (0.007) and $NH_4H_2PO_4$ with P- value (0.008) showed major effect on lipase production. In addition, predicted R2 of PB design was 99.63%, which is a good model fit. Three factors were considered at 3 levels for FFD, total 27 experiments were performed to evaluate the enzyme activity. FFD results showed that all independent variables are significant having the P value less than 0.05 (except molasses and $NH_4H_2PO_4$ interaction). From FFD we got 1.5ml for molasses, 0.2gram for $NH_4H_2PO_4$ and 1gram peptone as optimum value. There exist significant interactions between molasses and peptone and $NH_4H_2PO_4$ and peptone. R-sq of FFD design was 97.11% which confirm that our model is good fit.

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Phytosynthesis, Characterization and Antimicrobial Activity of Copper Oxide Nanoparticles from Cassia auriculata

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ABSTRACT

Nanotechnology is the art and science of manipulating matter at the nanoscale to create new unique materials with enormous potential to change society. Nanoparticles can serve as "magic bullets", which carry the active ingredient along with it, green synthesis or phytosynthesis of nanoparticle is an eco-friendly approach which is in common practice. In this present study, a mono dispersed Spherical shaped copper oxide nanoparticle was prepared successfully using *Cassia auriculata* flowers extract without using any harmful reducing agents. The synthesized nanoparticles were characterized by UV-VIS spectroscopy, Fourier Transform Infra-Red spectroscopy and Transmission Electron Microscopy. The antibacterial activity of synthesized copper nanoparticles was compared by agar well diffusion method and minimum inhibitory concentration was also calculated. The zone of inhibition varied in range of 10 to 30 mm. However, bactericidal effect of copper nanoparticles varies with respect to the organism tested. The phytosynthetic approach is a simple alternative to chemical and physical methods due to low cost and less use of toxic chemicals. This study presents a simple, fast, cheap and eco-friendly method for CuONPs synthesis. The method was based on the reduction of copper (II) sulfate pentahydrate salt by Cassia auriculate flowers ethanol extract. The copper oxide Nps synthesized using the green method showed excellent antioxidant, antibacterial antifungal activity. The exact mechanism and the cytotoxic nature of the nanoparticles should be investigated further for its effective application. These findings showed that green method could be used as a good alternative to the current physical and chemical methods associated with environmental toxicity.

KEY WORDS: ANTI-BACTERIAL; ANTI-FUNGAL; CASSIA AURICULATA FLOWER; COPPER OXIDE NANOPARTICLES; PHYTOSYNTHESIS.

INTRODUCTION

Now nanoparticles have many applications in the commercial world. Recently, the green synthesis of NPs using microorganisms and plants extracts has been achieved (Gunalan et al., 2012). Green synthesis procedures are very simple, rapid, nontoxic, inexpensive (Kumar et al., 2009). Conventionally, copper and its complexes have been used as water purifiers, antibacterial and antifungal agents (Stoimenov et al., 2002; Lee et al., 2009). Copper oxide nanoparticles (CuONPs) have been of great interest, due to their exclusive physical and chemical properties (Varshney et al., 2012). The growing interest of environmental supporting phenomenon for synthesis nanoparticles, phytogenic reduction methods (Phytosynthesis) are more suitable, effective and eco-friendly. *Cassia auriculata* Linn., an annual or biennial shrub found throughout India, belongs to the family *Caesalpiniaceae*. The flowers, leaves, stem, root and unripe fruit are used for treatment, especially in Ayurvedic medicine. The plant has been reported to possess antibacterial, antifungal, and anticancer,

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antipyretic, antihyperglycaemic, antiperoxidative and hepatoprotective activity (Ahamed et al., 2014; Joshi et al., 2019; Zangeneh et al., 2019; Siddiqi et al., 2020).

Image I: Flowers of Cassia auriculata Linn.

Phytosynthesis of copper oxide nanoparticles by various plant extracts has been reported so far. The major advantage of using plant extracts for copper oxide nanoparticles (NPs) synthesis is that they are easily available, safe, and nontoxic in most cases. Several reports have been proven that Copper oxide NPs has the highest antimicrobial activity compared to other metal oxides (Gebremedhn et al., 2019; Renuga etal., 2020). In this study copper oxide nanoparticles have been synthesized using *Cassia auriculata* flowers for the first time with the help of greener protocols. Synthesized CuONPs were characterized by UV-visible spectroscopy, FTIR, TEM and followed by antioxidant activity by DPPH method. Further its efficiency against bacteria and fungus were analysed using disc diffusion method.

MATERIAL AND METHODS

The flowers of the *Cassia auriculata* were collected from in and around Kanchipuram, Tamil Nadu, India. The taxonomic identities of this plant were determined. They were thoroughly rinsed using normal water, followed by distilled water and then dried in the shade at room temperature. The *Cassia auriculata* flowers were cut into small pieces and crushed with help of mortar and pestle. 20 grams of powder sample were subjected to Soxhlet extraction at 40-60°C for 8 cycles in 200 mL of ethanol. The mixture was filtered using Whatman no.1 filter paper and the filtrate solution was concentrated and evaporated on a rotary evaporator (Buchi, R–124 and Switzerland) to obtain a residue which was stored at 4°C to use further.

Antioxidant activity of extract was estimated on the basis of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging effect (Huang et al., 2005). The free radical scavenging activity (FRSA) calculated using the following equation:

FRSA (%) = 100 × (AC – AS) / AC where AC is the absorbance of DPPH without sample and AS is the absorbance of DPPH in the presence of sample. The concentration of sample required to scavenge 50% of DPPH radicals was measured as IC50.Various concentrations of copper (II) sulfate pentahydrate solution and the extract in different pH, temperature and incubation time were mixed. The reaction mixture was allowed to stand in a dark room to complete the reaction. The obtained precipitation was purified by repeated centrifugation at 12000 rpm for 20 min, dried in oven at 80o C for 8 h and stored in properly containers (Shiravand and Azarbani, 2017).

Image 2: Ethanolic Extraction and Preparation of CuONPs from Cassia auriculata flower

The reduction of copper ions in copper (II) sulfate pentahydrate solution to CuONPs was periodically monitored by ultraviolet–visible (UV-Vis) Spectrophotometer. UV-Vis spectral analysis was done by UV-1700 (Shimadzu, Japan) spectrometer at the range of 300–600 nm. The FT-IR spectroscopy were carried out for both the Cassia auriculata flowers ethanol extract and the synthesized CuONPs to identify possible biomolecules in the Cassia auriculata flowers extract that can participate in reduction process of copper ions and capping of the resulting CuONPs. The samples grinded with potassium bromide (KBr) and analyzed by Bruker fourier transform infrared (FTIR) Tensor- 27 spectrophotometer at range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1}. The sample morphology and size were examined with Tecnai G2 20 (FEI) S Twin model operating at 200 kV transmission electron microscope (TEM). The images were recorded to confirm the shape of newly synthesized CuONPs (Shiravand et al., 2017).

Antibacterial activity of synthesized CuONPs was evaluated against *Escherichia coli, Staphylococcus aureus, serratia species, Vibrio harveyi* by disc diffusion method. Then antifungal activity was evaluated against *Aspergillus niger* and *Aspergillus fumigatus*. Nutrient agar plates were seeded with overnight bacterial and fungal culture. 50 µL of different concentrations (250 –1000 µg/mL) of biosynthesized CuONPs were placed on the surface of the inoculated plates. After incubation at 37°C for 24 h, zone diameters were measured (mm) (Li et al., 2006).

RESULTS AND DISCUSSION

In the present study, the different concentrations of Ethanolic extract of *C. auriculata* flowers were subjected to DPPH free radical scavenging assay. The antioxidant capacity of the extract was compared with standard ascorbic acid. Results obtained showed that standard antioxidant had higher scavenging activity at all tested concentrations than the extracts. The result showed the Percentage of activity of standard ascorbic acid and extract for 1000 µg/ml as 97.3% and 82.32%, respectively when compared with DPPH assay activity. Therefore, the results indicated that *C. auriculata* flowers are a rich source of antioxidant compounds. Therefore, their ethanolic extract was used to reduce copper ions present in copper (II) sulfate pentahydrate solution and synthesis of CuONPs.

Figure 1: DPPH radical scavenging activity of ethanolic extract of *Cassia auriculata* flower in various concentrations

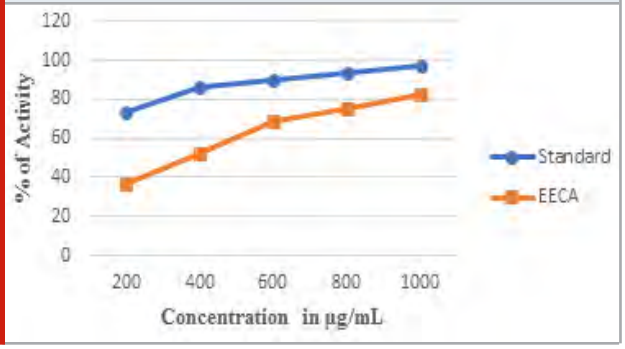
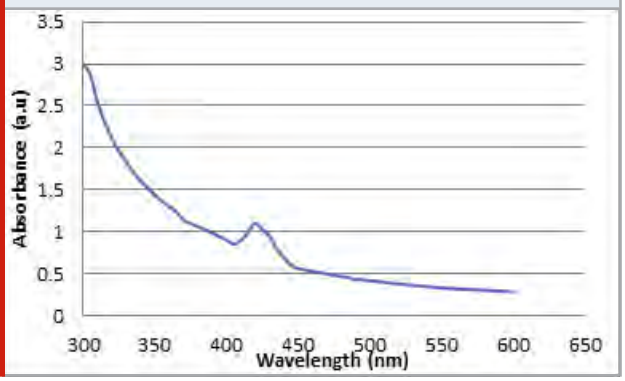


Figure 2: UV-Vis spectrum of CuO nanoparticles phyto-synthesized by *Cassia auriculate* flower.



Synthesis of CuONPs using ethanol extract of *Cassia auriculata* flowers were observed by UV-visible spectroscopy. Reduction of copper sulphate solution to CuONPs was confirmed by measuring the UV-Vis spectrum at the range of 300–600 nm. The UV-Vis absorption spectrum of sample is recorded and shown in Figure 2. As expected, CuO nanoparticles show an absorption peak between 400 and 500 nm i.e. 420 nm that can be contributed to the characteristic absorption of CuONPs. The peak was observed even after one week indicating the stability of CuONPs (Subashini et al., 2019).

CuONPs solution was centrifuged at 10,000 rpm for 30 minute and obtained solid residue was washed several times with distilled water followed by drying. The solid powder was used for FTIR analysis, which were performed on Bruker fourier transform infrared (FTIR) Tensor- 27 spectrophotometer. The FTIR peaks were identified and expressed in wave numbers (cm^{-1}). The refined CuONPs possessed absorption peaks at 3411, 2964, 2924, 2857, 1730, 1628, 1515, 1442, 1383, 1317, 1111, 879 and 779 cm^{-1} corresponding to hydroxyl group (OH) stretching, hydroxyl (-OH) bending, and C-O stretching, respectively. (Figure 3). It may be confirmed that the bioactive ingredients of *Cassia auriculate* flower was the probable reducing agent which was concerned in the Phyto-synthesis of CuONPs and might have organized a layer on the CuONPs (i.e., Phyto-capping) that may have delayed the agglomeration of the Nanoparticles would have stabilized them (Hassanien et al., 2018).

Figure 3: FT-IR spectrum of CuO nanoparticles phyto-synthesized by *Cassia auriculate* flower

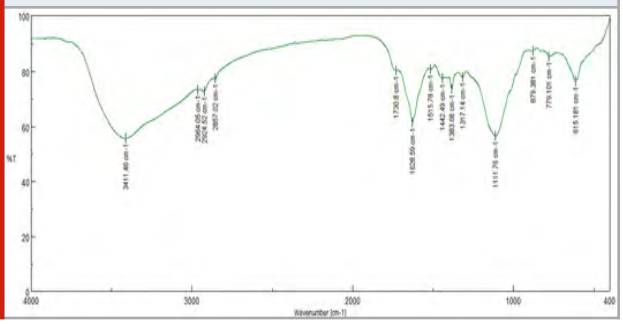
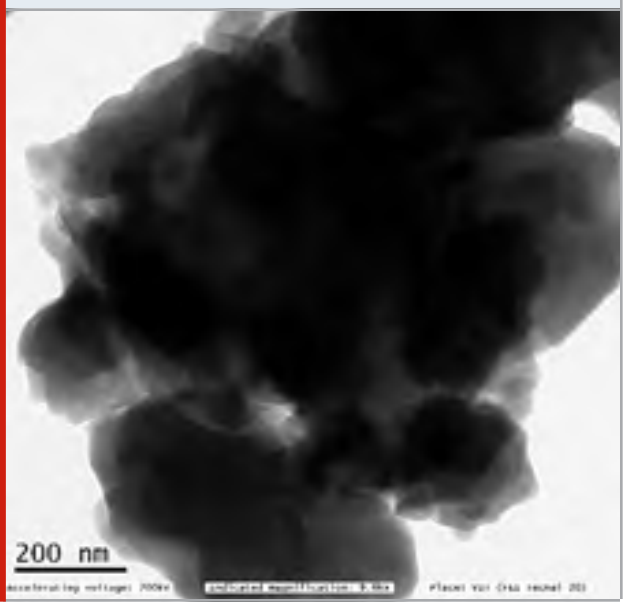


Image 3: Bright field TEM image of phyto-reduced copper nanoparticles. (Vellore Institute of Technology, Vellore, Tamil Nadu, India)



The shape and size of the synthesized CuO-NPs were analysed by TEM analysis. Image III shows the TEM image of biosynthesized CuONPs. The experimental results showed that the shape of prepared CuONPs was spherical with diameters that ranged from 200 nm and found in form of nanocluster. The larger copper particles may be due to the aggregation of the smaller ones, during the TEM analysis.

Table 1. Antibacterial activity of Phyto-synthesized CuONPs.

	Zone of Inhibition (mm)			
	Concentration (µg/mL)			
Bacterial Strain	Control	250	500	1000
E. coli	13	14	15	18
S. aureus	30	15	23	30
Serratia sp	33	10	12	17
V. harveyi	24	13	14	15

Figure 5: Antibacterial activity of Copper oxide nanoparticles

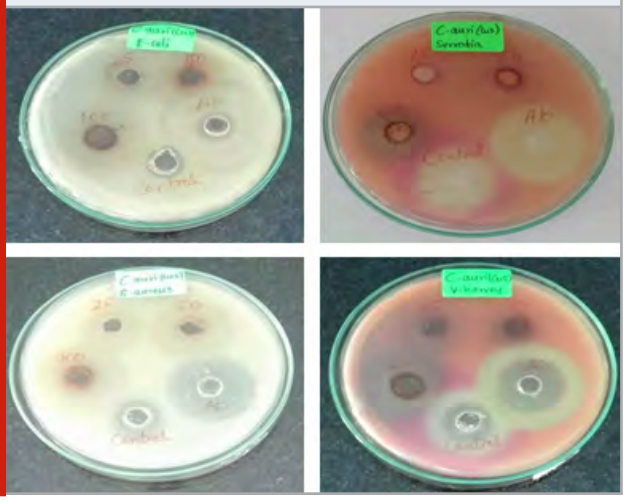
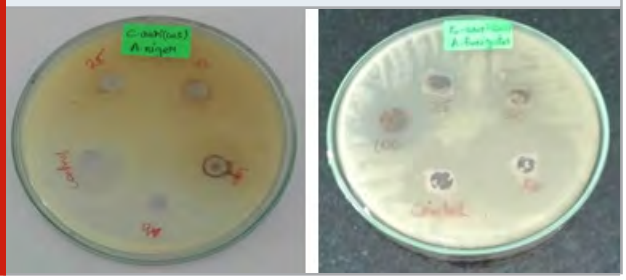


Figure 6: Antifungal activity of copper oxide nanoparticles



The antibacterial activity of synthesized CuONPs was evaluated against *E. coli, S. aureus, Serratia* Sp and *V. harveyi* bacteria. The CuONPs showed activity against all tested organisms (Table 1). It was found that the zone of inhibition increased with increasing the concentration of CuONPs (Figure 5). The exact mechanism behind the biocidal activity of CuONPs is not yet fully known. It was suggested that copper ions originating from the CuONPs may interact with phosphorus and sulfur-containing biomolecules such as DNA and protein to distort their structures and thus disrupt biochemical processes (Ruparelia et al., 2008; Wu et al., 2009). Effectiveness of CuONPs against both Gram-negative and Gram-positive bacteria proposing as broad-spectrum potential of nanoparticle. Bacterial colony stamp down by cell filaments formation influenced by CuONPs subjected to bacterial cell membrane destruction (Montes-Burgos et al., 2010; Saranya et al., 2020).

Table 2. Antifungal activity of Phyto-synthesized CuONPs.

	Zone of Inhibition (mm)			
	Concentration (µg/mL)			
Fungi	Control	250	500	1000
A. niger	16	9	11	13
A. fumigatus	35	16	16	18

The antifungal test of CuO nanoparticles were performed by allowing Aspergillus niger and Aspergillus fumigatus to grow on agar CD medium containing different concentration of CuO nanoparticles respectively (Figure 6). It was found that the growth inhibition of A. niger and Aspergillus fumigatus were observed in a concentration dependent manner (Table 2). Recent advances in the field of nanotechnology, particularly the ability to prepare metal oxide NPs of any size and shape, could lead to the development of new antifungal agents. The use of NPs suggests a new promising approach for fungal infection therapy.

CONCLUSION

The copper oxide Nps synthesized using the green method showed excellent antioxidant, antibacterial antifungal activity. The exact mechanism and the cytotoxic nature of the nanoparticles should be investigated further for its effective application. These findings showed that green method could be used as a good alternative to the current physical and chemical methods associated with environmental toxicity.

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Hypoglycemic and Antioxidative Potential of Coriandrum sativum Seed Extract in Alloxan Induced Diabetic Rats.

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ABSTRACT

Diabetes, an endocrine disorder, causes hyperglycemia along with oxidative stress that leads to diabetes-related complications. Diabetic nephropathy is one of the complications related to oxidative stress. Antioxidants play a pivotal role to protect body organs against damage caused by free radical species like Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). Nowadays, drugs that are used to treat diabetes mellitus cause serious side effects, leading to the gaining popularity of alternative herbal medicine. This study aims to investigate antioxidant potential concerning kidney tissue and hypoglycemic effect of *Coriandrum sativum* seeds in alloxan induced diabetic rats. The rats were divided into Normal, Diabetic and Coriander seed crude methanolic extract treated groups. Diabetes was induced by administering Alloxan at a dose of 100 mg per kg body weight. Diabetic rats were treated with crude methanolic Coriander seed extract at a dose of 500mg/kg bodyweight for 30 days using gavage. Blood serum was used for Glucose estimation whereas Kidney tissues were collected and stored in Tris Buffer for antioxidant assay. Glucose and antioxidant assays were carried out using previously reported methods with slight modifications. The results showed a significant ($p<0.05$) decrease in blood glucose level indicating its hypoglycemic effect. Besides, it caused a significant ($p<0.05$) increase in Antioxidant enzymes and compounds of kidney tissue such as Superoxide Dismutase (SOD), Glutathione-S-transferase (GST), Glutathione Peroxidase (GPx), Catalase (CAT), Glutathione Reductase (GSSG Red) and Reduced Glutathione (GSH) as compared to the diabetic group. Thus, it indicates that the crude methanolic extract of *Coriandrum sativum* seeds has the potential to combat hyperglycemia and oxidative stress-induced diabetic complications.

KEY WORDS: DIABETES, HYPOGLYCEMIC, ANTIOXIDANT, CORIANDRUM SATIVUM, OXIDATIVE STRESS.

INTRODUCTION

Diabetes, being, an Endocrine disorder having many complications (Adeyemi et al., 2010). Oxidative stress has been reported to play a key role in the initiation and progression of diabetes mellitus along with its complications. Diabetes-induced Hyperglycemia increases oxidative stress, which may be due to either increased free radical species production or a decrease in antioxidant defenses (Giacco and Brownlee, 2010). Studies indicate that oxidative damage caused due to oxidative stress is an important factor related to diabetic nephropathy (Zhang

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and Sun, 2015). Diabetic nephropathy, a chronic disease, caused due to diabetes mellitus, if left untreated, leads to end-stage renal failure (Chen et al., 2015; Ghaderian et al., 2015; Magee et al., 2017). Hyperglycemia and Oxidative stress together play an important role in the progression of Diabetic Nephropathy (Rehman and Akash, 2017). Chronic hyperglycemia is the prime factor of diabetes mellitus complications that modulates metabolism by elevating the production of reactive oxygen species (Rosca et al., 2005). Moreover, it has been reported that Diabetes mellitus is linked to elevation in oxidative stress (Ighodaro, 2018 Prabhakar et al., 2020).

Antioxidant enzymes such as Superoxide dismutase (SOD), Catalase (CAT), Glutathione reductase (GSSG Red), Glutathione Peroxidase (GPx), Glutathione Transferase (GST) and other antioxidant molecules such as Reduced Glutathione (GSH) scavenges the free radicals and provide protection to cells against oxidative damage (Bahadir et al., 2016). The antioxidant can decrease the oxidation rate of other molecules; various studies have indicated that antioxidants have the ability to suppress the complications of diabetes (Ayala et al., 2014 Deepa et al., 2018). Previous works have proved the efficiency of plants in the regulation of oxidative stress related to diabetes mellitus (Taleb et al., 2009). *Coriandrum sativum* (Family Apiaceae) also known as Cilantro is one of the extensively used herbs as traditional medicine for gastrointestinal disorders etc. Several workers have reported that *C.sativum* has antioxidant (Wangensteen et al., 2004), antihyperglycemic (Eidi et al., 2009) and antihyperlipidemic (Chithra and Leelamma, 1999; Sreelatha and Inbavalli, 2012). In the present study, an attempt has been made to further assess the hypoglycemic and antioxidative effect of crude methanolic extract of *Coriandrum sativum* seeds with reference to the kidney in case of *Diabetes mellitus*.

MATERIAL AND METHODS

The Plant part was authenticated by Prof. S. R. Padmadeo, Former Head of Department of Botany, Patna University. The seeds of *C.sativum* were purchased from the local market during the harvesting season of the plant. Seeds of *C. sativum* were carefully washed with distilled water 3-6 times to remove dirt and other contaminating material. The plant materials were shade dried at ambient temperature and pressure until no moisture was left in it. The plant material was converted to fine powder using kitchen grinder followed by sieving with the help of muslin cloth to remove coarse particles. The powdered form of seeds of *C.sativum* respectively was stored in a well-labeled airtight container for further use. The methanolic crude extract of *C.sativum* was prepared using Soxhlet apparatus (Riviera, India). 100 grams of fine powder of plant material was weighed using a digital weighing machine (Wensar, India) and placed in the cellulose thimble using gloves. The thimble was carefully placed in the extraction chamber of the soxhlet apparatus while 500 ml of Methanol (100%) was placed in the boiling flask attached to the heating mantle. The Soxhlet apparatus was run for 48 hours at 60°C to ensure that all phytochemicals in the plant material have dissolved in methanol (Nafisa et al., 2007).

After 48 hrs cycle, the methanolic extract was collected from the Soxhlet apparatus and was further filtered using Whatman filter paper to get rid of any solid particle. The methanolic extract was concentrated by Rotavapour (Popular, India) at 60 °C and reduced pressure to one-twentieth volume (5 ml). it was further lyophilized to get thick greenish brown coloured residue in case of *C. sativum* which were stored in a well-labeled vial at 4°C. Alloxan monohydrate used in this study was a product of Sigma Chemical Company, St Louis, MO, U.S.A. Gluco-one glucometer was a product of Dr.Morepen, Delhi, India. UV-Vis Spectrophotometer (Systronics, India) was used to analyse enzymes and molecules. All other chemicals and assay kits used were products of Sigma-Aldrich Inc. and Merck, Germany, respectively. Healthy Wistar male albino rats (100–150 g) were kept under well-ventilated standard environmental conditions (temperature 25±2 °C, relative humidity 50±5 %) with a 12 h light / dark cycle. Animals were allowed to acclimatize for 7 days before the commencement of the experiment. The experiments were designed and conducted as per the current ethical norms and guidelines approved by the Ministry of Social justices and Environment, Government of India (Nafisa et al., 2007).

The rats were fed on Laboratory prepared pellet having the composition suggested by Subcommittee on Laboratory animal nutrition, National Research Council, USA and water *ad libitum* to ensure proper growth and nourishment. The extra supplement that was given was carrot, sprouted Bengal gram and green gram. Alloxan monohydrate 100 mg/kg body weight dissolved in 0.9% sterilized NaCl solution of pH 7.0 was administered in the tail vein of rats to induce diabetes mellitus. After 48 hours, their fasting blood glucose levels were monitored using a glucometer by collecting blood from the tail artery of animals. Those rats having fasting glucose levels in the range of 250 and 400 mg/dl were considered diabetic and used for the experiment (Nafisa et al., 2007).

The pure breed rats were kept in new polypropylene cages and were categorized into the following groups:-Group I – Normal/Control., Group II – Alloxan treated Diabetic rat, Group III – Crude Methanolic Coriander seed Extract treated ratMethanolic crude *C. sativum* seed extract at a dose of 500mg/kg. body weight was prepared from the stock solution according to the weight of the rats by dissolving in olive oil. Oral administration of the desired herbal extract was made through oral gavages for 10, 20 and 30 days. For the present research work, blood samples were collected by tail clipping for fasting glucose estimation and after an interval of 10, 20, and 30 days rats were sacrificed for organ collection and preservation. For the entire research work, a tissue sample of the kidney for the antioxidant assay of different parameters was kept in Tris-buffer at -20 °C.

The kidney tissue was isolated, washed in 0.2 M Tris buffer solution, blotted dry and weighed. A 10% tissue homogenate was prepared in 0.2 M Tris buffer solution by a motor-driven Teflon pestle glass homogenizer. The tissue homogenate was centrifuged at 10,000 rpm for 20 min, to remove cell debris and then the supernatant was centrifuged at 15,000 rpm for 30 min. The supernatant obtained was used for various assays. The tissues collected at each interval were immediately processed and each tissue sample was analyzed separately (Rotruck et al., 1973).

Superoxide Dismutase (SOD) activity was measured by the method of Marklund and Marklund based on the inhibition of the auto-xidation of pyrogallol (Marklund and Marklund, 1974). Catalase (CAT) activity was determined by measuring the rate of decomposition of H_2O_2 by the method of Claiborne, 1985. The Glutathione Peroxidase (GPx) activity was determined using H_2O_2as a substrate according to the method of (Rotruck et al., 1973). Glutathione Peroxidase enzyme catalyzes the decomposition of H_2O_2 or other peroxides (-OH) with the simultaneous oxidation of GSH into GSSH (Rotruck et al., 1973). The tissue GSH content was estimated by the method of Beutler based on the development of a stable yellow colored complex, with 5,5'-dithio, bis-2, nitrobenzoic acid (DTNB) or Ellman's reagent (Beutler et al., 1967).The activity of GSH-R was measured by the oxidation of NADPH as described by Horn, 1963. The activity of GST was determined using 1-chloro 2,4-dinitrobenzene (CDNB) as substrate (Habig et al., 1974). Data were expressed as the Mean ± SEM. For statistical analysis of the data, group means were compared by one-way analysis of variance (ANOVA) followed by Tukey and Duncan post hoc test for multiple comparisons using Graph Pad Prism 8 software. $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Diabetes is a disease, if not controlled in time can lead to a secondary pathological condition due to a rise in oxidative stress during the progress of the disease. In the present study, an attempt has been made to investigate the ameliorating impact of Coriander seed extract on diabetic nephropathy. Diabetes was induced with the help of alloxan, alloxan caused diabetes by the destruction of pancreatic Beta cells (Jelodar et al., 2007). Alloxan treated rats at a dose of 100 mg/kg body weight caused elevation in blood glucose up to 489% as compared to control leading to loss of weight and lethargic activity.

Nevertheless, when the crude -coriander methanolic extract at a dose of 500 mg/kg body weight were administered to diabetic rat caused a significant decline in blood glucose level up to -74% (Fig.1). Kajal and Singh, (2019) reported similar findings through their work on petroleum ether extract of *C. sativum* seeds (Radenkovic et al., 2016; Kajal and Singh, 2019). The antioxidant effects of crude coriander were studied in terms of antioxidant enzymes like SOD, Catalase, GPx, GST and Glutathione Reductase along with antioxidant molecules like Reduced Glutathione (GSH) (Yasui and Baba, 2006; Kangralkar et al., 2010; Radenkovic et al., 2016).

The Superoxide Dismutase (SOD) belongs to the metalloenzyme group that forms defense against oxygen species (ROS) mediated injury by catalyzing the dismutation of superoxide anion free radical (O_2^-) into molecular oxygen and hydrogen peroxide (H_2O_2) thereby decreasing O_2^- concentration which harm cells (Yasui and Baba, 2006; Kangralkar et al., 2010; Radenkovic et al., 2016). In the diabetic rat group, SOD level considerably decreased (-84%), Nonetheless, Crude methanolic coriander seed extract treatment leads to a significant increase in enzyme activity (+100%) ($p<0.005$) as compared to the diabetic group suggesting a decrease in oxidation stress and ROS level (Landis and Tower, 2005) (Table 1). Catalase and Glutathione Peroxidase are other significant antioxidant enzymes that help to overcome stress by the elimination of H_2O_2 (Bagri et al., 2009).

There was a marked decline in catalase activity up to 98% in the diabetic group as compared to normal, which may be due to inactivation by superoxide radical and Glycation of Enzyme (Bagri et al., 2009). However, on treatment with plant extract for 30 days, catalase activity augmented 15.46 times as compared to the diabetic group showing a recovering trend ($P<0.05$) (Table 2), which may be due to the antioxidant potential of the plant. Similarly, GPx activity decreased substantially by 90% in the diabetic group. Reduced activity of GPx in the Diabetes group may be attributed to free radical induced inactivation and Glycation of the enzyme (Zhang and Tan, 2000; Rajasekaran et al., 2005).

Administration of crude methanolic *C.sativum* extract increased the activities of GPx by (+82%) on day 30 ($p<0.05$) with respect to the diabetic group (Table 3), which is in agreement with the result of (Sreelatha and Inbavalli, 2012). GST is multifactorial enzymes that play a key role in the detoxification of electrophilic metabolites (Hayes et al., 2005). GST activity in alloxan induced diabetes group illustrated a two-fold decrease with respect to normal on day 30. However, on treatment with methanolic crude coriander seed extract, there was an elevation in enzyme activity by 1.42 fold from day 10 to day 30 showing the beneficial effect of the extract (Table 4), which is similar to the reports of Rai et al., 2010.

The decreased activity of GST noted in the diabetic group may be due to deactivation caused by ROS. This suggests that plant extract may assist in neutralizing ROS (Andalu and Vardacharlu, 2003; Sreelatha and Inbavalli, 2012). Glutathione reductase (GSSH red) another key antioxidant enzyme assists in regenerating reduced glutathione (GSH) from the oxidized form of Glutathione that is produced due to oxidation of GSH and therefore ratio of cellular GSH: GSSG is maintained (Dym and Eisenberg, 2001; Taleb et al., 2009; Sato et al., 2011).

Glutathione reductase activity followed the declining trend in the case of the diabetic rat group (-58%) but on treatment with crude seed extract of coriander, there was 43% increase in enzyme activity as compared to the diabetic group on day 30 (P<0.05) (Table 5), which is in consensus with the work of (Taleb et al., 2009). Reduced Glutathione, a tripeptide antioxidant that protects the cellular system from the deleterious effect and scavenging free radicals besides being acting as co-substrate for detoxification by glutathione peroxidases (Anantham et al., 2004; Nain et al., 2012). In the present study, the GSH level was reduced to -24% in the diabetic group as compared to Normal that suggests increased oxidative stress (Table 6). Treatment with crude coriander seed extract leads to 25% increase in GSH level in the diabetic rat (p<0.05) in contrary to the diabetic group without treatment, which is in congruence with the findings of (Ozsoy et al., 2006; Hussien, 2008; Nain et al., 2012).

Figure 1: Effect of methanolic extract of crude coriander seeds on blood glucose

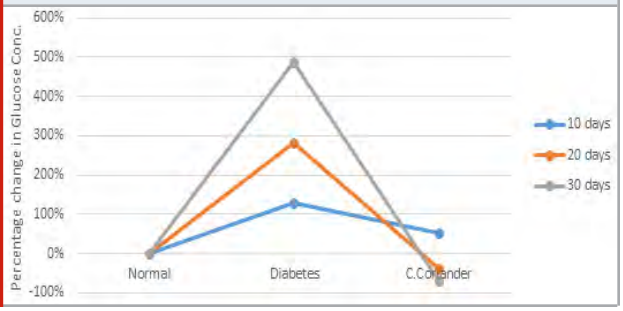

Table 1. Effect of Methanolic extract of crude Coriander seeds on SOD (U/ mg of protein) in Kidney Tissue

Days	Normal	Diabetes	C.Coriander
10 days	172.1567± 0.494821	130.3967± 0.471926*	4.243333± 0.024037*#
20 days	172.1567± 0.494821	97.06333± 0.087432*	7.256667± 0.016667*#
30 days	172.1567± 0.494821	27.59667± 0.800861*	55.30333± 0.104775*#

Values indicate mean ± SEM (n=3)
*p<0.05, compared with normal control values, # p<0.05, compared with Diabetic values

Table 2. Effect of Methanolic extract of crude Coriander seeds on Catalase (U/ mg of protein) in Kidney Tissue

Days	Normal	Diabetes	C.Coriander
10	413.7567± 57.7487	271.85± 1.66872	17.26± 0.355387#
20	413.7567± 57.7487	51.48± 0.931522	26.34667± 0.7349#
30	413.7567± 57.7487	8.703334± 0.275096	266.8567± 0.280496#

Values indicate mean ± SEM (n=3)
*p<0.05, compared with normal control values, # p<0.05, compared with Diabetic values

Table 3. Effect of Methanolic extract of crude Coriander seeds on GPx (U/ mg of protein) in Kidney Tissue

Days	Normal	Diabetes	C.Coriander
10 days	12.28667± 0.01453	4.26± 0.05*	1.233333± 0.082529*#
20 days	12.28667± 0.01453	2.093333± 0.027285*	1.94± 0.04*#
30 days	12.28667± 0.01453	1.23± 0.005773*	2.243333± 0.024037*#

Values indicate mean ± SEM (n=3)
*p<0.05, compared with normal control values, # p<0.05, compared with Diabetic values

Table 4. Effect of Methanolic extract of crude Coriander seeds on GST (U/ mg of protein) in Kidney Tissue

Days	Normal	Diabetes	C.Coriander
10 days	0.612667± 0.00491	0.481± 0.001155*	0.331± 0.001155*#
20 days	0.612667± 0.00491	0.462667± 0.00318*	0.749333± 0.001202*#
30 days	0.612667± 0.00491	0.311± 0.002309*	1.297± 0.006506*#

Values indicate mean ± SEM (n=3)
*p<0.05, compared with normal control values, # p<0.05, compared with Diabetic values

Table 5. Effect of Methanolic extract of crude Coriander seeds on Glutathione Reductase (U/ mg of protein) in Kidney Tissue.

Days	Normal	Diabetes	C.Coriander
10 days	0.774333± 0.001453	0.693± 0.003055*	0.202667± 0.000882*#
20 days	0.774333± 0.001453	0.462± 0.000577*	0.322± 0.000577*#
30 days	0.774333± 0.001453	0.323333± 0.001856*	0.463667± 0.001764*#

Values indicate mean ± SEM (n=3)
*p<0.05, compared with normal control values, # p<0.05, compared with Diabetic values

Table 6. Effect of Methanolic extract of crude Coriander seeds on GSH (U/ ml of sample homogenate) in Kidney Tissue

Days	Normal	Diabetes	C.Coriander
10 days	11.56967± 0.024333	10.21667± 0.006667*	5.241± 0.024*#
20 days	11.56967± 0.024333	9.613± 0.024*	5.966± 0.024*#
30 days	11.56967± 0.024333	8.767667± 0.041858*	10.94167± 0.041858*#

Values indicate mean ± SEM (n=3)
*p<0.05, compared with normal control values, # p<0.05, compared with Diabetic values

CONCLUSION

Diabetes mellitus shows its severity through complications that are caused by oxidative stress generated by ROS due to hyperglycemia leading to diabetic nephropathy. The study reveals that crude methanolic *Coriandrum sativum* seeds are effective in lowering blood glucose level and has the potential to alleviate diabetes mellitus related oxidative stress from organs such as the kidney. Thus, it can be concluded that it has both hypoglycemic and antioxidant potential. However, it needs further investigation to identify active components, as this study was performed in a small population group with limited resources.

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Conflict of Interest: The authors declare that there are no conflicts of interest regarding publication or any other activity related to this article.

Ethical Clearance Statement: The Current Research Work Was Ethically Approved by the Institutional Review Board (IRB) of the experiments were designed and conducted as per the current ethical norms and guidelines approved by the Ministry of Social justices and Environment, Government of India.

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Evaluation of Phytochemical, Antioxidant and Reducing Activity in Whole Plant Extract of Andrographis paniculata

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ABSTRACT

Andrographis paniculata (AP) is an annual herbaceous plant commonly known as Kalmegh, belonging to Acanthaceae family. It has enormous use in research in form of herbal preparations and products and hence its crude extract can be studied further. In the present study, phytochemical screening, antioxidant activity, polyphenolic activity and reducing power of *Andrographis paniculata* plant prepared in different solvents (methanolic, ethanolic and double distilled water) was assessed by different protocols.2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, Hydrogen peroxide (H_2O_2) radical scavenging activity, Polyphenolic contents and Reducing activity of the plant was evaluated by modified method. Phytochemical screening of plant showed the presence of carbohydrate, cardiac glycosides, amino acids, flavonoids, alkaloids, phenols, saponins, steroids and tannins. In DPPH free radical and H_2O_2 radical scavenging activity, methanolic extract of plant were most potent in activity with 50% inhibition at 333.34 µg/ml. and 398.12 µg/ml concentration respectively. Total phenolic (309 ± 0.81 mg/g of gallic acid equivalent) and flavonoid content (82.125 ± 0.85 mg/g of rutin equivalent) were maximum in the methanolic extract of plant. High reducing capacity of plant was observed in case of methanolic extract. A significant positive correlation was found between antioxidant activity and polyphenolic content (total phenols and total flavonoids). Moreover, a significant correlation was found between antioxidant activities and reducing potential of plant extract, depicting that reducers are important contributors to antioxidant. The study shows whole plant extract of *A. paniculata* as an important natural source of antioxidants and phytochemicals. Through this study we could able to determine the results that can act as a milestone supporting future studies in a progressive manner.

KEY WORDS: ANDROGRAPHIS PANICULATA, WHOLE PLANT EXTRACT, ANTIOXIDANT ACTIVITY, POLYPHENOLIC CONTENT, REDUCING POWER.

INTRODUCTION

Medicinal plant is the future of phytomedicines (plant-derived drugs) and serves as a rich source of food, fibres, and drugs. They have been used in folk medicine since ancient times for the prevention and treatment of the numerous diseases as they express a vast array of biological activities. Presently, research is focusing attention on medicinal plants as it is considered as the most sustainable alternative source of antioxidants to supplement the endogenous oxidative stress defense system in humans. Antioxidants obtained from the plants either in the form of crude extracts or their derived products is very effective to inhibit the destructive processes caused by oxidative stress (Zengin et al., 2011; Rahman et al., 2012).

Oxidative stress generates free radicals in form of reactive oxygen species (ROS) in the human body through aerobic

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respiration, ionizing radiation and pollution may increase the risk of chronic and degenerative diseases such as cancer, cardiovascular diseases, ageing and atherosclerosis. The human body generates antioxidant enzymes to neutralize free radicals, a diet rich in edible antioxidants is recommended to assist the human body to protect itself from food borne free radicals (Rimbach et al., 2005; Valko et al., 2007; Taïlé et al., 2020).

Phytochemicals have shown to possess antioxidant properties capable of scavenging free radicals, preventing cellular damages and related diseases via several mechanisms. Hydrogen peroxide (H_2O_2), superoxide ion (O_2^-) and hydroxide radical (OH^-) are considered as most common ROS. Antioxidants are the molecules which stabilize or deactivate free radicals, before they hit targets in living human cells (Nunes et al., 2012). Plants contain a wide variety of free radical scavenging molecules, such as anthocyanins, carotenoids, flavonoids, glutathione, vitamins, and endogenous metabolites (Zheng and Wang, 2001). The concentration of the phenolic compounds like phenolic acids, flavonoids, anthocyanins, and tannins etc. may be related to the antioxidant activity of medicinal plants (Djeridane et al., 2006). Natural antioxidants have gained interest in pharmaceutical research as an alternative for substitution of synthetic substances showing antioxidant activity (Huang et al., 2005). It is mainly because natural antioxidants are cost effective, easily available, non-toxic, eco-friendly, and sometimes more efficient than synthetic ones. Continuous efforts are required to characterize plants phytochemicals for their antioxidant potentials and mode of action for various therapeutic uses against oxidative stress-related diseases.

Andrographis paniculata (Burm. f.) Wall. ex Nees of Acanthaceae family is commonly known as Kalmegh/ King of Bitter. The plant is gregarious and grows abundantly in moist, shady waste area and dry forests. It is extensively cultivated in southern Asia, some parts of Europe and China. Traditionally it is used for treating common cold, bronchitis, diarrhoea, fever, hypertension, liver disease and sinusitis and snake bite (Gabrielian et al., 2002; Premchandran et al., 2011). Major constituents of *A. paniculata* are diterpenoids i.e., andrographolide, 14-deoxy-11,12-didehydroandrographolide, 14-deoxyandrographolide, neoandrographolide, flavonoids and polyphenols reported to possess the most potent hypotensive and vasorelaxing effect (Pholphana et al., 2013). The plant has been reported to exhibit multifarious pharmacological and biological properties like antibacterial, anticancer, antidiabetic, antifungal, anti-inflammatory, anti-HIV and antihepatotoxic.

The plant showed potential therapeutic action in curing liver disorders, common cough, and colds in humans (Nanduri et al., 2004; Akhtar et al., 2006; Geethangili et al., 2008; Mishra et al., 2009; Chandrasekaran et al., 2010; Nagalekshmi et al., 2011; Lim et al., 2012; Sule et al., 2012). The present study was therefore performed to study the antioxidant and polyphenols of whole plant extract of A. paniculata in three different solvents, which may prove to be beneficial against free radical generated disorders. Reducing potential of the plant was evaluated for the first time in methanolic, ethanolic and aqueous extracts derived from the plant.

MATERIAL AND METHODS

The chemicals used are, ascorbic acid, 2,2-Diphenyl,1-picryl hydrazyl (DPPH), gallic acid, rutin, trichloroacetic acid (TCA), potassium ferricyanide ($K_3Fe(CN)_6$), ferric chloride ($FeCl_3$), Folin-Ciocalteu reagent, aluminium chloride ($AlCl_3$), rutin, sodium potassium tartarate (Na-K tartarate), sodium carbonate (Na_2CO_3) was purchased from Hi-Media Ltd and solvent ethanol and methanol used were of analytical grade and purchased from Merck (Darmstadt, Germany).

For the flora collection and preparation of extracts, A. paniculata plant was collected from the campus of Banaras Hindu University, Varanasi. The plant was washed under running tap water to remove the soil and dust particles. The plant was authenticated at Botanical Survey of India (BSI), Allahabad. Collection number BHU-173 and voucher number-91924 was given by BSI to plant flora. Whole plant consisting of (root, stem, leaf, seed, flower) was shade dried for one week and kept in an oven at 40-45°C for 24 h, and then grinded in an electrical grinder to make coarse powder. Extraction was done from 20 g of plant powder in 200 ml of solvent by using a Soxhlet apparatus for 12 h. Methanol, ethanol and double distilled water were used as extraction solvents for extraction purpose. Extracts were then filtered and dried at 40°C in a rotary evaporator. Extracts were stored at 4°C till use. Percentage yield {PY, expressed in (w/w)} of crude plant extract was calculated by given formula: PY=(Wt of crude extract recovered)/ (Wt of powder used).

$$\text{formula: PY} = \frac{\text{Wt of crude extract recovered}}{\text{Wt of powder used}}$$

One gram of each extract was dissolved in 10 ml of respective extraction solvents to obtain a stock solution of concentration 100 mg/ml. Test plant samples were diluted in various concentrations according to the experiments. For the phytochemical screening, testing of the plant for various solvent extract was carried using a standard protocol (Harborne, 1973; Sofowora, 1993).

For the antioxidant assay through DPPH, the free radical scavenging activity of the extracts, based on the scavenging activity of the stable DPPH free radical, was determined by the method given by McCune and Johns, (2008) with some modifications. One ml sample of various concentrations (100-600 µg/ml) of plant extract (PE) was added to 3 ml methanolic solution of DPPH (0.004%) and shaken vigorously. The mixtures were incubated in the dark for 15 min at room temperature. Ascorbic acid was used as standard and methanol served as blank. The solution without sample was served as control. The absorbance of the samples was recorded at 517 nm by using a spectrophotometer (UV1, Thermo Scientific, US).

The experiment was expressed as the percent inhibition of free radicals by the sample and was

$$\text{calculated using the following equation: DPPH activity}(\%) = \frac{(C-S)}{(C)} \times 100$$

(C =Absorbance of control, S = Absorbance of sample)

For the hydrogen peroxide (H_2O_2) scavenging assay, the radical scavenging activity of methanolic, ethanolic and aqueous extracts of the plants to scavenge hydrogen peroxide (H2O2) was evaluated by the method of Ruch et al., (1989) with slight modifications. One ml sample of various concentrations (100-600 µg/ml) of plant extract (PE) were added to 2 ml of H_2O_2 (40 mM) prepared in (50 mM, pH-7.4) phosphate buffer. The test samples were incubated for 10 min at room temperature. The absorbance was measured at 230 nm (Thermo Scientific UV 1). Phosphate buffer without H_2O_2 was used for blank and hydrogen peroxide solution without extract served as control. Ascorbic acid was used as a standard. Hydrogen peroxide scavenging activity was calculated by following.

$$\text{formula:Hydrogen peroxide scavenging activity}(\%) = \frac{(C-T)}{(C)} \times 100$$

Where, C = absorbance of control, T= absorbance of test sample.

For the estimation of total phenolic content, the total phenolic content (TPC) was measured by Folin-Ciocalteu assay (McDonald et al., 2001). In brief, 0.5 ml Folin reagent (1:10 diluted with DDW) was added to 0.5 ml (200 µg ml^{-1}) PE and finally 4 ml (1M) aqueous sodium carbonate (Na_2CO_3) was added to this reaction mixture and incubated for 15 min at room temperature. Absorbance was recorded at 650 nm. Gallic acid was prepared in methanol and DDW (1:1) and used as standard. Total phenolic content was expressed in terms of gallic acid equivalent (GAE, mg/g of dry mass), which is a common reference compound (McDonald et al., 2001).

The total flavonoid content (TFC) was determined using the method of aluminium chloride ($AlCl_3$). The plant extract (1 ml, different concentration) prepared with different solvent (methanol, ethanol and water) was taken in which 100 µl $AlCl_3$ (10% w/v), 100 µl Na-K tartrate and 2.8 ml distilled water were added and kept for 30 min. Finally, the reaction mixture was diluted to 10 ml with double distilled water and the absorbance was measured at 415 nm. The results were expressed as mg rutin (RE)/g plant material (Chang et al., 2002).

For the estimation of reducing power capacity (RPC) of methanolic, ethanolic and aqueous plant extract was estimated by the method of Athukorala et al., (2006) with some modifications. In brief, 1ml of PE (50-300 µg/ml) prepared in different solvents were mixed with 2.5 ml of phosphate buffer solution (PBS, 0.2 M, pH- 6.6) and 2.5 ml potassium ferricyanide (30mM). The above reaction mixture was incubated at 50°C for 20 min. After that, 2.5 ml trichloro acetic acid (TCA, 0.6M) was added to the mixture to stop the reaction and centrifuged at 3000 rpm for 10 min. Then, 2.5 ml of supernatant was taken out and mixed with 2.5 ml double distilled water and 0.5 ml ferric chloride ($FeCl_3$) solution. Absorbance was recorded at 700 nm. Ascorbic acid was used as standard.

For the statistical analysis, all the above experiments were performed in quadriplate (n=4) and repeated thrice (x=3). Data were analyzed as mean ± SE by applying one way analysis of variance (ANOVA). Tukey's multiple range tests were used for separation of means when ANOVA was significant ($p< 0.001$) (SPSS 16.0; Chicago, IL, USA). IC_{50} was calculated through linear regression analysis. The graphs were drawn in sigma plot 11.0.

Table 1. Phytochemical screening of plant in different solvents

Phytochemicals	Test performed	Methanolic extract	Ethanolic extract	Aqueous extract
Carbohydrate	Fehling test	+	+	-
Phenols	Ferric chloride test	+	+	-
Flavonoids	Ammonia test	+	+	-
Alkaloids	Wagner's test	+	+	+
Steroids	Salkowski test	+	+	-
Tannins	Lead acetate test	+	+	-
Saponins	Frothing test	+	+	-
Glycosides	Nitroprusside test	+	-	-
Amino acids	Ninhydrin test	+	+	+

Note: + = Presence; - = Absence of phytochemicals

RESULTS AND DISCUSSION

Percentage yield and phytochemical screening in different solvents: Percentage yield of *A. paniculata* extract was found maximum (22%) in aqueous followed by methanol (18.4%) and ethanol (17.6%) was obtained. The percentage yield of extract differed in various extraction solvents and this may be due to various degrees of solubility of plant materials depending on polarity of solvents. A similar trend was seen in

leaves extract of *A. paniculata* (Banji et al., 2018). Our results highlight that methanolic and ethanolic extracts whole plant were enriched in phytochemicals like alkaloids, amino acids, carbohydrate, flavonoids, phenols, saponins, steroids and tannins while aqueous extract shows presence of alkaloids and amino acids only (Table 1). It may be due to poor solubility of these phytochemicals in the aqueous extract.

Antioxidant activity of plant extract by through DPPH assay: In the present study, the free radical scavenging ability of the crude methanolic, ethanolic and water extracts were determined through the degree of discoloration of the methanol solution of DPPH (Table 2). In *A. paniculata*, methanolic extract showed higher scavenging activity (IC_{50} = 398.31 µg/ml) than ethanolic (IC50 = 404 µg/ml) and aqueous extracts (IC50 = 483.29 µg/ml). The present study reveals that the best antioxidant activity in terms of DPPH scavenging strength was displayed by methanol extract followed by ethanol and aqueous extract. The higher antioxidative capacity of methanolic extract followed by ethanolic extract may be explained via the higher content of biologically active substances, such as e.g., polyphenol (Zwolan et al., 2020).

The antioxidant activity of the extract is first estimated based on their capacity to trap free radical DPPH. In the presence of an active free radical scavenger, the absorption vanishes and the resulting discoloration from deep violet to light yellow. The solution fades colour with increase in concentration of antioxidant as electrons are taken up by DPPH radical from the antioxidant of the extract (Calliste et al., 2001). Ascorbic acid was used as a standard antioxidant as used as a standard to determine the IC_{50} value of the extract in other plants (Sreekala et al., 2013). Ethanolic extract was characterized by higher free radical antioxidant activity than water extract in *Argyreia pierreana, Matelea denticulata* and *Nigella sativa* (Gudise et al., 2019; Zwolan et al., 2020).

Antioxidant activity by Hydrogen peroxide (H_2O_2) scavenging assay: Hydrogen peroxide (H_2O_2) scavenging activity of *A. paniculata* plant was observed higher in methanolic (IC_{50}= 377.074 µg/ml) followed by ethanolic (IC_{50}= 379.06 µg/ml) extract and aqueous extract (IC_{50}= 467.65 µg/ml) (Table-3). H_2O_2 scavenging activity relies upon the phenolic content of the plant extract by donating electrons to H_2O_2, thereby neutralizing it into water. The study suggests that aqueous extract will be required in relatively high concentration to show its effectiveness. The ethanolic extract of the Aesculus hippocastanum was capable of scavenging H_2O_2 in a dose dependent manner (Geetha et al., 2013). H_2O_2 radical scavenging activity was also reported from different extracts of *E. prostrata* (Sinha and Raghuwanshi, 2016a).

Table 2. Antioxidant activity of *A. paniculata* by DPPH free radical scavenging method in different solvents

Concentration (µg/ml)	Percentage inhibition (Mean±SE)			
	Methanolic	Ethanolic	Aqueous	Ascorbic Acid
100	23.89±0.68f	25.77±0.60f	17.07±0.34f	25.12±0.29f
200	30.52±0.63e	35.01±0.23e	24.71±1.9e	39.34±0.20e
300	43.09±0.68d	42.88±0.18d	34.10±0.59d	56.25±0.22d
400	49.92±0.82c	51.58±0.53c	43.69±0.72c	65.15±0.14c
500	59.11±1.04b	58.84±0.12b	51.94±0.51b	86.47±0.38b
600	63.81± 0.49a	63.65±0.19a	60.12±0.80a	95.22±0.32a
IC50	398.31	404.00	483.29	271.47

Data represented as mean ±SE (n=4). One way ANOVA followed by Tukey's test. All data are significant at p<0.001; a, b, c, d, e, f = different letter shows significant difference between means.

Total phenolic and flavonoid content: Total phenolic content was reported as mg/g of GAE in reference to standard curve (y=0.001x+0.05, R^2=0.997). In *A. paniculata* plant, maximum TPC (309.00±0.816 mg/g) was found in methanolic extract followed by ethanolic (290.5±1.29 mg/g) and aqueous extracts (189.25±0.957 mg/g) respectively. Total flavonoid content was calculated by standard curve (y=0.0008x+0.198, R^2=0.994) and reported as mg/g of RE. *A. paniculata* plant showed maximum TFC (82.125±0.853 mg/g) in methanolic extract followed by ethanolic (61.375±1.10 mg/g) and aqueous extracts (37.80±0.731 mg/g) (Table 4). Methanol extract of *A. paniculata* shows important antioxidant activity because it contains phenols and flavonoids (Kurzawa et al., 2014). Similar, higher phenolic content in organic solvent has also been reported (Zaman et al., 2011). Presence of active metabolites like phenol and flavonoid contents in plant extract depend on solvent used (Sulaiman et al., 2011; Kurzawa et al., 2014).

Phenolic compounds present in plant contain an aromatic ring bearing one or more hydroxyl groups. Flavonoids are the largest group of naturally occurring phenolic compounds, which occurs in different plant

parts in form as aglycone and glycosides. It has two benzene rings separated by a propane unit. Their ideal structural chemistry nature helps them to scavenge injurious free radicals such as super oxide and hydroxyl radicals (Younes and Siegers, (1981). Therefore, acting as antioxidants for their scavenging activity (Das and Pereira, 1990) or chelating process, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory actions and giving protection against cardiovascular disease, certain forms of cancer and age-related degeneration of cell components. Flavonoids might show higher antioxidant activity in organic solvent due to structure and substitution pattern of hydroxyl group (Clavin et al., 2007; Kurzawa et al., 2014).

Table 3. Antioxidant activity of *A. paniculata* by H_2O_2 radical scavenging in different solvents

Concentration (µg/ml)	Percentage inhibition (Mean±SE)			
	Methanolic	Ethanolic	Aqueous	Ascorbic Acid
100	21.07±0.79f	27.66±0.50f	20.57±0.70f	25.86±0.38f
200	31.68±0.63e	35.63±0.62e	27.88±0.62e	36.80±0.30e
300	43.17±0.56d	43.68±0.60d	36.00±0.34d	48.33±0.31d
400	51.81±0.83c	51.80±0.38c	44.62±0.49c	57.07±0.29c
500	56.69±0.50b	55.67±1.98b	54.75±0.85b	61.87±0.39b
600	62.87±0.78a	63.01±0.49a	59.62±0.87a	74.16±0.38a
IC50	377.07	379.06	467.65	342.56

Data represented as mean ±SE (n=4). One way ANOVA followed by Tukey's test. All data is significant at p <0.001. a, b, c, d, e, f = different letters show significant difference between means.

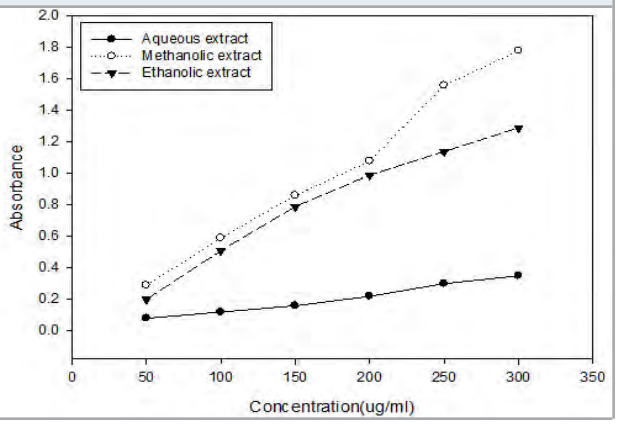
Table 4. Total phenolic and flavonoid content of *A. paniculata* in different solvents

Total polyphenolic content (*A. paniculata*)		
Plant extract	TPC (mg/g GAE)	TFC (mg/g RE)
Methanol	309 ± 0.816a	82.125±0.853a
Ethanol	290.5±1.290b	61.375±1.108b
Aqueous	189.25±0.957c	37.805±0.731c

Data represented as Mean ±SE (n=4); One way ANOVA followed by Tukey's test. All data is significant at p <0.001; a, b, c letters show significant difference between means.

Reducing Potential: The reducing power of the extracts (methanolic, ethanolic and aqueous) of *A. paniculata* (Fig. 1) plant increased in a concentration dependent manner from lower to higher concentrations. Similar results reported by in which the reducing power of *Ziziphus mauritiana* extract increased with the increase of their concentrations. Maximum reducing power was observed in the methanolic extract the plant. In reducing potential assay, after the addition of the extract, the yellow colour of the test solution changes from yellowish green to blue. The colour change of sample solution indicates the reducing power of extract of plants. High absorbance shows high reduction potential of the plant. These reducers show their antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Thus, it is concluded that both polyphenolic compounds and reducers present in the extracts are major determinants of antioxidant capacity of extracts (Abdallah et al., 2016).

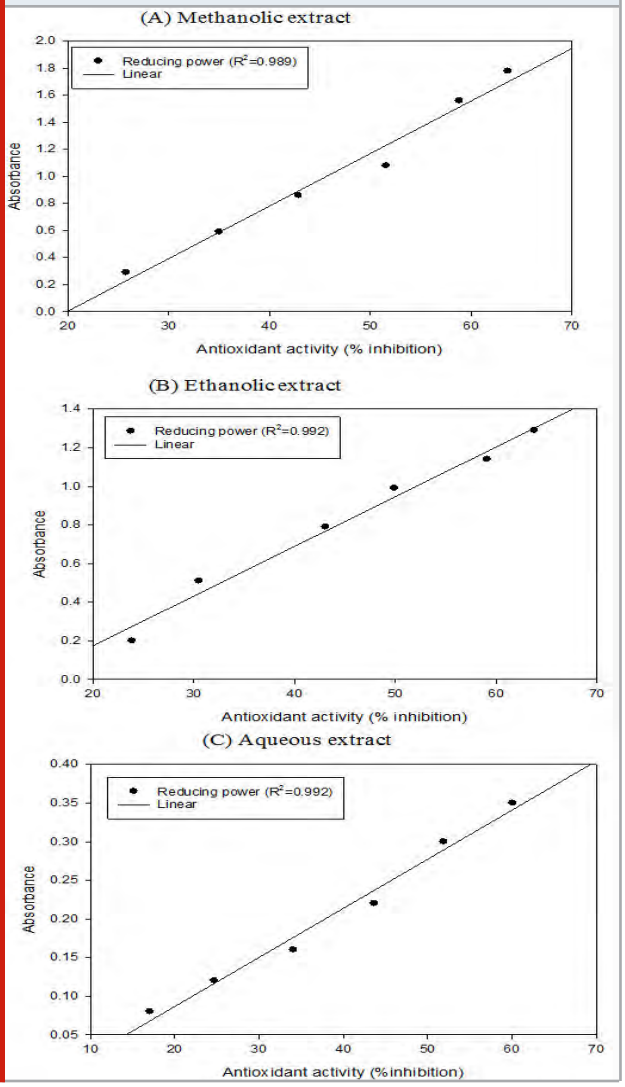
Figure 1: Reducing potential of *A. paniculata* plant extracts in different solvents



Correlation between antioxidant activity and reducing potential of A. paniculata plant: Correlation between total antioxidant activity and reducing power was obtained through linear regression analysis. A significant correlation was found between total antioxidant activities and reducing potential in *A. paniculata* extract (Fig. 2). In, *A. paniculata*, correlation coefficient (R^2) between antioxidant activity and reduction potential was (R^2 =0.989) for methanolic, (R^2 =0.992) for ethanolic and (R^2 =0.992) for aqueous extract. In our result, there is significant positive correlation between antioxidant activity and reducing power of the plant. Koleva et

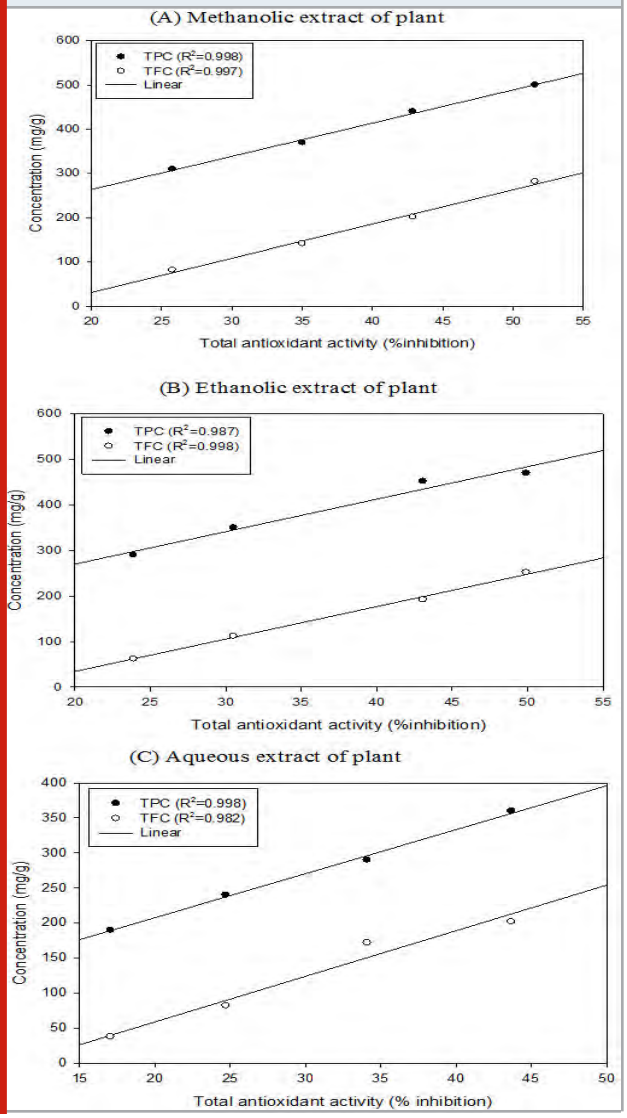
al., (2002) also reported positive correlation between antioxidant activity and reducing potential. Similar studies are seen in *E. prostrata* and *Ocimum americanum* (Sinha and Raghuwanshi, 2016a; Jaiswal et al., 2019).

Figure 2: Correlation between antioxidant and reducing potential in (A) Methanolic, (B) Ethanolic and (C) Aqueous extract of *A. paniculata* plants



(TPC & TFC) of aqueous and methanolic extracts of Chinese medicinal plant and Jordanian plant species are well reported (Cai et al., 2004; Tawaha et al., 2007; Akilandeswari et al., 2020).

Figure 3: Correlation between antioxidant activity and polyphenols (TPC and TFC) (A) Methanolic (B) Ethanolic (C) Aqueous extract of *A. paniculata*



Correlation between antioxidant activity and polyphenolic compounds: A positive, significant, and linear correlation was found between total antioxidant activity and polyphenolic contents (TPC & TFC) of various extracts. Correlation coefficient (R^2) values of different extracts showed a very close correlation between antioxidant activities and polyphenolic contents (TPC and TFC content). Positive and linear correlation (R^2, ranges from 0.982-0.998) was found in *A. paniculata* in the present experiment (Fig. 3). In the present work, we found a strong correlation between antioxidant activity and total phenolic contents (TPC & TFC). High correlation coefficient ($R^2 \geq 0.946$) values showed close correlation between them. Correlation coefficient (R2) between antioxidant activity and polyphenolic contents

Phenolic compounds play an important role as antioxidants and a good correlation exists between the concentration of plant phenolics and the total antioxidant capacity (Sinha and Raghuwanshi, 2016a). The phytochemicals present in the plant and food products are generally nontoxic and contain many medicinal properties. Generally, antioxidants and polyphenolic compounds are mutually related with each other for their activities. *A. paniculata* is a good source of phytochemicals like phenolics, flavonoids, antioxidants, alkaloids, and tannins etc. These phytochemicals play an important role in promoting pharmaceutical drug preparation and are used for curing various health

ailments (Usman and Osuji, 2007; Akilandeswari et al., 2020).

CONCLUSION

Our study reports that the whole plant extract of *A. paniculata* plant is a rich source of natural antioxidants. The antioxidant property, reducing potential and polyphenolic components like total phenols and flavonoids varied significantly in the different extraction solvent. The organic solvent i.e., methanol and ethanol gave better results than aqueous one. This revealed that the whole plant extract contains rich number of antioxidants i.e., phenolic, and flavonoid contents with good free radical scavenging activity. Thus, bioactive compounds present in the extract of this plant may develop into antioxidant agents in the form of plant-based drugs that may have applications in human health in form of food additive or nutraceutical and biopharmaceutical industries.

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In Silico Identification of Protein in Ralstonia solanacearum, A Bacterial Wilt Pathogen for Drug Target by Subtractive Genomic Analysis

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ABSTRACT

Ralstonia solanacearum is a devastating pathogenic soil borne bacterium causing Bacterial Wilt disease in 450 plant species belonging to 54 botanical families and it severely impairs global solanaceous crop production. The loss of crop may go up to 90% depending upon the environmental suitability. The bacterium is very robust and can survive in diverse host plants, soil, water and even in weeds. It possesses an arsenal of secretory molecules like diverse virulent factors, exopolysaccharide, cell wall degrading enzymes to subvert host defense mechanisms. The wilt pathogen is also very efficient to overcome existing control measures rendering it extremely difficult to control. Understanding of molecular mechanism of pathogenesis through genome analysis and identification of novel drug target could be an effective alternative. In this study, subtractive genome analysis of *Ralstonia solanacearum* GM1000 strain having total 5106 proteins obtained from Uniprot database was done and 4972 non paralogous sequence of proteins were selected applying CD-HIT tool. A total of 465 essential proteins were then identified using BLASTp tools of DEG database. Functional pathway assessment of 424 essential proteins revealed 117 metabolically active proteins using KAAS server and a total of 7 non homologous proteins exclusive to the pathogen were identified using BLASTp algorithm. After screening the druggability of 7 proteins in DrugBank Database, 4 proteins were shortlisted and further analyzed for subcellular localization using PSORTb tool. After survey of the existing literature, type II secretory pathway gspe-related protein has been identified and predicted to be the best possible target for drug designing. The present work reports for the first time that type II secretory system could serve as drug target and therefore, opens a new avenue for in silico screening of novel molecules for effective control of bacterial wilt in future.

KEY WORDS: DRUG DESIGN, *RALSTONIA SOLANACEARUM*, SUBTRACTIVE GENOME ANALYSIS, WILT DISEASE.

INTRODUCTION

Soil born bacterium *Ralstonia solanacearum* is the most devastating plant pathogenic bacteria that causes wilt diseases in many wide varieties of plants (Yuliar, Nion, and Toyota, 2015). The strains of this pathogen can infect 450 plant species distributed in 54 botanical families, including potatoes, tomatoes, brinjal, tobacco etc. (Wicker et al., 2007). It invades through the wounded roots or natural opening and colonize in the vascular tissues and release viscous exopolysaccharide that causes obstruction in xylem conduction and lead to fatal wilting disease symptoms in the plants (Schell MA, 2000). Direct yield losses by *R. solanacearum* vary widely according to the host, cultivar, climate, soil type, cropping pattern.

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It has been reported that it accounts for 80% loss in tobacco, 100% in banana, and up to 20% in the groundnut (Elphinstone, 2005; Somani et al., 2010). *Ralstonia* infection causes more than 50% crop loss in India and that may reach up to 75% in some parts of Karnataka (Gadewar et al., 1991). Bacterial wilt disease affects potato cultivation in different parts of India and accounts for 30 to 70 % crop loss in these areas (Somani et al., 2010). The control of bacterial wilt pathogen is very challenging. Difficulties are associated with controlling this pathogen due to its abilities to grow endophytically, long survival in the soil especially in the deeper layers, travel along water, and its relationship with weeds (Wang et al., 2005; Mansfield et al., 2012; Santana et al., 2020; Yan and Gao, 2020).

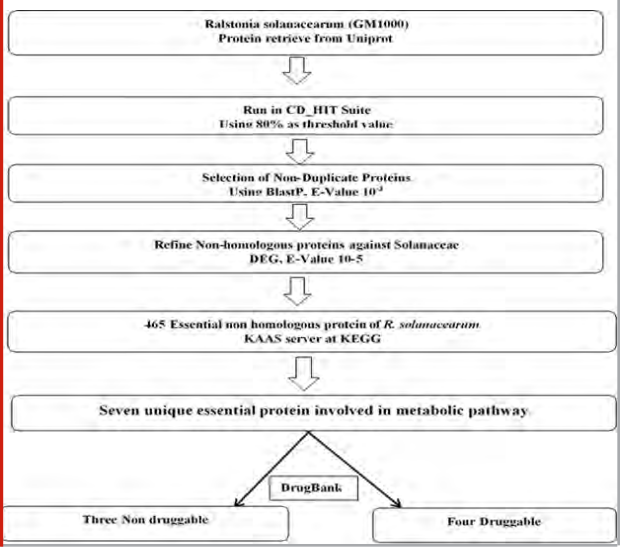
The bacterial pathogen often undergoes VBNC (Viable but not culturable) state under unfavorable condition (Van Elsas et al., 2001). Furthermore, many environmental stresses weaken the defense systems of the plants allowing to proliferate *Ralstonia* and other bacterial endophytes inside the host. Conventional disease management practice such as preventive measures, cultural practices are inefficient to pre-existing infection and because of the pathogen's diverse host range and persistence in the weeds and soil (Mbaka et al., 2013). Chemical pesticides such as algicide (3-[3-indolyl] butanoic acid), fumigants (Metam sodium, 1, 3-dichloropropene, and chloropicrin), and plant activators (validamycin A and validoxylamine) inducing systemic resistance in the tomato have been used to control bacterial wilt but with limited success (Ishikawa et al., 2007; Yuliar et al., 2015; Coutinho et al., 2017).

Copper compounds (copper hydroxide (CH), copper hydroxide-oxadixyl, and copper oxychloride-dithianon), and essential oils (Cinnamon oil, Clove oil) have been partially effective to control bacterial wilt. (Elphinstone, 2005; Lee et al., 2012). Many bactericides such as triazolothiadiazine [0.5 to 12 mM, in solution), streptomycin sulfate [400 mg kg–1 of soil] have been employed to control bacterial wilt pathogen with average rate of success (Khanum et al., 2005; Lin et al., 2010). Additionally, emergence antibiotic resistance and environmental pollution due to long-term use of chemical pesticides rendered bacterial wilt disease management very difficult. Although, there are many studies have been done employing biocontrol strategy to control bacterial wilt but of limited success due to inefficient colonization, narrow range and requirement of high inoculum of biocontrol agents. Therefore, identification of novel pathogenic target protein and discovery of its corresponding drug could be an attractive alternative for controlling bacterial wilt disease (Whipps and Gerhardson, 2007; Coutinho et al., 2017).

Rapid advancement in the field of biotechnology enabled us to have vast genomic data from the prokaryotic whole genome projects that in turn may be exploited for finding novel drug targets and virulent factors in microbes. With the availability of whole genome sequence, subtractive genome analysis has been evolved as a very efficient tool to identify novel drug targets and virulent factors in pathogenic microbes (Miesel et al., 2003; Amineni et al., 2010; Keshri et al., 2014). Subtractive genome analysis is a smart technique to identify essential metabolic gene present exclusively in the pathogen having no homologue in the host and therefore, the targeted drug developed against the pathogenic essential metabolic gene will impair only the metabolic function of the pathogen leaving the host metabolism undisturbed (Vetrivel et al., 2011; Barh et al., 2011). Many possible drug targets have been identified in human pathogenic bacteria (Barh et al., 2011; Sudha et al., 2019; Santana et al., 2020; Yan and Gao, 2020).

However, there are very few reports regarding drug target identification in plant pathogenic bacteria using in silico techniques (Allen et al., 2009; Silver, 2011). Subtractive hybridization technique has been exploited to underpin drug targets in rice bacterial pathogen, *Xanthomonas* by some researchers (Keshri et al., 2014; Prava et al., 2019). Although, the complete genome sequence of *Ralstonia solanacearum* is available in the database, but there is no report available so far that have tried subtractive genome analysis to identify drug targets in this bacterium. Therefore, the present work is attempted to identify possible drug targets in *Ralstonia solanacearum* through subtractive genome analysis and other *in silico* analysis tools (Prava et al., 2019).

Figure 1: The conceptual framework showing the methodology followed for the analysis



MATERIAL AND METHODS

Subtractive genomic approach was applied for the identification of essential proteins in the *Ralstonia solanacearum* (GM1000) which were then analyzed for the identification potential drug targets. The identified drug target was then screened through DrugBank database to evaluate druggability scope. Network based analysis was done for the identification of metabolic activity of target protein (Yu et al., 2010).

The complete proteome of *Ralstonia solanacearum* GM1000 strain was retrieved from UniProt (http://www.uniprot.org). The UniProt Knowledgebase is the central hub for the collection of functional information on proteins, with accurate, consistent and rich annotation (The UniProt Consortium, 2019). Identification of nonhomologous protein and essential gene of *Ralstonia solanacearum* - Paralogous sequences were excluded from the complete proteome of *Ralstonia solanacearum* GM1000 strain by using CD-HIT at 80% threshold. BLASTp was performed for the remaining proteins against Solanaceae using threshold expectation value (E Value) 10^{-3} as parameter. Non homologous protein sequences were then subjected to BLASTp against the database of essential genes (DEG) assessed at DEG database (http://tubic.tju.edu.cn/deg/) using E-Value cut off of 10^{-5}, to screen out essential gene proteins (Li et al., 2001).

Table 1. Unique metabolic pathway essential proteins

Sl no	DEG ID	UNIPROT ID/ DRUGGABILITY	METABOLIC PATHWAY
1	DEG10570448	Q8XW91 Druggable	QUORUM SENSING
2.	DEG10570275	Q8XX10 Druggable	BACTERIAL SECRETION SYSTEM
3.	DEG10570247	Q8Y3B8 Druggable	PEPTITOGLYCAN BIOSYNTHESIS BETA LACTAM RESISTANCE
4.	DEG10570255	Q8XVI1 Druggable	BETA LACTAM RESISTANCE PEPTIDOGLYCAN BIOSYNTHESIS
5.	DEG10570232	Q8XQ85 Not Druggable	BACTERIAL CHEMOTAXIS
6.	DEG10570446	Q8XVG2 Not Druggable	QUORUM SENSING
7.	DEG10570220	Q8XX15 Not Druggable	BACTERIAL SECRETION SYSTEM

Sub Cellular Localization

Name of Protein	Uniprot ID	Location
Probable conjugal transfer protein trbb	Q8XW91	cytoplasmic
Probable type II secretory pathway gspe-related protein (RSc2308)	Q8XX10	cytoplasmic
Peptidoglycan D, D-transpeptidase MrdA	Q8Y3B8	cytoplasmic
Peptidoglycan D, D-transpeptidase FtsI	Q8XVI1	Cytoplasmic Membrane

KEGG Automatic annotation Server (KAAS) was accessed to analyze the metabolic pathway of the essential proteins of *Ralstonia solanacearum* GM1000 strain for the identification of potential drug target. The server performs BLASTp comparisons of the query protein against Kyoto Encyclopedia of Genes and Genomes (KEGG) Genes Database (Moriya et al., 2007). Sub Cellular localization of non-homologous essential proteins of bacteria illustrates their potential of becoming the possible drug targets. Therefore PSORTb tools at Expasy server was utilized to identify the subcellular localization of non-homologous essential protein sequences (Yu et al., 2010). The modulation of the activity of a protein target with a small molecule of a drug accounts for its prospective druggability. DrugBank Database was accessed to calculate the druggability potential of each identified drug target (Knox et al., 2011). BLASTp with default parameters was used to align the potential drug targets from *Ralstonia solanacearum* against the list of the of compounds found within the DrugBank (Szklarczyk et al., 2019).

Selected indispensable proteins were then subjected to STRING database (http://string.embl.de) to construct protein-protein interaction network (Li, Jaroszewski and Godzik, 2001). Interactors with confidence score greater than or equal to 0.700 alone included here in the protein network and with low and medium confidence score were eliminated to avoid false positive and false negative results. Target protein with more interactors is considered as a metabolically active protein which could serve as appropriate Drug target (Peyraud et al., 2017; Szklarczyk et al., 2019).

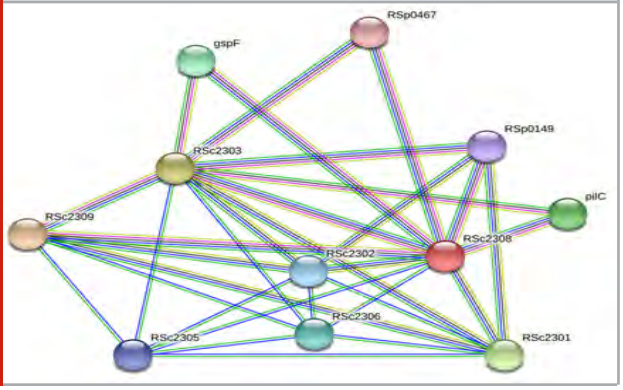
RESULTS AND DISCUSSION

The main goal of the subtractive genomic analysis was to examine *Rolastonia solanacearum* GM1000 strain critical proteins as a possible drug target for future strategic drug discovery. Total 5106 proteins of total proteome were originally obtained from Ralstonia solanacearum GM1000 Uniprot database. The CD-HIT tool was used to differentiate paralogous and non-paralogous proteins. 134 paralogous proteins were screened and 4972 non paralogous sequence of proteins were selected for further analysis.

Table 2. Non homologous essential protein of *Ralstonia solanacearum* strain similar to binding pattern of FDA approved drugs against DrugBank database using BLASTp

Sl. no	Protein name	DrugBank ID	Uniprot ID
1.	Conjugal transfer protein trbb	DB02930 DB04395	Q8XW91
2.	Type II secretory pathway gspe-related protein (RSc2308)	DB04395 DB02930	Q8XX10
3.	Peptidoglycan D, D-transpeptidase MrdA	DB01413, DB00438, DB14879, DB01598, DB01329, DB01327, DB01163, DB01163, DB01328, DB01413, DB01415, DB00948, DB00438, DB00303, DB00671, DB01326, DB00923DB00355, DB00493, DB04570, DB01413, DB01147, DB09050, DB06211, DB14879 DB04918DB00274, DB00430, DB01607, DB01000 DB02443, DB02968, DB04041, DB01603, DB00417	Q8Y3B8
4.	Peptidoglycan D, D-transpeptidase FtsI	DB06211, DB14879DB04918, DB00267, DB01413, DB01147, DB09050, DB01416, DB01329, DB01327, DB01331, DB01328, DB01413, DB01415, DB00430DB05659 DB00535, DB04918, DB01150DB03190	Q8XVI1

Figure 2: Interaction among Type II secretory pathway gspe-related protein (RSc2308) and other proteins of *R. solanacearum*.



The selected proteins were assessed against Solanaceae proteome in BLASTp, with an E-value cut off 10^{-3}. Selected non homologous proteins were employed for the identification of essential gene using BLASTp tools of DEG database at default parameter settings. The analysis identified 465 essential proteins. There are 41 hypothetical proteins were identified which were finally excluded in this study. The essential proteins of bacteria are expected to be involved in housekeeping and are important for the survival of pathogen.

Total no of protein	5106
Duplicate (>80% identical) in CD-HIT	134
Essential proteins in DEG (E-value 10-5)	465
Number of hypothetical proteins as essential proteins	41
Essential proteins involved in metabolic pathway	117
Unique metabolic pathway essential proteins	7
Essential proteins found to be druggable	4

Functional pathway assessment of 424 essential proteins were conducted using KAAS server. Among 424 proteins 117 proteins were found to be involved in different metabolic pathway of the pathogen. These 117 proteins were further analyzed by the BLASTp algorithm for the comparison of metabolic pathway in *Ralstonia solanacearum* proteome and *Solanum tuberosum* proteome as a reference organism of Solanaceae family to exclude the common pathway. Total seven pathogen specific pathways of *Ralstonia solanacearum* GM 1000 were identified by KEGG which were absent in Solanaceae family.

Table 3. Interaction among Type II secretory pathway gspe-related protein (RSc2308) and other proteins of *R. solanacearum* and their combined score.

node1	node2	node1 string id	node2 string id	combined_score
RSc2300	RSc2308	267608.RSc2300	267608.RSc2308	0.762
RSc2301	RSc2308	267608.RSc2301	267608.RSc2308	0.922
RSc2302	RSc2308	267608.RSc2302	267608.RSc2308	0.886
RSc2303	RSc2308	267608.RSc2303	267608.RSc2308	0.955
RSc2304	RSc2308	267608.RSc2304	267608.RSc2308	0.867
RSc2305	RSc2308	267608.RSc2305	267608.RSc2308	0.884
RSc2306	RSc2308	267608.RSc2306	267608.RSc2308	0.887
RSc2307	RSc2308	267608.RSc2307	267608.RSc2308	0.845
RSc2309	RSc2308	267608.RSc2309	267608.RSc2308	0.981
RSc2310	RSc2308	267608.RSc2310	267608.RSc2308	0.869
RSp0143	RSc2308	267608.RSp0143	267608.RSc2308	0.772
RSp0149	RSc2308	267608.RSp0149	267608.RSc2308	0.884
RSp0467	RSc2308	267608.RSp0467	267608.RSc2308	0.882
RSp0474	RSc2308	267608.RSp0474	267608.RSc2308	0.715
gspD	RSc2308	267608.RSc3114	267608.RSc2308	0.756
gspF	RSc2308	267608.RSc3116	267608.RSc2308	0.895
pilC	RSc2308	267608.RSc2826	267608.RSc2308	0.896
pilD	RSc2308	267608.RSc2827	267608.RSc2308	0.790

Total seven nonhomologous proteins were identified that are thought to be essential and involved in pathogens unique metabolic pathway. Therefore, new drugs may be designed to target these essential proteins to inhibit one or more of these metabolic pathways thereby controlling the growth and viability of the pathogenic strain *Ralstonia solanacearum* omit GM 1000. The total seven non homolog essential proteins (Table1) so obtained were verified within DrugBank Database for possible druggability and four essential non homologous proteins (Table 2) were identified to have druggability potential. Thereafter, the four selected proteins were then subjected to PSORTb for their sub cellular localization.

Earlier, 20 proteins of *Ralstonia solanacearum* were targeted for drug design having Protein Data Bank (PDB) ID of 3ZI8, 4I68, 4KF9, 4FDB, 3UMB, 3TMB, 3TOT, 3TOU, 3NPN, 3NPQ, 3LOP, 3GG9, 3GHY, 3EN2, 2QGU, 2CHH, 2BT9, 2BS5, 2BS6, 1UQX. (Kotaki and Saikia, 2015). Peptidoglycan D, D-transpeptidase MrdA, Peptidoglycan D, D-transpeptidase FtsI, Type II secretory pathway gspe-related protein were identified as the best predicted protein for drug target in this study. Type II secretion system is a virulent factor of *R. solanacearum* (Peeters et al., 2013). Inhibition of Quarum sensing protein can only prevent biofilm formation of pathogenic bacteria without any apparent direct effect on survivability. However, Peptidoglycan D, D-transpeptidase MrdA, Peptidoglycan D, D-transpeptidase FtsI protein as drug targets have already been reported and efforts have been taken for drug design in many human pathogenic bacterial strains, but these drug targets are inapplicable for *Ralstonia solanacearum* strains as β lactam antibiotics are less effective in controlling bacterial wilt disease (Souvage and Terrak, 2016; Waack et al., 2017).

Different secretion systems of bacteria are very attractive targets for alternative therapeutics because their inactivation interferes with the delivery of secreted virulence factors. There are many cell walls degrading enzymes are secreted through Type II secretory system (T2SS) in *Ralstonia solanacearum*. Therefore, inhibitor of Type II secretory system (T2SS) could be a good alternative for drug design. Rsc2308 (UniProtKB ID-Q8XX10) is the Type II secretory pathway gspe-related protein of *Ralstonia solanacearum* associated with secretory system of bacteria which is responsible for pathogenicity. Therefore. Type II secretory pathway gspe-related protein (RSc2308) of *Ralstonia solanacearum* could be a promising drug target for future drug design that has not been properly addressed so far. Network based analysis showed that this protein Rsc3208 is interconnected with eighteen proteins in network with combined score greater than 0.7 (Table3) (Salanoubat et al., 2001; Waack et al., 2017).

So, it may be assumed that this Type II secretory pathway gspe-related protein is a highly metabolically active protein and inhibition of this protein may arrest the growth of the bacteria. Therefore, the present work opens a new avenue for searching novel drug compounds that may interact with the target Type II secretory pathway gspe-related protein (RSc2308) and may pave the path for new control strategy (Souvage and Terrak, 2016).

CONCLUSION

Subtractive genome analysis revealed possible drug targets in many human pathogenic bacteria and only few in plant pathogenic bacteria. *In silico* identification of possible drug target in *Ralstonia solanacearum* is completely lacking. Therefore, the present work probably is the first report underpinning the druggability of type II secretory pathway gspe-related protein of *Ralstonia solanacearum* through subtractive genome analysis. The gspe-related protein is essential in type-2 secretion pathway for secreting cell wall degrading enzymes that are key to host penetration and colonization. Therefore, targeting the protein with new drugs may prevent host colonization and survival in the weeds thereby offering a good strategy for controlling the pathogen in future.

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Developing Social Communication Skills in Saudi Arabian Female University Students using Forum Software for Collaborative E-Learning

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ABSTRACT

Many female students use social communications as a part of their *digital literacy*. There is no doubt that social communication skills, along with mental abilities – represent efficiency and effectiveness among university youth. Any defect in these communication skills may lead to an inability to adapt to the university environment. Consequently, students may lose many opportunities, and suffer academic progress. The aim of this study was to investigate the effectiveness of *collaborative E- learning* in developing social communication skills in the "Research Seminars " course among students of the seventh level of the College of Education at King Khalid University -Abha city - Saudi Arabia. The study followed the semi-experimental approach, which is the design of pre-measurement and post-measurement for two groups: the experimental group and the control group. The experimental group uses the *collaborative learning* method of network through the course forums to activate social communication with the pre and post application of the search tool on the two research groups. The researchers applied the tool (social communication scale) on a sample consisting of (25) students from the College of Education. The result confirmed the effectiveness of *collaborative E-learning* in developing the social communication skills of the experimental group. The study recommends, based on its results, to take advantage of the *collaborative -networked learning* method to develop innovative thinking skills among university students. as well, it is necessary to hold training courses for university faculty members to develop their skills in the use of online *collaborative learning* tools such as blogs and discussion forums in the educational process. Moreover, there is a necessity to educate faculty members about the importance of *collaborative -networked learning* method in developing social communication among university students.

KEY WORDS: EFFECTIVENESS - COLLABORATIVE -E- LEARNING - SOCIAL COMMUNICATION SKILLS.

INTRODUCTION

The E-learning has become an entry and starting point for the strategic development of the educational process and educational institutions. A new concept emerged based on scientific and theoretical foundations related to e-learning such as virtualization, networking. They used various web technologies and tools to provide a *collaborative electronic learning* environment of a social nature that increases the effectiveness of social communication between teachers and students. Social media have become an indispensable necessity in our life and in addition to meeting the need for communication, some students have begun social media intensive use in order to meet their psychological, social and academic needs, (Ozad & Uygarer 2014., Pokrovskaia, Leontyeva, Ababkova, Cappelli, D'Ascenzo, 2021).

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E-learning is applied to a group of learners in a collective collaborative manner called the " *collaborative E-learning* style". These collaborative *E-learning environments* contributed to improving individuals 'awareness of their self-efficacy and their research skills within the framework of effective social interaction and communication with others. Social media has changed the learning path in higher education, as many students reported that social media is useful and beneficial in their studies and motivates them to cooperate and interact with others through these sites, (Zaitoun.2005, Arshad, Akram, Arshad & Nazir 2014, Lacka ,Wong, Haddoud.2021).

Downes (2012) also showed that the social and participatory feature is the hallmark of collaborative e- learning based on Web 2 tools. In the past few years, there has been an increase in the use of computer networks in education and training, but despite this, the *e-learning* or virtual learning environments do not include any systematic collaborative. However, the ideas of computer-aided collaborative learning have been increasingly applied in different methods of Internet-enabled learning. They usually include capabilities for documents sharing and a variety of specific tools for communication and networking via the network where synchronous communication tools such as chatting, dialogue, voicemail and video conferencing can be used. Asynchronous communication tools such as discussion forums also indicate that the use of collaborative learning through the Internet and computers will further enhance and expand methods of interaction and communication between students and teachers, leading to the development of educational practices and support for students at the level of learning and interaction, (Hamada and Ismail 2014) (Quintana and Osuna 2020).

In light of the above, the researcher considered the necessity to encourage interaction between students in the networked collaborative learning groups, and to provide appropriate support and conditions that help them increase effective social communication. This is the researcher's starting point in studying the effectiveness of *collaborative networking* in developing social communication among students of the College of Education for Girls at King Khalid University Abha KSA.

MATERIAL AND METHODS

Subject and study design: The purpose of this study was to answer these questions regarding *E-Learning* and *collaborative digital communications*. All participants were given a survey (document included) to complete prior to attending the Research Course weekly meeting which was conducted for a period of 16 weeks (academic semester), and all completed an exit survey at the end of the term. The study community consisted of female students from the College of Education at King Khalid University. The research sample consisted of (50) undergraduate female students in the, Research Seminars course at the College of Education in Abha at King Khalid University. The participants were divided into two groups of (25) each. The experimental group received 2 hours of weekly online instruction via Blackboard Forum Software. The control group received 2 hours of weekly traditional classroom instruction.

Collaborative e-learning does not achieve the effectiveness of interaction by simply placing students in groups and assigning them to learning tasks, as it is required to study the variables related to the environment of interaction and collaborative learning itself, which are related to strategies, tools, levels and types of interaction, to reach the best conditions under which interaction and collaborative learning achieve its maximum effectiveness. Through her experience in academic education, the researcher found that there is an urgent need to identify the effectiveness of e-learning in its part on network sharing through its effect on developing the social communication skills of Education College students at King Khalid University via the Internet in the blackboard system with e-learning.

Therefore, this study seeks to answer the question that states, "What is the effectiveness of *online collaborative learning* in developing social communication skills among students of the College of Education at King Khalid University?" To answer this essential question a test will be conducted on the validity of the two following hypotheses the first of which states that "There is a statistically significant difference between the pre and post measurement of the experimental group on the social media scale in favor of the post measurement". While the second hypothesis states,

"There is a statistically significant difference between the experimental and control groups on the social communication scale (in the course forums after using the *collaborative learning* method in the network) in the post-measurement for the benefit of the experimental group." The quasi-experimental approach was used, which is the design of the pre- and post-measurement for two groups, the experimental group, and the control group. The experimental group uses the collaborative network learning method through the course forums to activate social communication with the pre and post application of the two research tools on the two research groups.

RESULTS AND DISCUSSION

The present study intended to determine the effectiveness of *collaborative E- learning* in developing social communication skills The arithmetic average scores of the experimental group in the pre and post measurements were compared on this scale as an overall score. The following table illustrates the findings related to this assumption:

It is evident from the data in Table (1) that there is a statistically significant difference between the pre and post measurement of the experimental group on the social media scale, where the value of "T" was (25.82) at a level

of significance (0.01), in favor of the post measurement. This result can be interpreted as that the improvement and change of the members of the experimental group for the better is attributed to the use of the *collaborative learning* method in the network through scientific discussion forums in the course on the blackboard system. It contributed to supporting communication and interaction among them and to developing the spirit of cooperation, and thus supporting positive trends towards *collaborative learning* environments via the Internet in raising their level of social interaction.

Table 1. Shows the significance of the difference between the pre and post measurement of the experimental group on the social communication scale

Comparison group	Number	Average	Standard deviation	T value	Significance level
Pre	25	26.84	4.20	25.82	significant at 0.01
Post	25	52.72	3.63		

Table 2. illustrates the significance of the difference between the experimental and control groups on the social media scale after completing the networked participatory learning method

Comparison group	Number	Average	Standard deviation	T value	Significance level
Control	25	26.68	6.66	17.167	significant at 0.01
Experimental	25	52.72	3.63		

In fact, the students of the experimental group found in the forums a fertile *electronic learning* environment that helped them to deal with each other in the course of the "Research Seminar". This increased their interaction and social communication in a positive way, which indicates the success of these educational forums in achieving their educational goals, and confirms the effectiveness of network *collaborative learning* in developing the social communication skills of the students of the experimental group in the course of the Research Seminars ". This result is consistent with the studies of (Kabuli, 2013), (Ali, 2016), (Harb, ; Khamis,; Abu Jahjouh . 2013), and (Al-Dukhani, ; Faraj, Khamis,. 2015), (Al-Muaither and Abdullah 2020) in that the use of collaborative networking led to social communication in a large and effective manner among the members of the research sample.

It is evident from the data of Table (2) that there is a statistically significant difference between the two groups of experimental and control research on the scale of social communication after the completion of the use of the collaborative learning method in the network in the total score where the value of "T" is (017.167) in favor of the experimental group. This indicate the students of the experimental group, as indicated by the statistical evidence shown in Table (2), have benefited from the use of the collaborative online learning method in communication and social interaction in the course of the " Research Seminars ", which confirms the effectiveness of *collaborative online learning* in developing social communication skills in the course of the " Research Seminar " Seventh-level students at the College of Education at King Khalid University.

This result is in consistent with the findings of the study of Ali (2016), aimed at measuring the effect of some social *e-learning* environments based on social media platforms on developing educational electronic communication skills among students of the College of Education at Al-Baha University. there were statistically significant differences between the arithmetic average scores of the three research groups in the post application of the observation card in favor of the two experimental groups.

The study of Alsurehi & Youbi (2014) sought identifying and studying the factors that influence students' academic performance using SNSs. Suggested factors affecting student performance are interactions with colleagues, interactions with teachers, participation, and cooperative learning. The primary research goal of this case study is to determine the factors that affect students' academic performance while using SNSs. The results indicate that *SNSs* has a significant positive effect on interactions with colleagues, interactions with teachers, participation, *collaborative learning*, and student performance. In addition, interactions with peers and educators while using SNSs simplify communication between students and educators, which leads to enhanced collaboration, knowledge exchange, improvement, and development of the learning process, and provides many learning opportunities.

Abu Jahjouh's study (2020) investigated the impact of the *e-social learning* strategy on the development of scientific achievement, the development of the skill of scientific communication, the development of positive

trends towards learning based on social media, and the detection of the relationship between the three dependent variables. There is no statistically significant difference between the arithmetic average scores of trends and scientific communication due to the GPA variable.

Al-Muaither (2020) has identified the impact of the Edmodo social learning network environment on developing cognitive achievement and dialogue skills among the students of the College of Education in a course based on s*ocial constructive learning* in projects. The results revealed an impact of the e-learning environment integrated through the Edmodo network on developing dialogue and communication among students. which proved that social communication among students leads to enhancing their learning.

CONCLUSION

In this study, there was an effectiveness of using the *collaborative E-learning* in developing the social communication skills of the experimental group compared to the control group, which led to enhancing their learning of the subject Moreover, there is a statistically significant relationship between using the collaborative -E learning and developing the social communication skills. Therefore, it is important to encourage university students to have self-confidence in their abilities and capabilities to implement scientific research steps with skill and accuracy, as well as supporting the method of *collaborative E- learning* in teaching courses to achieve high quality learning outcomes.

Conflict of Interest: Author declares no conflicts of interests to disclose.

Ethical Clearance Statement: The Current Research Work Was Ethically Approved by the Institutional Review Board (IRB) of King Khalid University, Abha, Saudi Arabia.

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Toxicological Communication

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Sublethal Toxic Effects of Herbicides on a Non-Target Organism, Archachatina marginata

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ABSTRACT

Indiscriminate anthropogenic application and release of herbicides can induce oxidative stress to the detriment of living organisms. In this study, the deleterious effects of three commonly used herbicides (Paraeforce®, Cotrazine® and Force Uron®) in the Niger Delta area on a non-target environmental receptor - *Archachatina marginata* was assessed using biochemical indicators - malondialdehyde, superoxide dismutase and catalase. The results of sublethal exposure indicated that the values of the oxidative product, MDA in the tissues of the organisms in the treatment groups were higher (1.19 ± 0.10 to 3.80 ± 0.91 mmol/MDA) than the control (0.28 ± 0.03 mmol/MDA). The sublethal treatments were also associated with inhibition of SOD activities (24.64 ± 2.01 to 50.87 ± 6.83 unit/mg protein) relative to the control (57.02 ± 6.96 unit/mg protein). The CAT levels in the exposed species (12.84 ± 0.33 to 21.45 ± 2.46 unit/mg protein) were lower that the controls (22.78 ± 2.13 unit/mg protein). The alterations in biochemical indices indicated toxicity at sublethal levels below and at the safe limit (10% of EC50), which could result in likely environmental consequences on these non-target species highly consumed by human.

KEY WORDS: HERBICIDES; NON-TARGET ORGANISMS; OXIDATIVE STRESS; SUBLETHAL EFFECTS.

INTRODUCTION

In the last few decades, there has been an increase in the application of a myriad of herbicides to control weed and pests ravaging farmland and stored food crops. Similarly, due to high resistance of these herbicides in completely eliminating destructive weeds and pests, more enhanced products and formulations emerges yearly without recourse to testing the deleterious consequence these herbicides would impact on target, non-target species and humans. In the same vein, a lot of these chemicals (herbicides), have not been evaluated for their ecological impact on environmental receptors since some herbicides could accumulate to toxic levels in soils and become harmful to soil dwelling organisms, plants, wildlife and subsequently man, who consumes products from such soils (Micuti et al., 2018).Exposure of living species to pesticides (herbicides) and industrial chemicals have been identified amongst the major factor that can induce oxidative stress, which is an imbalance between oxidant and anti-oxidant mechanisms (responses) (Gurvinder, 2019).

Wang et al., (2018) noted the toxic manifestation induced by atrazine, mesotrione, and joint activity of herbicides on pigment, oxidative and antioxidant enzyme activities

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in their study. The effects of herbicides, which may be associated with enhanced generation of reactive oxygen species (ROS), may lead to oxidative stress in vulnerable living receptors. It has been reported that ROS production impairs tissue and cell functioning by inducing peroxidation, protein damage and DNA breakage (Valko et al., 2007).Malondialdehyde (MDA) values can be used to evaluate lipid peroxidation, which is referred to as the oxidative degradation of lipids. It is the process in which free radicals capture electrons from the lipids in cell membranes, thus resulting in cell damage (Gueraud, et al., 2010).

The main system of defense against damage from free radicals is the enzymatic system that opposes oxidation, which includes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). They are major anti-oxidant enzymes that prevent oxidative stress damage to tissues.Some commonly used herbicides in the Niger Delta area of Nigeria include the three evaluated in this study - paraquat, atrazine and diuron. Paraquat dichloride (1,1-dimethyl-4,4- bipyridinium dichloride) is a non-selective broadleaf weed control herbicide known to be highly toxic to human and animals. High paraquat concentrations has been found to induce ROS (Suntres, 2002; Xiaolong et al., 2014; Wang, et al., 2018).

Bakry et al., (2016), demonstrated the oxidative-stress inducing potential of paraquat in land snails.Atrazine (2-chloro-4-ethylamino-6-isoprioylamino-1,3,5-triazine) is a selective herbicide linked to generation of ROS which could lead to induction of oxidative stress (Gao et al., 2016). Diuron or DCMU (3-(3,4-dichlorophenyl)-1,1-dimethlurea) is a selective and a potent inhibitor of photosynthesis. Diuron herbicide has been linked to the cause of cytotoxicity and generation of ROS in treated HepG2 cells and zebrafish embryos (Kao et al., 2019).

The aim of this study was to assess the deleterious effects of three herbicides (paraquat, atrazine and diuron) exposed to a non-target environmental receptor (*Archachatina marginata*) at sublethal levels (2% and 5% and 10% of the EC50) of the herbicides using lipid peroxidation (LPO), superoxide dismutase (SOD) and catalase (CAT) as biomarkers. This is with the view of estimating the likely influence of oxidative stress induced by the test herbicides on the exposed species.

MATERIAL AND METHODS

Test Chemicals: Three herbicides commonly used by farmers and non-farmers namely Paraeforce®, Cotrazine® and Force Uron® were used in this assessment. Sublethal concentrations of 2%, 5% and 10% of the EC_{50} was used for this study to estimate the likely influence of oxidative stress that may be induced from exposure to the herbicides, that is around and below the safe limit of 10% (Table 1).

Table 1. EC_{50} and concentrations used for this assessment

Test chemical	Herbicide formulation (active ingredient)	Effective concentrations (EC_{50}) mg/kg	2%	5%	10%
Paraeforce®	paraquat dichloride	0.51	0.0102	0.0255	0.051
Cotrazine®	atrazine	0.41	0.0082	0.0205	0.041
Force Uron®	diuron	0.48	0.0096	0.024	0.048

Test specie: The test specie for the study was the giant African snail (*Archachatina marginata*). Snails are considered excellent indicators of ecosystem health and since they are particularly sensitive to changes in their environment, they can act as early warning sentinels of habitat deterioration. They are easy to sample and identify, abundant all year round and easy to breed under controlled conditions. In addition, they are consumed by man and other higher organisms since they are used as a rich source of protein and health treatments (Ogeleka et al., 2016).

Bioassay for biochemical indices in Archachatina marginata: *Archachatina marginata* of seven-day old with length 1.23 ± 0.5 cm and weight 0.81 ± 0.05 g, were collected from Songhai farms (cultured) in Delta State at latitude 5o34'N and longitude 5o50'E and used for the evaluation. The species were exposed to the test chemicals for 28 days using the International Organization for Standardization (ISO), #15952 protocol (ISO, 2006), after which they were removed, rinsed and used for the biochemical bioassay. Homogenates of the snails were prepared by homogenizing 0.5 g of the snails tissues in ice-cold phosphate buffer at pH 7.2. The homogenates were centrifuged at 4000 rpm for 10 minutes and the supernatant were used for the biochemical analysis.

2.4 Lipid peroxidation assay: Peroxidation was estimated using the method of Buge and Aust, (1978) based on malondialdehyde assay. Malondiadehyde, a product of lipid peroxidation, when heated with 2-thiobarbituric under acid conditions forms a pink coloured product which has a maximum absorbance at 532nm. MDA content was expressed as mmol/MDA wet tissue;

Antioxidant enzymes (superoxide dismutase and catalase): The activity of superoxide dismutase in *Archachatina marginata* was estimated spectrophotometrically using the method of Nishikimi et al., (1972). The assay of SOD is an indirect method based on the inhibitory effect of SOD in the initial rate of epinephrine (adrenaline) auto-

oxidation. SOD was expressed in unit/mg protein tissue. The activity of catalase was determined in the tissue homogenates by the method of Ramos-Vasconcelos and Hermes-Lima (2003). It was based on the measurement of the rate of decomposition of hydrogen peroxide (H_2O_2) after the addition of the material containing the enzyme. Catalase was expressed in unit/mg protein tissue.

Statistical Analysis: Values of the enzymological results were expressed as mean ± standard deviation (mean ± SD). The obtained data were statistically analyzed for the significant differences between treated and control groups using Student's t test in analysis of variance (ANOVA). P values ≤ 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The results for enzymological analyses are given in Table 2 and Figures 1-3. The results from Table 2 indicated that there was significant differences between the activities of the anti-defensive systems of the organisms exposed to the test herbicides with respect to the control. The results indicate that alteration in MDA (as lipid peroxidation), increased with increase in concentrations while SOD and CAT activities decreased with increase in concentration of the test herbicides. In addition, the weight of the organisms decreased significantly at levels of $P < 0.05$ after the 28 day exposure in all test concentrations with respect to the control groups.

Table 2. Mean values ± standard deviation of enzymatic analysis for *Archachatina marginata*

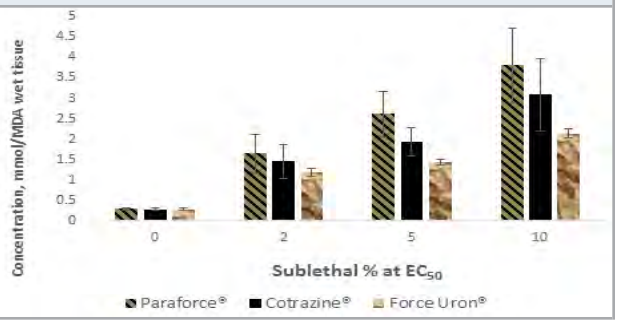
Test chemicals	% of EC_{50}	Concentration, mg/kg	MDA (mmol/MDA)	SOD (Unit/mgprotein)	CAT (Unit/mgprotein)
		Control	0.28 ± 0.03[a]	57.02 ± 6.96[a]	22.78 ± 2.13[a]
Paraeforce	2	0.0102	1.64 ± 0.47[a]	40.43 ± 8.29[b]	19.82 ± 0.03[a]
	5	0.025	2.62 ± 0.55[b]	33.48 ± 1.57[b]	16.32 ± 0.87[b]
	10	0.05	3.80 ± 0.91[b]	24.64 ± 2.01[b]	13.63 ± 2.26[b]
Cotrazine	2	0.0082	1.45 ± 0.41[a]	41.01 ± 3.26[b]	21.45 ± 2.46[a]
	5	0.0205	1.94 ± 0.35[a]	37.42 ± 0.78[b]	16.79 ± 0.60[b]
	10	0.041	3.09 ± 0.89[b]	32.03 ± 2.96[b]	12.84 ± 0.33[b]
Force Uron	2	0.0096	1.19 ± 0.10[a]	50.87 ± 6.83[a]	19.76 ± 2.15[a]
	5	0.024	1.43 ± 0.07[a]	42.46 ± 0.50[b]	16.14 ± 1.36[b]
	10	0.48	2.14 ± 0.10[b]	31.29 ± 4.95[b]	13.55 ± 0.62[b]

Values are means ± standard deviations of triplicate determinations. Values not sharing a common superscript on the same column differ significantly ($P < 0.05$).

Malondialdehyde activities: The MDA values increase with increase in the concentration values of the test herbicides with respect to the control ($P < 0.05$) which implied that the effects was concentration dependent (Figure 1). The effect of MDA induced by the test herbicides was more at 10% of the EC_{50} and decreased down from 5% to 2%, which implied that as you move further down away from the safe limit the effects of the test herbicides with respect to oxidative stress increases.

Superoxide dismutase activities: Superoxide dismutase catalyzes superoxide radicals (O^{2-}) to molecular oxygen (O^2) and hydrogen peroxide (H_2O_2) to defend cells from reactive oxygen. Superoxide dismutase activity in the organisms was observed to decrease as the concentrations of the test herbicides increased (Figure 2). The effects SOD induced by the herbicide was more at the lowest concentration of 2% and decreased down to 5% and 10%, which implied that as you move away from the safe limit of 10%, the more reduced the levels of SOD and the likely inability of the organisms to overcome oxidative stress.

Figure 1: Malondialdehyde concentrations (mean ± SE) in *Archachatina marginata* exposed to sublethal levels of test herbicides



Catalase activities: Catalase is an enzyme that decomposes hydrogen peroxide (H_2O_2) to water (H_2O) and molecular oxygen (O^2). Catalase activity showed decrease as the concentrations of the test herbicides increase which is an indication of high level of toxicity of the test herbicides when compared to the control groups. Similarly, the effect CAT induced by the test herbicides was more at the lowest concentration of 2%

and decreased up to 5% and finally 10%, which implied that as you move up away from the safe limit, the more reduced the levels of CAT and more likely the increase in oxidative stress (Figure 3).

Figure 2: Superoxide dismutase concentrations (mean ± SE) in *Archachatina marginata* exposed to sublethal levels of test herbicides

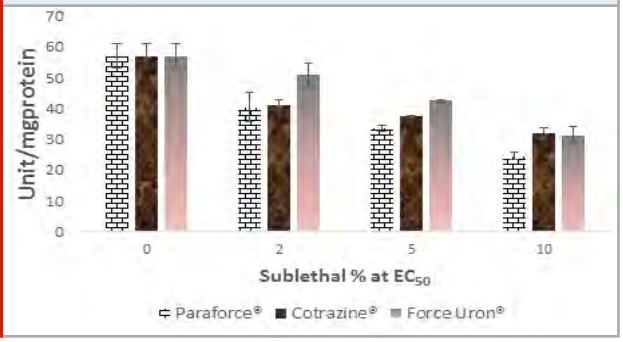
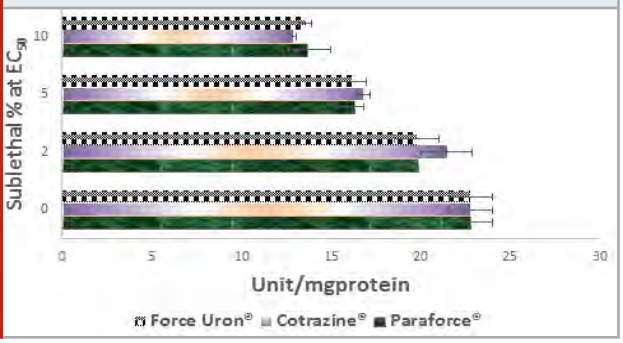


Figure 3: Catalase concentrations (mean ± SE) in Archachatina marginata exposed to sublethal levels of test herbicides



Oxidative stress induced by hazardous herbicides and chemicals could negatively impact environmental species. Over the years, anthropogenic agricultural activities have altered the environment to the detriment of these vulnerable organisms. In this assessment, there was an increase in the levels of MDA in the exposure organisms for all test chemicals with respect to the control which was an indication of lipid peroxidation. The increase in the values of MDA is often associated with high levels of free radicals generated by the presence of toxicants in the environment such as herbicides. An evidence that oxidative stress has been induced in the snails by the herbicides was corroborated in the reports of Belhaouchet et al., 2012 and Siwela et al., 2010.

The decline in activity of the SODs in this assessment can be linked to its role in oxidizing the free radicals generated by the presence of the toxicants (herbicides) (Barondeau et al., 2003). It has been reported that toxicants can decrease the activity of SOD and there was significant inhibition in SOD activity when compared to the control. This was in line with the study reported by Bakry, et al., (2013). Decrease in CAT activities could be due to decrease in the rate of reaction as a result of the excess production of peroxide (H_2O_2).The values of the anti-defensive systems with respect to the control for the different concentrations of the test herbicides could be as a result of the defensive mechanisms trying to get rid of the free radical species generated by the presence of the herbicides in the exposed test organisms, (Kono and Fridovich, 1982, Al-Fanharawi et al., 2018).

The present study showed reduction in SOD and CAT activities with significant increase in lipid peroxidation activities represented as MDA in the tissue of snails treated with the test herbicides. Hence as you move up away from the safe limit of 10%, the more reduced the levels of the anti-oxidant defense mechanisms (SOD and CAT) and thus a more enhanced probability of oxidative stress, which some species may likely not be able to overcome. However, since no mortality was reported at the different concentrations of the EC_{50} evaluated in this study, but rather sluggish movement and immobilization / lack of burrowing, it means that SOD and CAT was still available so they can continue to scavenge free radicals before permanent damage occurs (Al-Fanharawi et al., 2018). Similarly, if the concentrations of the test herbicides increase beyond the safe limit of 10% of the EC_{50}, oxidative stress damage / lipid peroxidation generation may overwhelm the anti-oxidant (SOD and CAT) defense mechanisms to the detriment of the exposed vulnerable species in the environment. This may possibly result in permanent alteration in some of their activities including immobilization and subsequently death of the exposed non-target biological receptors.

CONCLUSION

Exposure of non-target environmental receptor - *Archachatina marginata* to herbicides (Paraeforce®, Cotrazine® and Force Uron®) at sublethal concentrations had deleterious effects measured by MDA, SOD and CAT. The eco-toxicity potential of these herbicides is indicated. The present study considered specifically MDA, SOD and CAT in a bid to ascertain changes induced by the test herbicides on the exposed species, subsequently further studies by the authors will consider hematological indices amongst others.

Competing Interest:The authors declare that they have no competing interest.

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Authors' Contributions: This work was carried out in collaboration between all authors. Doris Fovwe Ogeleka and Felix Ebhodaghe Okieimen designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Beatrice

Oghenetega Peretiemo-Clarke managed the analysis of the study and literature searches. All authors read and approved the final manuscript.

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Biotechnological Communication

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Antifungal Activity of Bacteria Isolated from Rotten Fruits and Vegetables: their Partial Morphological and Biochemical Characterization

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ABSTRACT

Anti-metabolites are produced by the microorganisms and have the highest potential as the agents of bio-preservation. The aim of the present research work was to study the antimicrobial activities of some selected antimicrobial and anti-metabolites producing microorganisms, against the microorganisms responsible for food spoilage. In addition to this, we tried to extract and isolate these microorganisms from natural sources available. In the present research work, a total of 75 bacterial cultures were extracted and isolated from different food samples and later they were purified and screened to record their antimicrobial activity against some food spoiling standard bacterial cultures and fungi which were isolated from spoiled vegetables and fruits. The isolated bacteria were kept and maintained on MRS medium which is a fast growing mesophile with low generation time. Czapec-Dox agar media was used to keep and maintained the fungi isolated from several spoiled vegetables after purification. In order to test the antimicrobial activity of the supernatants from the isolates after the span of 18 hours of incubation against the fungi isolated from rotten vegetable like Tomato (*Solanum lycopersicum*), Cucumber (*Cucumis sativus*), Brinjal (*Solanum melonngena*) and rotten fruits like Orange (*Citrus x sinensis*), Grape (*Vitis vinifera*) and Apple (*Malus domestica*) the paper disk assay method was used. The antimicrobial activity of the supernatant was evidenced by the clear zone of inhibition ranging from 1.9 -3.5 cm by using 50 µl soup. It was found that out of seventy-five (75) isolates, three isolates, IP-1 (isolated from rotten peach), IVP-2 (isolated from vermicompost) and IRF-1(isolated from a teleost fish Rohu, *Labeo rohita*) have most prominent and potent activity against standard bacterial cultures and fungi isolated from spoiled vegetable and fruits. It is evident from the present research that bio-control can be a potent method for food preservation.

KEY WORDS: BIO-PRESERVATIVE, LAB, ROTTEN VEGETABLE AND ROTTEN FRUITS.

INTRODUCTION

Food products that are likely to be perished, require protection from spoilage during the process of their preparation, storage and distribution. One of the biggest concerned and challenge for the food industry is safety and quality of food product, as there is huge demand for processed fresh food products across globe after the food industry being globalised. There is constant threat of contamination of these food products by microbes. Many of these microorganisms can cause undesirable reactions that deteriorate flavor, odor, color, sensory, and textural properties of foods.

The development and survival of common spolage and pathogenic microorganisms such as *Clostridium perfringens, Escherichia coli, Staphylococcus aureus, Aspergillus niger, Listeria monocytogenes*, *Saccharomyces*

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cerevisiae, Salmonella, Bacillus cereus and *Campylobacter* are catalysed and affected by a variety of intrinsic factors like presence of oxygen and pH and extrinsic factors and conditions which includes relative humidity, time and temperature (Appendini and Hotchkiss 2002; Brewer, et al., 2002; Davidson et al., 2014). To prevent growth of spoilage and pathogenic microorganisms in foods, several physical and chemical preservation techniques, such as heat treatment, salting, acidification, and drying have been applied in the food industry (Frakas, 2007; Gálvez et al., 2007). Benzoic acid, sulphur dioxide, sodium nitrite, sorbates, ethylene diamine tetra acetic acid, citric acid and butylated hydroxytoluene (BHT) are some of the examples of synthetic preservatives commonly used for enhancing the shelf life of edible materials, but their full compatibility with the human system is still questionable (Gálvez et al., 2007; Gutierrez et al., 2008; Davidson et al., 2014 Oechslin, 2018).

Moreover, sulphur dioxide causes breathing difficulties; sodium nitrite and BHT are reported to be carcinogenic (Gálvez et al., 2007; Gutierrez et al., 2008; Davidson et al., 2014). A few years back, concern regarding synthetic chemicals, additives were increased because of the great consumer awareness and foods preserved with natural additives became quite popular. Natural preservatives are thought to be better alternatives. Traditionally, herbal drugs were utilized in the form of mixture of different plants. Plant-based preservatives are biodegradable, renewable, and safe for non-target organisms and have diverse biological effects. They provide less chance of resistance development to microbes. The antimicrobials can be used in different ways in order to control undesirable microorganisms, it can be directly added into the product formulation, coated on its surface or incorporated into the packaging material.The result of direct incorporation of active agents into foods is immediate but short-term reduction of bacterial population, on the other hand the antimicrobial films can control their activity for a longer period of time (Hanušová et al., 2009).

The essential oil derived from plants (e.g., basil, thyme, oregano, cinnamon, clove, and rosemary), enzymes obtained from animal sources (e.g., lysozyme, lactoferrin), bacteriocins from microbial sources (natamycin, nisin,), organic acids (e.g., citric acid, sorbic, propionic,) and naturally occurring polymers (chitosan) are important natural compounds that can be used for food preservations.Essential oils from plants have started to gain wide interest from food industry as decontaminating agents, as they are Generally Recognized as Safe (GRAS). The active components found in essential oils have wide wide spectrum of antimicrobial activity, against food-borne pathogens and spoilage bacteria (López-Pedemonte et al., 2003; López-Malo, et al., 2005; Davidson et al., 2014). Another method that is widely receiving interest is the lactic acids bacteria, which are bacteriocin producing or somewhat more or less purified form of the same (López-Pedemonte et al., 2003; López-Malo, et al., 2005; Davidson et al., 2014).

This bacteriocin is usually smaller in kinds and show strong anti-microbial potential activities. This bacteriocin is even gram positive, has a larger spectrum of activities present against the various kinds of lactic acids, and has even able to achieve the GRAS standards across 50 countries worldwide as food preservatives (Rydlo, Miltz, and Mor, 2006; Sobrino-López and Martín-Belloso, 2006; Kilcawley and O'sullivan, 2017). The modernization in the lifestyles of the human has also resulted in various consequences that contribute to many kinds of outbreaks being caused by the food diseases. One of the most continuous concerns of the health of the public, economic and social cause is the infectious diseases that are caused in the intestinal of the human body by the various kinds of bacteria. Hence, one of the most promising tools that have emerged is biological preservation of the food (Kilcawley and O'sullivan, 2017).

Table 1. Composition of MRS Agar Medium

Ingredients	Grams/Litre
Universal peptone	10.0
Meat extract	5.0
Yeast extract	5.0
D (+)-Glucose	20.0
Dipotassium hydrogen phosphate	2.0
Diammonium hydrogen citrate	2.0
Sodium acetate	5.0
Magnesium sulfate	0.1
Manganous sulfate	0.05
Agar	2%

The use of endolysins is considered to be safe as they do not create gene transduction issues or contribute to the emerging problem of resistant bacteria. Although there are concerns about the application of phages such as the emergence of phage-resistant bacteria and gene transductionendolysins do not create such problems; therefore, endolysins are promising biocontrol agents that could be applied in the field of food safety (Bakhshinejad et. al., 2014; Oechslin, 2018). The sole purpose of current investigation is to isolate the bacteria from spoiled food sample which produces antimicrobial substances and purified them. There will be study made in the characteristics of these antimicrobial producing microbes and analysis will be made to know the application for the controlling the pathogens of that spoil the vegetable and fruits (Oechslin, 2018). To check the inhibitory effect of antimicrobial producing bacteria against food pathogenic fungi, the pathogenic food fungi will be isolated, purified and preserved for same. It has been revealed by the literature review that there has not been enough research done in this field. Bio-preservation is a promising strategy to control spoilage risk against lightly preserved food industries (Oechslin, 2018).

MATERIAL AND METHODS

For the isolation of the microorganisms (bacteria), which

are anti-metabolite producing using the techniques of crowded plate from the natural sources. The first step was to collect various kinds of sample from the market, i.e., the spoiled food like spoiled fish, spoiled products of milk, spoiled Idli, and spoiled products of meat. Then sterilized one gram of each of the sample and kept suspended in 10 ml of sterilized distilled water which is kept in a test tube. Now the sample needed for the process of inoculation, was spread on the MRS agar medium and left for incubation at 37o degrees centigrade for around 4-7 days. Soon after the process of incubation, it was seen that several colonies were appearing on the plates of the MRS agar (Table-01). The clear zones were surrounded by the colonies and were isolated and maintained on the slants of the MRS agar as the pure cultures.

Table 2. Details of 75 Isolated colonies

Sl. No.	SAMPLES	ISOLATED PURE COLONIES	No. of colonies
01	Fish intestine	A) F1(white).	
		B) F2(Yellow)	
		C)F3(Off white)	
		D)F4 (Gummy white)	4
02	Peach	A) Peach1 (cream colour) (IP-1)	
		B) Peach 2 (gummy)	2
03	Idli	A) Idli1(pure white)	
		B) Idli2 (Whitish)	2
04	Salted fish (Rohu)	A) Salted fish1(White)	1
05	Salted (3% NaCl) Cabbage after 3 days	A) Cab1 (Slight redish)	
		B) Cab2 (Cream color)	2
06	Guava	A) Guava1	
		B) Guava2	2
07	Goat milk	A) Goat milk1 (White)	
		B) Goat milk2 (Whitish)	2
08	Cucumber	A) Cucumber1 (slight yellow)	
		B) Cucumber2 (Offwhite)	
		C)Cucumber3 (Cream)	3
09	Apple	A) Apple1(Maroon)	
		B) Apple2 (White)	2
10	Pineapple	A) Pineapple1	
		B) Pine apple 2	
		C)Pineapple 3	3
11	Sosage	A) Sosage1 (Offwhite)	
		B) Sosage2 (Yellow)	2
12	Papine	A) Papine1 (Dark yellow)	
		B) Papine2 (Pure white)	
		C)Papine3 (Cream)	3
13	Salted Cabbage after 9 days of storage	A) Cab1 (Yellow)	
		B) Cab2 (White)	2
14	Mango	A) Mango1 (White)	
		B) Mango2 (Faint white)	2
15	Dosachatni	A) Dosa chatni1 (Slight yellow)	
		B) Dosachatni (White, glossy)	2
17	Vermi compost	A) Vermicompost1 (White)	
		B) Vermicompost (Slight yellow) (IVP-2)	2
18	Soya sausage	A) Soya sosage1	
		B) Soya sosage2	2
19	Salted cabbage after 16 days of storage	A) Cab1 (Pure yellow)	
		B) Cab2 (Pure White)	2

Continue Tanle 2

20	Papine (3 days old)	A) Pap1 (maroon)	
		B) Pap2 (Cream)	2
21	Taal (Palm)	A) Taal1 (Gummy white)	
		B) Taal2 (white, tiny)	
		C)Taa3(Off-white)	3
22	Without salted cabbage	A) Without salted cab1	
		B) Without salt6ed cab2 (Slight yellow)	
		C)without salted cab3 (Cream)	3
23	Sambar	A) Sambar1	
		B) Sambar 2	
		C)Sambar 3	3
24	Fish	A) Fish1 (Dark yellow)	
		B) Fish2 (Yellow)	
		C)Fish3 (White)	3
25	Rohu Fish	A) Rohufish (Yellow) (IRF -1)	
		B) Rohu fish2 (White)	2
26	Food grain	A) Food grain1(yellow)	
		B) Food grain2 (Whitish)	2
27	Milk	A) Milk1 (White)	
		B) Milk2 (Tiny white)	
		C)Milk3 (White droplet like)	3
28	Doi	A) Doi1 (White round)	
		B) Doi2 (White, Gummy)	2
29	Cabbage after 28 days	A) Cab1 (White)	
		B) Cab2 (Cream)	2
30	Naspati	A) Naspati1 (Yellow)	
		B) Naspati 2 (White) (IN-1)	2
31	Bamboo chatni	A) Bamboo cahtni1 (White)	
		B) Bamboo chatni2 (Off white)	
		C) Bamboo chatni3 (Off white)	3
32	Soya sausage (After 14 days of storage)	A) Soya1 (Yellow)	
		B) Soya2 (White)	
		C)Soya (Gray)	3
33	Soil from vat	A) Soil1 (White)	
		B) Soil2 (Yellow)	
		C) Soil 3(Gray)	3
34	Donkey Milk	A)Milk (White)	
		B) Milk (yellowish)	2
Total number of isolated colonies		75	

Figure 1: Growth curve of IRF -1

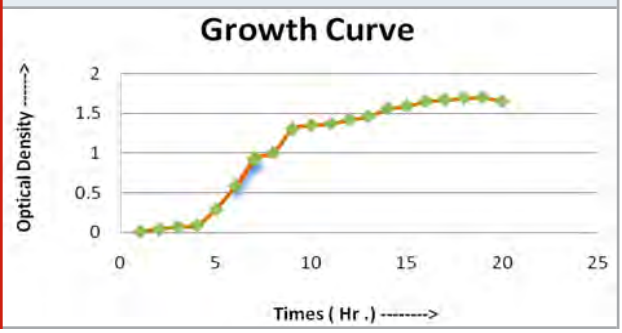
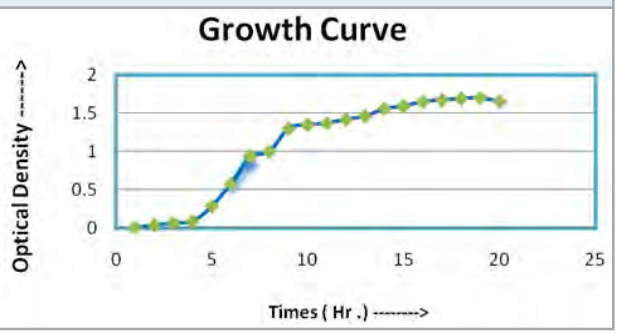


Figure 2: Growth curve of IP -1



For the screening of the anti-microbial activity against the standard cultures of the bacteria, the bacterial colonies which were isolated by previous step were maintained on MRS agar slants and same were inoculated in MRS broth and kept in incubator for overnight at 370C. Broth culture of each isolated organisms amounting 1.5 ml, after

overnight incubation was taken in sterilizeeppendorf and centrifuged at 10,000 rmp for 15 min. After centrifugation, 50 µl cell-free culture supernatant was applied into the well on agar plates previously inoculated with indicator organisms (MTCC 3041, *Lactococcus lactis* subsp. lactis) and then plates were incubated at 37°C for 24 hours. The place was observed for the presence of zone of inhibition after incubation process. Out of 75 isolates (Table -02), bacteria isolatedIP-1 (isolated from rotten peach), IVP-2 (isolated from vermicompost) and IRF-1(isolated from Rohufish) had shown antimicrobial activity against the three indicator organisms. After this the diameters of zone of inhibition were pen downed.

To find the solution of food spoilage fungi from different spoiled fruits and screening of their sensitivity by well diffusion method, three types of rotten vegetables and three types of rotten fruits like Tomato (*Solanum lycopersicum*), Cucumber (*Cucumis sativus*), Brinjal (*Solanum melonngena*) and Orange (*Citrus x sinensis*), Grape (*Vitis vinifera*) and Apple (*Malus domestica*) respectively were collected from local market of Salt Lake, Kolkata, West Bengal. Samples of all six individual items were taken in a test tube per sample 1 gm and suspended in 10 ml sterilized distilled water. This mixture was then taken to be incubated at 37 ^{0}C while being streaked on the Czapec-Dox agar plate for around 3-5 days. After 3-5 days it was seen that Czapec-Dox agar plates were filled with fungi and these fungi was later maintained and isolated on the slants of the Czapec-Dox agar plate as the pure culture. Furthermore, a culture was prepared in a test tube containing 5 ml of each of MRS broth medium and Czapec-Dox agar. MRS broths were inoculated with the four test isolates and incubated under shaking condition for about 18 hours at 37 ^{0}C.

The supernatant of each sample was then collected by centrifugation at 10000 rpm for 12 min. Six Czapek-Dox agar plates were prepared and six fungal cultures were inoculated on six different plates. Four wells were made on each Czapek-Dox agar plate and filled with 50 ul supernatant of the four isolates. Then the plates were incubated at 37 ^{0}C for 3 to 5 days. Observations were made and the effects of the supernatants on the spoilage organisms were recorded. For the morphological and biochemical characterization of IRF-1 and determining the growth curve, IRF-1 was inoculated in 100 ml MRS broth medium and incubated in a rotary shaker at 37 ^{0}C. At 1-hour intervals, the absorbance of the culture was monitored in a colorimeter at 540 nm.

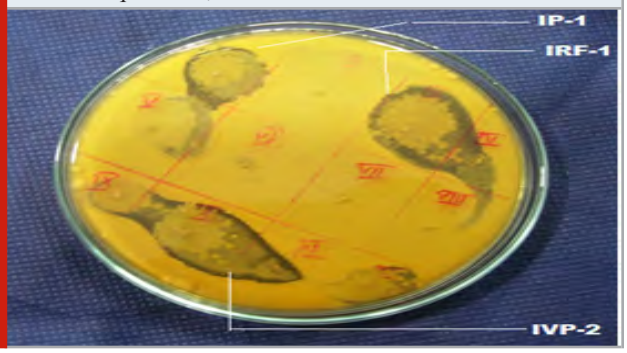
RESULTS AND DISCUSSION

Since long time microorganism like LAB are used in fermentation because of their beneficial properties and major role on nutritional enhancement, organoleptic, and shelf –life characteristic. These organisms help in causing a speedy acidification through the process of producing anti-microbial substances inside the raw materials that results in preserving the nutritional values of the edible products via increasing the shelf life of these products and inhibiting the spoilage that is caused by the pathogenic bacteria. Apart from these, there are several lactic acid bacteria that are known to be food grade found and used in the fermentation of food (Kilcawley and O'sullivan, 2017; Oechslin, 2018).

In the current investigation it was observed that the isolate IRF-1, IP1 and IVP-2 can inhibit the growth of the pathogenic fungi isolated from rotten vegetables/fruits. The supernatant of isolate IRF-I, IP-1 and IVP-1 after 18 hours of growth produced clear zone of inhibition against spoilage fungi isolated from Tomato (*Solanum lycopersicum*), Cucumber (*Cucumis sativus*), Brinjal (*Solanum melonngena*) and rotten fruits like Orange (*Citrus x sinensis*), Grape (*Vitis vinifera*) and Apple (*Malus domestica*). The range of inhibition zone diameter was between 1.9-3.5 cm. Morphological, physiological and biochemical characters of IRF-1 was studied and recorded. By 16S rRNA sequencing analysis isolate IRF-1 was identified as *Bacillus subtilis* subsp. inaquosorum (Identified from IMTECH Chandigarh) (Kilcawley and O'sullivan, 2017; Oechslin, 2018).

Biochemical, Morphological and Physiological characters of IRF-1: The IRF-1 had its growth curve, which represented that the IRF-1 (Figure -01). Its lag phase occured during the 0-4 hours, it experienced its log phase during the 5-18 hours, experienced the stationary phase during the period of 18-21 hours and later after the 21 hours mark, the death phase started. The Figure – 02 of stain IP-1 showing the growth curve that it experienced its lag phase during the 0-5 hours mark, it experienced its log phase around 5-19 hours mark, experienced the stationary phase at around the 19-22 hours mark and after the 22 hours mark, it experienced the death phase. By 16S rRNA sequencing analysis isolate IRF-1 was identified as one of bacteria belongs to *Bacillus* group of bacteria. After 16S rRNA sequencing analysis isolate IRF-1 was identified as *Bacillus subtilis subsp. inaquosorum* (Identified from IMTECH Chandigarh) (Oechslin, 2018).

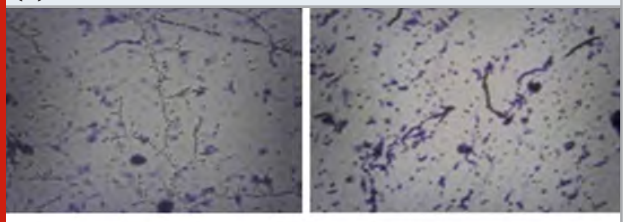
Picture 1: Anti-microbial activity of 14 hours grown culture of IRF-1, IP-1 and IVP-2 against MTCC 3041(*Lactococcus lactis subsp. lactis*.)



Screening for antimicrobial activity against standard bacterial cultures and against fungi isolated from spoiled fruits: There were seventy-five (75) morphological different bacterial colonies selected from different food

samples. Furthermore, the paper disc method was implied in order to screen each individual isolated strain for the antagonistic activity that might have taken place against the cultures of the standard bacteria. Out of those isolated bacteria only IP-1, IRF-1 and of the IVP-2 was able to reflect antimicrobial activities against the standard bacterial cultures (MTCC 3041) (Picture -01).

Picture 2: Morphological structure after 12 hrs (A) IRF-1 (B) IP-1



(A) IRF-1 (B) IP-1

Table 3. Temperature, pH and Salt Concentration of two isolates (IP-1 and IRF-1).

Sample	Parameter	Variation	After 24 hrs	After 48 hrs
IP -1	Temp.	4°C	-	-
		10°C	-	-
		30°C	+	+++
		37°C	++	+++
		45°C	+++	+++
	pH	3	-	-
		5	-	-
		7	+	+
		9	-	-
	Salt Concentration	5%	-	0.02
		10%	-	0.01
		15%	-	0.0
		20%	-	0.0
	Temp.	4°C	-	-
		10°C	-	+
		30°C	++	+++
		37°C	++	+++
IRF-1		45oC	+++	+++
	pH	3	-	-
		5	-	-
		7	+	+
		9	-	-
	Salt Concentration	5%	-	1.16
		10%	-	1.01
		15%	-	0.5
		20%	-	0.0

The characterization was done using the biochemical and the morphological processes of the IRF-1 and was performed using the cultures that were kept for 24 hours. The process of Gram straining along with the observation of the microbes aided in identifying that the IRF-1 is gram positive and is a rod-shaped bacterium existing in chain formations. It was observed from the result that the IP-1 and the IRF-1 was able to grow at around 30 ^{0}C, 37 ^{0}C and 45 ^{0}C and the maximum growth potential was recorded at around temperature of 37 ^{0}C and they were unable to grow at 4 ^{0}C and 100C. Regarding the tolerance of the pH levels, the IRF-1 and IP-1 were only able to grow at the pH -7 and were not able to grow at the pH - 3, pH - 5, and pH – 9. Regarding the tolerance of salt concentration, it has been observed in case of IRF -1, at 5% the growth went up to 1.16, at 10% went up to 1.01, at 15% negligible growth took place and at 20% there was no growth at all. In case of IP-1, at 5% the growth went up to 0.02, at 10% went up to 0.01, at 15% extremely negligible growth took place and at 20% there was no growth at all (Table -03).

Table 4. Utilization of different carbon sources was studied of two isolates

Carbon sources	IRF -1 After 24 hrs	IP-1 After 24 hrs
Mannitol	+	-
Sucrose	+	+
Lactose	+	+
Rhamnose	+	+
Cellobiose	-	-
Fructose	+	+
Galactose	+	+
Dextrose	+	+
Trehalose	+	+
Inositol	+	+
Maltose	+	+

Testing was also done to indentify the ability of the isolates to utilize various sugars in order to grow using them as the sole carbon sources in the MRS broth medium using various sugars (Mannitol, Sucrose, Lactose, Rhamnose, Cellobiose, Fructose, Galactose, Dextrose, Trehalose, Inositol and Maltose) (Table -04) (Oechslin, 2018).

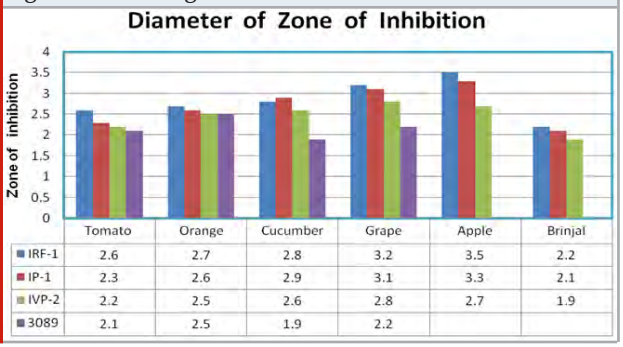
The findings showed that the IRF-1 was capable to utilize all the sugar but cellobiose and in the case of the IP-1 the exceptions of the sugars, which it was not able to utilize, was the mannitol and the cellubiose. The fungi were isolated from three rotten vegetables namely Tomato (*Solanum lycopersicum*), Cucumber (*Cucumis sativus*), Brinjal (*Solanummelonngena*) and three rotten fruits namely Orange (*Citrus x sinensis*), Grape (*Vitis vinifera*) and Apple (*Malus domestica*). Out of the three isolates that were being observed, IRF-1 was able to reflect the maximum antimicrobial activities against the fungi, which was isolated from the rotten Rohu Fish. The zone of inhabitation ranges from 2.2 cm to 3.5 cm for the IRF-1 and for the IP-1 the ranges is from 2.1cm to 3.3 cm and for the IVP-2 isolates the range

is from 1.9 cm to 2.8cm for the *Lactobacillus plantarun* (MTCC3089, indicator organism) were observed (Table– 5 and Picture from 03- 08) (Kilcawley and O'sullivan, 2017; Oechslin, 2018).

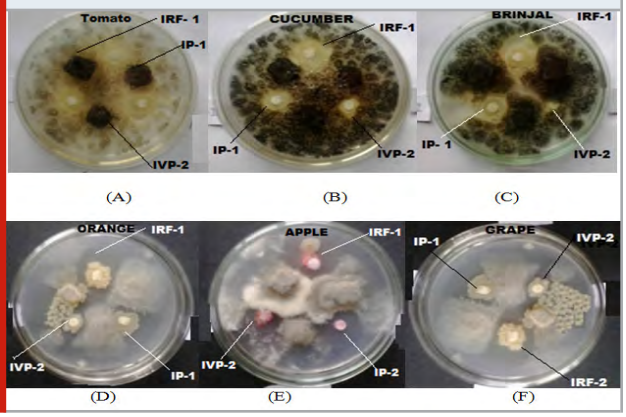
Table 5. The clear zones surrounding the wells (the zone of inhibition) were observed and the results were recorded

Serial No.	Source of spoilage fungi	Supernatant of the Isolates	Presence of zone of inhibition	Diameter of zone of inhibition in cm
1.	Tomato	IRF-I	+	2.6
		IP-1	+	2.3
		IVP-2	+	2.2
		3089	+	2.1
2.	Orange	IRF-I	+	2.7
		IP-1	+	2.6
		IVP-2	+	2.5
		3089	+	2.2
3.	Cucumber	IRF-I	+	2.8
		IP-1	+	2.9
		IVP-2	+	2.6
		3089	+	1.9
4.	Grape	IRF-I	+	3.2
		IP-1	+	3.1
		IVP-2	+	2.8
		3089	+	2.2
5.	Apple	IRF-I	+	3.5
		IP-1	+	3.3
		IVP-2	+	2.7
		3089	-	-
6.	Brinjal	IRF-I	+	2.2
		IP-1	+	2.1
		IVP-2	+	1.9
		3089	-	-

Figure 3: Graphic representation of Zone of Inhibition against all 6 fungi



Pictures 3: Inhibition zone observed in the plates containing different pathogenic fungus culture,
A = Tomato: B = Cucumber: C = Brinjal: D = Orange: E = Apple: F= Grape



CONCLUSION

The present study has found that the isolate IRF-1, IP1 and IVP-2 can inhibit the growth of the pathogenic fungi isolated from rotten vegetables and fruits. The current research also revealed that in future bio-control could be a major part of food industry for preservation of food items, because preservation of food by means of biological ways can be highly accepted as it would be non-toxic in nature.

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Conflict of Interest: The authors declare that there exist no commercial or financial relationship that could, in any way, lead to potential conflict of interest.

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Combinational Impact of Chelerythrine and S-Allyl Cystine on Metastasis melanoma of liver : An In vivo Analysis

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ABSTRACT

Metastatic melanoma, the highly fatal and aggressive disease, has yet to any effectual remedies. Several evidences suggested delicate responsibility of oxidative/cytotoxic stress in the modulation of tumor microenvironment leading to metastasis. Therefore, conditioning of reactive oxygen species in tumour and its adjacent arena may play a guardian role for restricting metastatic melanoma. Well-known active biocomponents like S-allyl Cysteine and Chelerythrine as nontoxic dietary phytochemicals are recently documented as potential anti-tumorigenic and anti-inflammatory therapeutics but their role in metastatic melanoma still remains elusive. Therefore, present study was carried out to investigate the efficacy of S-allyl Cysteine and Chelerythrine against metastatic melanoma to the hepatic tissue. Status of liver function was estimated by performing ALT, AST, GGT and ALKP assay. ROS accumulation was determined by estimating the altered DCF fluorescence in hepatic tissue lysates. GSH and TBARS content were measured as a marker of anti-oxidant and cytotoxicity level after the treatment. Analysis on the marker proteins like Caspases, CytochromeC, BCl_2, Bax, VEGF, MMP9 and NF-κβ depicted the triggering of p-p53 nuclear translocation and significant increase in Bax expression that in-turn induced CytochromeC-Caspase9-Caspase3 apoptotic axis after drug administration. Data also illustrated notable reduction in tumor nodules at liver along-with normalization of liver function as demarcated by the level of biomarkers in the treated groups. Restoration of enzymatic and non-enzymatic anti-oxidants as well as suppression of VEGF and MMP9 expression as an effect of attenuated NFkb nuclear localization by S-allyl Cysteine and Chelerythrine effectively delimited extracellular matrix remodeling as well as angiogenesis, two major prerequisites for metastasis. Combinatorial administration of S-allyl Cysteine and Chelerythrine further portrayed better efficacy in metastatic tumor regression and tissue restoration by sustaining ROS/antioxidant balance and stabilization of p53 through its phosphorylation, that can be considered as future directives for the development of novel remedial strategy against metastatic melanoma in liver.

KEY WORDS: METASTATIC MELANOMA, ROS, ANTIOXIDANT, S-ALLYL CYSTEINE, CHELERYTHRINE.

INTRODUCTION

Melanoma, a predominant skin cancer, originates from melanocyte. Surgical removal followed by popular therapies with chemo/radiation-based drugs can cure primary melanomas. Due to its high aggressive nature and lack of complete effective therapeutic strategy, it can able to metastasize into local as well as distant organ following invasion and this in turn reduces the chances

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of survivability of the patients. Therefore, majority of melanoma related morbidity is due to metastasis (Eggermont et al., 2020). Recent studies suggested that metastatic melanoma is responsible for 80% of skin cancer related fatality (Jones et al., 2020). According to the reports only 14% of malignant melanoma patients can able to survive more than 5-year (Sandru et al., 2014; Enninga et al., 2017).

Evidences from several clinical studies demonstrate distant metastasis of melanoma cells from their primary subcutaneous location (Zbytek et al., 2008 and Tan et al., 2019). Previous works also identified liver (58.3%) as the second most common target organ for metastatic melanoma (Bostanci et al., 2014; Ruini et al., 2020). Metastatic progression encompasses an array of interrelated events such as dissemination, migration and establishment of new foci, finally they then grow to develop fetal metastatic tumor silently (Palmer et al., 2011; Hao et al., 2019).

Presently many expensive therapeutic approaches like surgery followed by chemotherapy, radiation therapy and immunotherapy etc. are well practiced as remedial measures; but success rate is not significant (Sundarajan et al., 2020). Even most of them are coming up with adverse side effects (Schirrmacher et al., 2019). On this context, many phytochemicals form traditional medicine are practiced as potential anti-tumourogenic medicine considering their efficacy to suppress cancer cell proliferation by triggering apoptosis through altering the status of reactive oxygen species (ROS)- the crucial player for the sustenance of tumour microenvironment and malignant behavior of tumour cells leading to unfettered tumour progression as an effect of imbalance in pro- and anti- apoptotic proteins (Wang et al., 2012 and Kapinova et al., 2019). S-allyl Cystine (SAC) and Chelerythrine (CHEL) are two well-known naturally occurring bioactive phytochemicals with effective anti-tumourogenic effect against prostate cancer, liver cancer, breast cancer, oral cancer, neuroblastoma and non-small cell lung cancer (Kanamori et al., 2020; Yang et al., 2020).

Previous reports did not come out with any grade of subsequent toxicity in therapeutic application of those drugs in *in vivo* cancer treatment of nude mice model (Chmura et al., 2000 and Ng et al., 2012). SAC is an aged garlic derived water soluble, stable organo-sulfur compound. Studies suggested the ability of SAC in the suppression of cell growth, cell proliferation, metastasis and induction of apoptosis by modulating cellular ROS dependent activities (Shang et al., 2019). CHEL is a benzo-phenanthridine alkaloid commonly isolated from herbal plants like *Cheludonium majus, Macleaya cordata* and *Sanguinaria canadensis* (Kumar et al., 2013). Various reports suggested anti-diabetic, anti-microbial, anti-inflammatory, anti-fungal and even anti-cancerous properties of CHEL (He et al., 2018).

Previous reports also exhibited CHEL mediated apoptosis in prostate and renal cancer cells through activating ROS mediated intrinsic cell death pathway (Wu et al., 2018; He et al., 2020). SAC was also able to inhibit the proliferation of melanoma cell lines in a dose-dependent manner and demonstrated significant cytotoxic effects on Sk-mel3 melanoma cell line as observed in *in vitro* assay (Hakimzadeh et al., 2010). Reports demonstrated the provoking role of CHEL in the development of apoptotic response in uveal melanoma cells (Kemeny-Beke et al., 2006).

Moreover, potential role of CHEL in the suppression of proliferation and metastasis of human prostate cancer cells via modulating MMP/TIMP/NF-κB system as well as inhibition of the migration and invasion of Hep3B cells in a dose-dependent manner along with change of cell structure were reported (Yang et al., 2020). While a series of *in vitro* experiments including MTT, colony-forming, wound-healing, invasion, apoptosis and cell cycle assays demonstrated anti-proliferative and anti-metastatic effects of SAC on the metastatic HCC cell line MHCC97L (Ng et al., 2012).

SAC treatment also significantly reduced the migration of A2780 cells, and markedly decreased the expression of key proteins such as Wnt5a, p-AKT and c-Jun, involved in proliferation and metastasis (Xu et al., 2014). Observations indicated that oral administration of SAC not only inhibited the growth of primary tumors but also reduced the occurrence of lung and adrenal metastases by without causing notable toxicity. This metastatic suppression was accompanied by a distinct reduction of viable circulating tumor cells, supporting the potential use of SAC as an E-cadherin up-regulating antimetastatic agent for the treatment of androgen-independent prostate cancer (Kanamori et al., 2020).

Although, therapeutic efficacy of SAC and CHEL on metastatic melanoma, categorically in liver, still remains elusive. Here, in this study, we aimed to investigate therapeutic effect of SAC and CHEL, individual as well as in combination, on the ectopic metastatic mice melanoma tumor model. Experimental results depicted that SAC and CHEL administration-maintained ROS/antioxidant balance and stabilized p53-axis through its phosphorylation resulting significant increase in Bax expression that in-turn turned on intrinsic apoptotic pathway.

Data also first time illustrated the reduction in tumor nodules at liver and normalization of liver function in the treated groups. Analysis on related molecular status suggested effective delimitation of extracellular matrix remodeling and angiogenesis by SAC and CHEL via suppressing VEGF and MMP9 expression as an effect of reduced level of NFκ translocation towards nucleus. Hence in the summary, our findings first time described the novel therapeutic role of SAC and CHEL against B16F10 induced metastatic melanoma in *in vivo* and their potential anti-metastatic properties need to be scrutinized in other *in vivo* and clinical studies on urgent basis to give a probable ray of hope in the designing of therapeutics against metastatic melanoma in future.

MATERIAL AND METHODS

Unless and until mentioned all chemicals and reagents were purchased from Merck-Millipore, USA. B16-F10, a well-established murine mice melanoma cell line, were collected from IICB, Kolkata, originally purchased from ATCC, Manassas, Virginia and cultured in Dulbecco's modified Eagles medium (HiMedia, Mumbai, India) supplemented with 10% fetal bovine serum (HiMedia, Mumbai, India), 1% PenStrep (Life BioSciences, USA) and 0.1% Fungizone (Life BioSciences, USA) at 37 ^{0}C and 5% CO2 containing humidified air (Chowdhury et al., 2019 B). Five-week aged male Balb/C mice of 12-15gm weight were purchased and housed in micro-isolator cages with 12h day/night cycle under hygienic condition. Animal house was maintained at 27$\pm$3^{0}C with a relative humidity of 50-62%. Mice were free access of standard pellets as food and water through *ad libitum*. All animal experiments were designed following Institutional Animal Ethical Committee Guidelines to reduce the animal sufferings without hampering the requisites of statistical analysis (Sengupta et al., 2017 A).

B16F10 melanoma cells were injected subcutaneously to the left thigh at a dose of 2X10^6 cells in 200µl phosphate buffered saline (PBS). Primary tumour was first visualized after 5-6 days of cell inoculation and progression was noticed day by day. This group was labelled as ST. Only PBS injected control animals were simultaneously maintained as a separate group (n=5) with same diet and were tagged as Con. After five weeks of B16F10 cell exposure, a group (n=5) of ST animals were treated daily with individual SAC (ST+SAC) and CHEL (ST+CHEL) at a dose of 250mg/kg b.w. and 5mg/kg b.w., respectively, through gavage for 30days and 60days, sequentially. Again, another ST group of animals (n=5) received daily 250mg/kg b.w. SAC in combination with 5mg/kg b.w.

CHEL through gavage for 30days, 45days and 60days and were denoted as ST+SAC+CHEL. Three separate groups (n=5) of Con mice were treated with 250mg/kg b.w. SAC, 5mg/kg b.w. CHEL and 250mg/kg b.w. SAC+5mg/kg b.w. CHEL for 60days as negative control and were marked as Con+SAC, Con+CHEL, Con+SAC+CHEL, respectively (Kumar et al., 2015; Chatterjee et al., 2019). Alterations in morphology were identified as dark patches of melanin synthesis and were evaluated by calculating tumour numbers in total ten quadrates (Bostanci et al., 2014). Number of tumour nodules was represented in 4x4mm2 area. Blood was collected from treated as well as untreated control, B16F10 infused, individual and combined drug treated animals and serum was isolated according to the standard protocol (Chatterjee et al., 2019).

Prepared serum was used as protein source for evaluating the activities of aspartate transaminase (AST), alkaline phosphatase (ALKP), alanine transaminase (ALT) and γ-glutamyl transferase (GGT). Assays were performed by following respective manufacturing kit protocols at room temperature. ALT and AST (TECO Diagnostics, CA, USA) activity was measured by estimating NADH oxidation at 320nm of wavelength for 30s intervals up to 2min. ALKP and GGT analysis absorbances were measured at 405nm for 30s intervals. Results were evaluated by determining mean absorbance change per minute. Values were portrayed in IU/L (Chatterjee et al., 2019). To evaluate TBA reactive substrate (TBARS) content, liver tissue was lysed in 10mM TRIS-HCl lysis buffer pH 7.4 and was used for experimental analysis. 0.4mg of protein in 100µl lysis buffer was added with 2ml TBA-TCA and was boiled for 20min at 100 ^{0}C water bath. Solution was centrifuged at 1000g for 10min at room temperature and absorbance of supernatant was measured at 532nm. Values were represented as nmol/mg protein (molar extinction coefficient: 1.56x105 M^{-1} cm^{-1}) (Chatterjee et al., 2019).

Hepatic tissue samples were deproteinized with 35% metaphosphoric acid (Ref). Extracted samples were neutralized with 0.3M Na2HPO4. 0.6mM DTNB, 0.5U GR and 0.2mM NADPH were added as final concentration to the reaction mixture. Formation of reduced glutathione-DTNB conjugate within supernatant was then measured spectrophotometrically at 412nm (Sengupta et al., 2017 A). Data were presented in mole/mg protein. Superoxide dismutase (SOD) activity was measured from chloroform methanol extract following the standard protocol (Sengupta et al., 2014).

Values were quantified spectrophotometrically (UV-1240 Pharma Spec, Shimadzu, Kyoto, Japan) by calculating the changes in pyrogallol auto-oxidation at 420nm in presence of catalase enzyme. One unit of SOD activity is equal to the 50% suppression of superoxide mediated oxidation of pyrogallol. Results were represented in unit/mg protein. Catalase activity was evaluated through spectrophotometrically (UV-1240 Pharma Spec, Shimadzu, Kyoto, Japan) by measuring degradation of H2O2 in presence of tissue lysate as a source of enzyme. Values were quantified by measuring absorbances at 240nm in 10s intervals. Data were represented in unit/mg protein (Sengupta et al., 2017 A).

Cells of the liver were isolated from experimental groups by Collagenase-IV digestion. Intracellular ROS was measured by incubating 5% cell suspension with 5µM 2,7-dichlorofluorescein diacetate (DCFDA) (Sigma-Aldrich, St. Louis, Missouri, USA), a fluorogenic dye, at 37 ^{0}C for 15min. After diffusion it was deacetylated by cellular esterase to a non-fluorescent compound which was later oxidized by ROS into highly fluorescent 2,7-dichloroflurescin (DCF). Emitted fluorescence (Ex: 485nm/ Em: 535nm) was estimated in RF-6000 Fluorescence Spectro-fluorometer (Shimadzu, Kyoto, Japan). Values were presented in Relative Fluorescence Unit (RFU) (Sengupta et al., 2014).

Liver tissue of experimental groups was lysed by lysis buffer following kit (Bio Vision, USA) protocol and was used as protein source for Caspase3 as well as Caspase9 activity assay (Sengupta et al., 2017 A). 100µg of protein in 50µl lysis buffer was loaded in Caspase3p17 antibody (capture antibody) coated microtiter plate, for Caspase3

analysis and in cleaved-Caspase9p37 antibody (capture antibody) coated microtiter plate, for Caspase9 analysis. After 4h incubation at 4^0C 5µl 4mM DEVD-pNA was added as substrate solution and was again incubated at 37 ^{0}C for 2h. Activities of Caspase3 and Caspase9 were evaluated by measuring the released pNA absorbance at 405nm and were represented in pmol pNA/min/mg protein (Chatterjee et al., 2019; Chao et al., 2019).

Bcl2, Bax, CytochromeC, phospho-p53/Ser15, NFκβ/p65, VEGF and MMP9 were quantified by using commercially available specific Elisa kit (R&D Systems Inc. Minneapolis, USA) following respective manufacturer instruction. Cytosolic fractions were prepared according to the standard kit protocol (Nuclear/Cytosol fractionation kit, K266, Cell Biolabs) and were used as protein source for quantitative analysis of cellular Bcl2, Bax, CytochromeC, VEGF and MMP9. While nuclear fractions were prepared following standard kit protocol (Nuclear/Cytosol fractionation kit, K266, Cell Biolabs) and were similarly used for the estimation of nuclear NFκβ/p65 and phospho-p53/Ser15 content (Li et al., 2018). Values were measured at 450nm and were calculated through provided standard curve. Results were represented in ng/ml protein. Data were mean±SD. Differences between treated and untreated groups were evaluated by student's t test in GraphPad Prism software, La Jolla, CA, USA. P≤0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

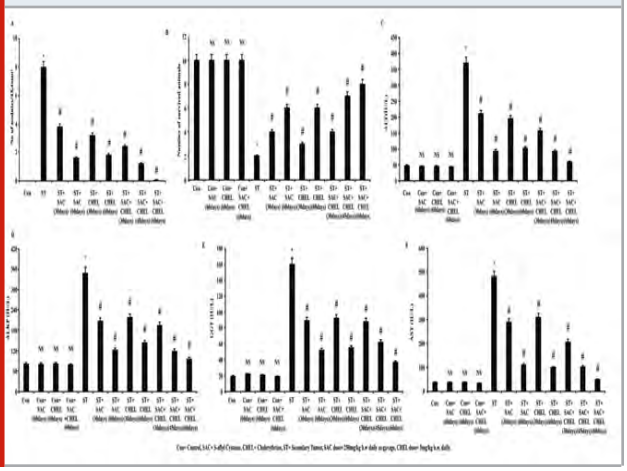
Model development, morphometric and survivability analysis: Herbal medicine is a potent remedial measure against neoplastic liver (Li et al., 2011; Yang et al., 2020). Previously, some *in vitro* analysis documented anti-metastatic effect of SAC against, prostate cancer, neuroblastoma, hepatocellular carcinoma and ovarian cancer, but not in the field of metastatic melanoma. Remedial efficacy of SAC against primary melanoma cell lines was previously described by researchers, and noted as a potent restricting player for the melanoma cell proliferation in *in vitro* system (Hakimzadeh et al., 2010; Xu et al., 2014). Similarly, researchers established the therapeutic advantage of CHEL administration against dalton's lymphoma, liver cancer, breast cancer and renal cancer in both *in vitro* and *in vivo* condition (Kumar eta al., 2015).

According to the reports benefit of CHEL administration was studied on OCM-1, the well-known melanoma cell line (Chen et al., 2016). Data suggested the discrete role of the treatment in DNA fragmentation as well as apoptosis induction of this primary cancer cells. Thus, previous reports pointed out the efficacy of individual administration of SAC and CHEL in the attenuation of tumor progression and healing of several primary cancers like prostate, liver, colorectal, lung and skin but their impacts on metastasis to distant organs (*in vivo* studies) specially in melanoma still remain unknown (Xu et al., 2014).

Moreover, no previous reports were found about the importance of combined treatment in this regard. Therefore, to deduce the therapeutic efficacy of SAC and CHEL against metastatic melanoma, spontaneous metastatic animal model was developed by injecting 2X106 B16F10, the perpetual melanoma cell line, at left thigh region of Balb/c mice (Bostanci et al., 2014). Nascent primary tumor was visualized after 5/6 days of cell inoculation at the mentioned site. Also, sharp and gradual increase in primary tumor volume was detected. Secondary tumor was first noted at liver after 21 days of cell inoculation. Unusual dark patches, due to accumulation of melanin within melanoma cells, was visualized and was considered as secondary growth of subcutaneous melanoma at liver. Nodule numbers were increased along with the increase in days and 7-9 metastatic nodules were noted per 4X4 mm2 of liver (Fig.1A) (ST) after 35 days of inoculation. 250mg/kg b.w. SAC (ST+SAC) and 5mg/kg b.w. CHEL (ST+CHEL) significantly regressed tumor number after 30 and 60 days of post treatment; while remedial efficacy was increased effectively in SAC+CHEL combinatorial approach ($p<0.01$).

Figure 1: SAC and Chelerythrine treatment restored liver morphology and function in B16F10 melanoma cell induced metastatic tumor bearing mice

Number of nodules at liver collected from control, B16F10 cell injected and individual 250mg/kg b.w. SAC, 5mg/kg b.w. CHEL as well as 250mg/kg b.w. SAC+ 5mg/kg b.w. CHEL co-treated groups of mice, were estimated and represented in average number of nodules/4X4mm2 (A). Survivability analysis (B) was measured and represented in number of survival animals. Liver stress specific bio markers ALT (C), ALKP (D), GGT (E) and AST (F) were estimated in blood serum isolated from untreated and treated control, B16F10 cell injected and individual 250mg/kg b.w. SAC, 5mg/kg b.w. CHEL as well as 250mg/kg b.w. SAC+ 5mg/kg b.w. CHEL co-treated groups of mice. Values were represented in IU/L. Data were expressed as mean±SD and were obtained from six independent experiments (n=5). NS,*p<0.01 vs Control, #p<0.01 vs ST. Con=Control, SAC=S-allyl Cystine, CHEL= Chelerythrine, ST= Secondary Tumor.



Following 45days of 250mg/kg b.w. SAC treatment in combination with 5mg/kg b.w. CHEL (ST+SAC+CHEL) reduced number of tumor nodules effectively than 60days individual drug treatment ($p<0.01$). Results also depicted more or less elimination of all metastatic melanoma nodules as well as that eventually restored hepatic morphology ($p<$NS vs. Control) (Fig. 1A) more or less similar to control mice after 60days of combined treatment. Comparative drug trials use risk of survival analysis during the assessment of clinical efficacy (Dahal et al., 2019). Considering this technical perspective experiments were designed and values from survivability analysis depicted a sharp decrease in numbers of survived animals in B16F10 inoculated ST groups after 95days of experimental schedule.

One month treatment with either of the single drug (ST+SAC/ST+CHEL) and in combination (ST+SAC+CHEL) demonstrated considerable increase in survivability rate ($p<0.01$). Combined therapy (ST+SAC+CHEL) for 45days and especially for 60days depicted distinct reduction in mortality rate than 60days of individual drug treated groups ($p<0.01$) (Fig.1B). Non-significant alterations in survivability rate (Fig.1B) were noticed in individual and combinatorial treatment for 60days in control group of mice.

Estimation of biochemical stress markers specific for liver function after S-allyl Cystine and Chelerythrine treatment in B16F10 induced metastatic melanoma at liver: Alanine aminotransferase (ALT), alkaline phosphatase (ALKP), γ-glutamyl transferase (GGT) and aspartate aminotransferase (AST) activity in plasma are well-known serum biomarkers for the estimation of liver injury and function (Lala et al., 2020). Altered activities of those stress specific biomarkers are the indicative of abnormalities in liver homeostasis. Data illustrated significant increase in plasma ALT, AST, ALKP and GGT level in ST groups. Values also depicted a trend of gradual regression of all the biomarkers after individual SAC or CHEL treatment (ST+SAC/ST+CHEL) for 30days and 60days.

Analysis suggested that ALT, AST, ALKP and GGT activities ($p<0.01$) after 45days of co-treatment with SAC+CHEL (ST+SAC+CHEL) were in a range of individual SAC or CHEL treatment for 60days. Whereas most significant efficacy ($p<0.01$) was noticed after 60days of combined (ST+SAC+CHEL) therapy (ALT=58.7892$\pm$5 IU/L, AST= 48.7892$\pm$5 IU/L, ALKP=79.7892$\pm$5 IU/L, GGT=36.7892$\pm$5 IU/L) ($p<0.01$). No considerable alterations were documented in either of ALT, AST, AKLP and GGT levels in individual or combined treatment for 60days in control mice (Fig.1C, 1D, 1E, 1F) indicating no such effectual toxic impact of the said drugs both in individual and combined schedule.

During metastasis migratory tumor cells invade into distal location and establish tumorigenic growth (Fares eta l., 2020). In the present study aggressive melanoma cell B16F10 demonstrated metastatic migration and colonization, proliferation and finally development of secondary tumors in the liver. Our data suggested that incidence of metastatic melanoma hampers normal liver function and physiological activity as observed other fatal liver injuries (McGill et al., 2019).

Disease severity as well as liver injury was gradually progressed with the increase in nodule numbers similar to the previous observations on liver cancer (Simoes Eugenio et al., 2020). Various contemporary researchers already portrayed SAC and CHEL as an effective drug against hepato-cellular carcinoma, liver cirrhosis and other liver diseases (Chmura et al., 2000; Ng et al., 2012). In our study, results first time noted noteworthy improvement of animal survivability by restoring liver morphology and homeostasis in B16F10 infused mice after SAC and CHEL combined treatment. Hence, combinatorial practice can able to heal metastatic melanoma at liver and this finding pointed us to look over the underlying aspects behind this remedial effect.

Evaluation of tissue specific stress and associated factors in B16F10 infused mice: According to the reports, SAC and CHEL are potential free radical scavengers and are able to modulate various ROS dependent biological activities like: cell survivability, cell proliferation, metabolic activity, apoptosis, etc. (Chen et al., 2016; Sengupta et al., 2017). Serum markers are also modified by the interactions of ROS with the antioxidant system present in the tissue microenvironment (Sengupta et al., 2017). Here elevated levels of liver stress specific biomarkers instigated us to study about the enzymatic as well as non-enzymatic anti-antioxidants and accumulation of the oxidized products/ reactive oxygen species since the imbalance between ROS and anti-oxidants, similar to previous studies, might be responsible for the development of liver stress in our study.

Analysis of enzymatic antioxidant pool was performed by measuring SOD and Catalase activity in liver isolated from control, ST and SAC/CHEL treated as well as co-treated (ST+SAC+CHEL) groups of animals. Results depicted reduced SOD (16.89234 Unit/mg protein) and very low catalase (5.8990234 Unit/mg protein) activities in liver of ST group of mice; although SOD and Catalase activities were repleted along the course of SAC and CHEL treatment for 30 and 60days. 45days of co-treatment demonstrated the values in a range of 60days of individual treatment while most effective repletion was noticed after 60days of co-treatment (ST+SAC+CHEL) ($p<0.01$) (Fig. 2A, 2B). Non-enzymatic antioxidant assay comprises of reduced glutathione (GSH) analysis.

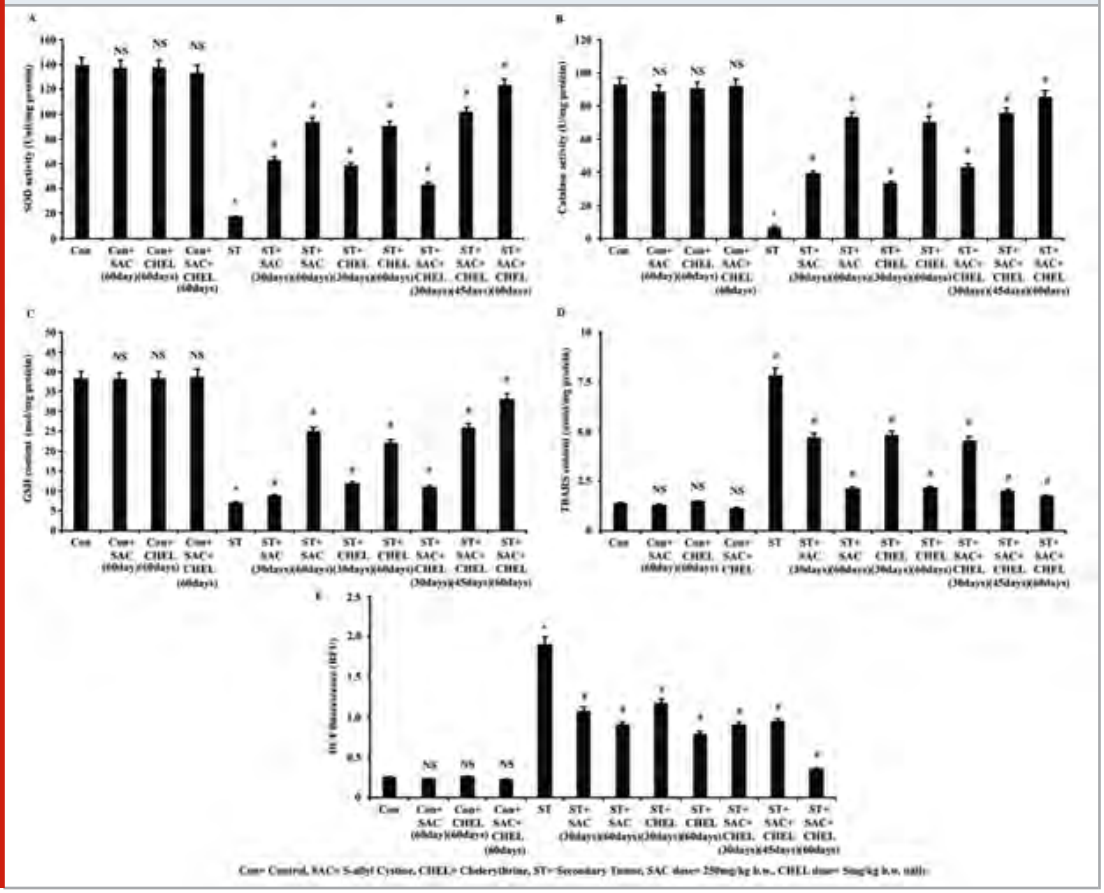
Data suggested reclaiming of GSH content after both 30 as well as 60days of individual treatment and co-treatment category portrayed similar improvement as before specially after the schedule of 60days (Fig. 2C). No considerable changes were revealed in SOD (Fig. 2A), Catalase (Fig. 2B) activities and GSH level (Fig. 2C) after 60days of individual or co-treatment of control mice. Thiobarbituric acid reactive substance (TBARS) content was estimated as an indication of cytotoxic stress (Chatterjee et al., 2019). Data indicated notable

enhancement of TBARS in liver collected from ST mice (7.79203405 nmol/mg protein) and value was normalized after individual and co-treatment with SAC and CHEL.

Observation further indicated the most significant reduction only after 60days of co-treatment (ST+SAC+CHEL) (1.892345 nmol/mg protein) ($p<0.01$) (Fig. 2D). Direct estimation of ROS was performed using DCFDA as fluorescent probe. Analysis depicted a noteworthy enhancement of DCF fluorescence in liver collected from ST group ($p<0.01$) with a trend towards normalization of cellular ROS in the treated groups. Values illustrated effective suppression of ROS in co-treated (ST+SAC+CHEL) group after 45 days and highest level of reduction was noticed after 60days ($p<0.01$) of treatment. No significant alteration was noted in DCF fluorescence and TBARS content after 60days of individual or combined treatment of control mice (Fig.2E).

Figure 2: Estimation of alteration in reactive oxygen species, TBARS content and antioxidant system after SAC and Chelerythrine administration

Lysates were processed from liver of untreated and treated control, B16F10 cell injected and individual 250mg/kg b.w. SAC, 5mg/kg b.w. CHEL as well as 250mg/kg b.w. SAC+ 5mg/kg b.w. CHEL co-treated mice. Status of enzymatic antioxidant level was estimated through measuring SOD (A) and catalase (B) activities and was represented as Unit/ mg protein. GSH (C) and TBARS (D) content were measured and were represented as nmol/mg protein. Level of reactive oxygen species was estimated by measuring DCF fluorescence (E) and was represented in RFU. Values were expressed as mean ±SD and were obtained from six independent experiments (n=5). NS,*p<0.01 vs Control, #p<0.01 vs ST. Con=Control, SAC=S-allyl Cystine, CHEL= Chelerythrine, ST= Secondary Tumor.



According to the reports ROS is generated in the form of highly reactive free radical superoxide which is dismutated into oxygen and hydrogen peroxide through enzymatic activity of SOD (Sengupta et al., 2017 A). Catalase further scavenges H2O2 to convert it into water and O2; in this way accumulation of ROS is averted (Sengupta et al., 2017 A). According to the present study tumour site of the liver in secondary melanoma showed very poor SOD and catalase activity along with a significant suppression of GSH content that probably nourished tumor microenvironment and helped in metastatic tumor formation.

SAC and CHEL treatment effectively augmented both enzymatic as well as non-enzymatic antioxidant system and accumulated ROS was neutralized by elevated catalase activity, finally hydrolyzed to non-toxic substance water and oxygen. Increase in GSH content

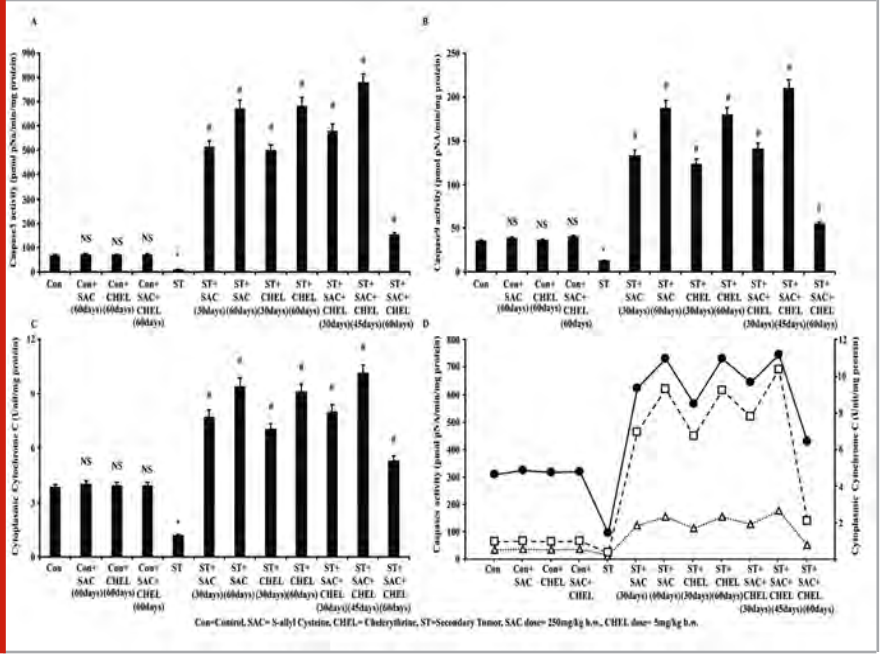
also added further protection to the tissue from oxidative stress. By scavenging ROS these anti-oxidant drugs also suppressed TBARS content similar to the effects as noted in other carcinogenic studies (Sang et al., 2019). This in turn probably helped to restrict metastatic tumor progression. So, the study experimentally proved the tuning role of SAC and CHEL in the maintenance of a balance between anti-oxidants and accumulated ROS leading to modulation of hepatic physiology as reflected in biomarker analysis, most significantly in combined approach (Sang et al., 2019).

Assessment of growth associated regulatory factors in B16F10 induced metastatic melanoma at liver: Cancer cell proliferation and tumour establishment are associated with negative modulation of the apoptosis (Connor et al., 2019). Reports suggested significant association of ROS with the regulation of apoptosis in cancer cells (Sang et al., 2019). Researchers further demonstrated the impacts of ROS in the reduction of growth of various primary tumors originated at colon, breast, liver, lung etc. in both *in vivo* and *in vitro* condition (Sengupta et al. 2017). Previous studies recommended chief executive role of caspases in the conduction of apoptosis (Phillips et al., 2020).

Considering the instructive responsibility of ROS in guide lining the Caspases here activities of Caspase9 as initiator and Caspase3 as executioner were studied for mechanistic analysis (Li et al., 2020). Results depicted significant enhancement in the activity of both Caspase9 (Fig. 3B) and Caspase3 (Fig. 3A) ($p<0.01$) in the liver of ST group of mice after individual as well as combined SAC and CHEL treatment. Interestingly activities of the Caspases were increased after 45days of combined therapeutics against individual treatment of either of the drugs ($p<0.01$); while continuation of the treatment up to 60days revealed distinct reduction in activities of both of the enzymes towards the level of control animal (Fig. 3A, 3B).

Figure 3: Evaluation of changes in CytochromeC distribution-caspase activity after SAC and Chelerythrine treatment

Estimation of Caspase3 (A) and Caspase9 (B) activity in whole cell lysate of liver isolated from untreated and treated control, B16F10 infused and individual 250mg/kg b.w. SAC, 5mg/kg b.w. CHEL as well as 250mg/kg b.w. SAC+ 5mg/kg b.w. CHEL co-treated group; were represented in pmol pNA/min/ mg protein. Cytoplasmic CytochromeC level (C) in cytoplasmic fraction of the above-mentioned lysates was quantified and were represented in Unit/mg protein. Data were mean±SD and were obtained from six independent experiments (n=5). NS,*$p<0.01$ vs Control, #$p<0.01$ vs ST. Comparative analysis in-between Caspase3, Caspase9 activity with released CytochromeC was portrayed in figure 3D. Values were mean±SD and were obtained from six independent experiments (n=5). Con=Control, SAC=S-allyl Cystine, CHEL= Chelerythrine, ST= Secondary Tumor.



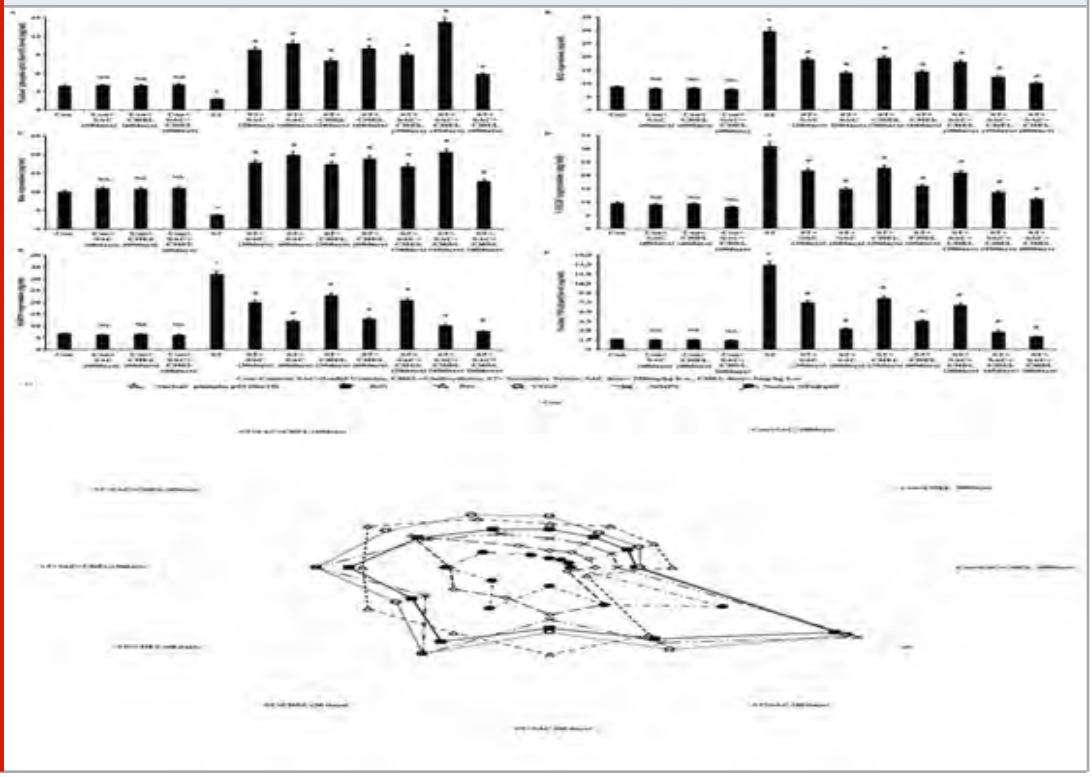
Caspase9 activity is dependent upon the release of mitochondrial CytochromeC to the cytoplasm which is again dependent upon accumulation of intracellular ROS as suggested (Li et al., 2020). Data demonstrated significant increase in CytochromeC content in cytoplasmic fraction of liver isolated from individual

and with a peak in 45days of combined drug treated group ($p<0.01$). Although similar to Caspase9 activity of CytochromeC was markedly reduced towards control level after 60days of combined treatment (Fig 3C). No such noteworthy changes were visualized in Caspase3 (Fig. 3A), Caspase9 (Fig. 3B) activities and cytoplasmic CytochromeC distribution (Fig. 3C) after 60days of individual or combined treatment to control mice (Lin et al., 2020).

Combined ray diagram of CytochromeC level in cytoplasm with the activities of both of the Caspases portrayed similar trend in the changes following the scheduled treatment suggesting a potential role of cytosolic CytochromeC in the activation of Caspases in metastatic melanoma tumor containing area of liver of B16F10 infused mice (Fig 3D). It is well evident that Cytochrome C- Caspase axis is turned off in cancer cells that in turn helped in tumor progression and metastasis. In our study SAC and CHEL administration effectively tuned on ROS dependent CytochromeC-Caspase axis and significantly induced apoptosis in colonized metastatic melanoma cells at liver. Similar effects were reported in the primary cancers like colorectal cancers, liver cancer and breast cancer (Chen et al., 2016 and Sengupta et al. 2017). Therefore, the proposed therapeutics significantly reduced symptomatic impacts in the B16F10 infused mice by triggering caspase mediated cell death at secondarily developed melanoma in liver as observed in our study.

Estimation of the status of biomolecules responsible for SAC and CHEL induced apoptosis, tissue degradation and angiogenesis: According to the previous reports cytoplasmic level of CytochromeC is significantly lower in the colony of cancer cells (Yau et al., 2019). Release of CytochromeC into cytoplasm is harmonized by proapoptotic-antiapoptotic balance and generally increased level of cytoplasmic CytochromeC level indicates an imbalance between Bax and Bcl2, the well-known proapoptotic and antiapoptotic proteins, respectively (Sengupta et al. 2017).

Figure 4: Estimation of the status of apoptotic, angiopoietic and ECM degrading guardian factors after SAC and Chelerythrine treatment
Bcl2 (B), Bax (C), VEGF (D), MMP9 (E) expression were measured in whole cell lysate and level of phopho-p53-Ser15 (A) and NFκβ/p65 (F) were estimated from nuclear fraction of the liver isolated from untreated and treated control, B16F10 cell injected and individual 250mg/kg b.w. SAC, 5mg/kg b.w. CHEL as well as 250mg/kg b.w. SAC+ 5mg/kg b.w. CHEL co-treated group; were represented in ng/ml protein. Results were mean±SD and were obtained from six independent experiments (n=5). NS,*$p<0.01$ vs Control, #$p<0.01$ vs ST. Comparative analysis of phopho-p53-Ser15, Bcl2, Bax, VEGF, MMP9 and NFκβ/p65 levels among control, B16F10 cell injected and individual 250mg/kg b.w. SAC, 5mg/kg b.w. CHEL as well as 250mg/kg b.w. SAC+ 5mg/kg b.w. CHEL co-treated groups and was represented in spider chart (G). Con=Control, SAC=S-allyl Cystine, CHEL= Chelerythrine, ST= Secondary Tumor.



Various studies also suggested the imperative role of stabilized p53 in the up-regulation of Bax expression leading to CytochromeC release into cytoplasm from mitochondria (Somade et al., 2020). Recent reports further suggest the wobbly level of p53 in the colonized cancer cells (Capaci et al., 2020). Present analysis demonstrated the elevated level of Bax in cytoplasm (Fig. 4C) and phospho p53-Ser15 in nuclear fraction (Fig. 4A), the stabilized from of p53 and a potential transcriptional factor of Bax, in both individual SAC and CHEL as well as 45days of combined treatment of ST animals ($p<0.01$). Further continuation of the combined treatment for another 15days showed a downfall of Bax (Fig. 4C) and phospho p53-Ser15 (Fig. 4A) level towards control animal.

While Bcl2 expression was significantly decreased along the course of the schedule both in individual and combined treatment and value after 60days of SAC+CHEL treatment demonstrated a range nearing to control mice ($p<0.01$) (Fig. 4B). No significant change was revealed after 60days of individual or combined treatment to control group of mice ($p<0.01$) (Fig. 4A, 4B, 4C). Therefore, SAC and CHEL treatment appreciably induced p53-Bax axis which was responsible for the activation of Cytochrome C-caspase pathway as observed in our study and probably took a part in controlling the melanoma cell progression in the liver as secondary site. Angiogenesis and modification in extracellular matrix by the factors secreted from cancer cells alter tumor microenvironment and help in metastatic invasion (Fares et al., 2020). It is evident that ROS can able to increase angiogenic processes and ECM remodeling via uplifting the expression of assisting factors like matrix metalloproteinases (MMPs) in the carcinogenic foci of a tissue (Bockmann et al., 2020).

Recent studies mentioned that attenuation of angiogenic regulatory (Ang-I, Ang-II, VEGF, etc.) and extracellular matrix degrading (MMP-2, MMP-3, MMP9, etc.) factors perform the crucial job related to the suppression of metastasis and reduction of tumor volume in liver, colorectal and prostate carcinoma as well as retinoblastoma, etc. (Chan et al., 2020). Result illustrated reduction of VEGF ($p<0.01$) (Fig. 4D), a well evident prime nourishing protein for angiogenesis and MMP9 ($p<0.01$) (Fig. 4E), factor that helps in the progression of metastatic tumor through restructuring extracellular matrix expression after individual SAC or CHEL as well as combined treatment categorically after 60days of SAC+CHEL treatment to ST group of mice (Fig. 4D, 4E). No effective alterations were suggested after individual and combined treatment to control animal (Fig. 4D, 4E).

Our studies further demonstrated significant nuclear localization of NFκβ/p65 in the metastatic site of the liver in B16F10 infused mice. According to the reports nuclear translocalization of NFκβ/p65 trigger VEGF and MMP9 expression to the tumorigenic site in parallel to ROS accumulation that in turn generates a a microenvironment favorable to further invasion and sustenance of the metastasis (Viswnadha et al., 2020). Nexus between the ROS and NFκβ/p65 nuclear localization along with VEGF and MMP9 expression guided us to investigate the status of NFκβ/p65 after SAC and CHEL treatment in our experimental model.

Present study evidently portrayed effective repression in nuclear localization of NFκβ/p65 both after individual as well as combined treatment with a most significant reduction after 60days of SAC+CHEL treatment and the value was nearly the level of control animal ($p<0.01$) (Fig. 4F). No such distinct alterations in NFκβ/p65 nuclear localization were noted after 60days of individual and combined treatment of control mice (Fig. 4F). Data presentation in spider chart of phospho p53-Ser15 and NFκβ/p65 level within nucleus, Bcl2, Bax, VEGF and MMP9 expression demonstrated their association with the alterations in liver of ST group of mice.

In summary categorical reduction in phospho p53-Ser15 nuclear localization and Bax expression as well as corresponding augmented Bcl2 expression in parallel to enhancement in NFκβ/p65 nuclear localization leading to increase in VEGF and MMP9 expression helped in development of a microenvironment favourable for the establishment of secondary melanoma in the liver of B16F10 infused mice. Analysis suggested increase in phospho p53-Ser15 nuclear localization-Bax expression, reduced Bcl2 expression, reduction in NFκβ/p65 nuclear localization, VEGF as well as MMP9 expression in individual and distinctly after 45days of combined treatment. Cumulatively the said scenario was probably responsible for the reduction of the tumorigenic growth at the metastatic sites of the liver in our model.

Moreover, 60days of SAC+CHEL treatment demonstrated a range of all parametric values tending towards control mice. Values indicated probable restoration of the tissue along with significant reduction of morphological alterations of the liver developed due to metastatic melanoma in ST group of mice (Fig. 4G). In the end it can be stated that our *in vivo* study was first time designed to evaluate anti metastatic property of individual as well as combined therapeutic effect of SAC and CHEL against metastatic melanoma in liver. The results clearly stated the efficacy of this therapeutic approach in the suppression of metastatic melanoma and normalization of native liver physiology.

Better efficacy in switching on the p53- Bax-CytochromeC axis along with reduction in NFκβ/p65 dependent VEGF and MMP9 expression after 45days of SAC+ CHEL administration pointed out the importance of combined therapy. Way of normalization of the mentioned apoptotic signaling as well as VEGF/MMP9 level with the sustained antioxidant balance after 60days of SAC+CHEL treatment indicated effectual restoration as also suggested by the liver specific biomarker assay (Viswnadha et al., 2020).

CONCLUSION

In summary, our work confirmed the role of SAC and

CHEL as effective anti-metastatic agents that were able to target p53, as well as NFκβ dependent signaling orchestras and cured metastatic melanoma at liver by calibrating tissue ROS/anti-oxidant malady. Data further asserted improved remedial efficacy along-with no such toxicity effects in combined therapeutics. Therefore, SAC and CHEL administration in combination may be considered for formulating effectual therapeutics to treat metastatic melanoma at liver.

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Declaration of competing interest: The authors declare that there are no conflicts of interests.

Author Contribution: SC and DP performed the experiments, maintained cell line, developed experimental mice model, contributed in research designing, assisted in data analysis and manuscript preparation. PG and SB helped in sample preparation, enzyme analysis, protein quantification and also involved in manuscript preparation. KDC participated in research idea development, contributed in experimental designing and execution, data analysis and manuscript preparation. PC participated in data representation and manuscript preparation. AB and GCS participated in research idea development, experiment designing, data analysis and editing of manuscript.

Ethical Clearance Statement: All animal experiments were designed following Institutional Animal Ethical Committee Guidelines to reduce the animal sufferings without hampering the requisites of statistical analysis

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Applying Machine Learning Algorithms in Predicting Skills in-Demand for Technological Occupations in Saudi Arabia

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ABSTRACT

Many female students use social communications as a part of their digital literacy. There is no doubt that social communication skills, along with mental abilities – represent efficiency and effectiveness among university youth. Any defect in these communication skills may lead to an inability to adapt to the university environment. Consequently, students may lose many opportunities, and suffer academic progress. The aim of this study was to investigate the effectiveness of collaborative E- learning in developing social communication skills in the "Research Seminars " course among students of the seventh level of the College of Education at King Khalid University -Abha city - Saudi Arabia. The study followed the semi-experimental approach, which is the design of pre-measurement and post-measurement for two groups: the experimental group and the control group. The experimental group uses the collaborative learning method of network through the course forums to activate social communication with the pre and post application of the search tool on the two research groups. The researchers applied the tool (social communication scale) on a sample consisting of (25) students from the College of Education. The result confirmed the effectiveness of collaborative E-learning in developing the social communication skills of the experimental group. The study recommends, based on its results, to take advantage of the collaborative -networked learning method to develop innovative thinking skills among university students. as well, it is necessary to hold training courses for university faculty members to develop their skills in the use of online collaborative learning tools such as blogs and discussion forums in the educational process. Moreover, there is a necessity to educate faculty members about the importance of collaborative -networked learning method in developing social communication among university students.

KEY WORDS: EFFECTIVENESS - COLLABORATIVE -E- LEARNING - SOCIAL COMMUNICATION SKILLS.

INTRODUCTION

Violence at work is one of the major deterrents affecting "health and safety" of workers in all sorts of occupations (Pouryaghoub et al., 2017). Aggressive behavior of patients and their attendants towards dental health care providers is an increasingly significant yet an under reported problem (Franz et al., 2010; Duxbury & Whittington., 2005; Cooper & Swanson., 2002). The term 'aggression' is used to refer to hostile behavior of varying intensity that

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In-vitro Phytochemical Screening and Bioactivity of Moringa oleifera Accessions

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ABSTRACT

There is a huge demand of plant with substantial phytocompounds due to their health benefits. *Moringa oleifera* is an important and fast-growing plant species with beneficial immuno-modulating properties. *Moringa oleifera* can withstand high temperatures, drought and mild frost conditions and hence it can be widely grown across the world. Large numbers of reports are available on nutritional properties of this plant however; research work on bioactivity of wild *Moringa oleifera* in India is still scanty. Hence, the present study was aimed to identify and quantify important phytochemicals with potent bioactivity as nowadays microorganism is adopting multidrug resistance and to cope up with these difficulties there is a need to identify plant-based drug for the betterment of society. *Moringa oleifera* accessions were collected from different regions of Gujarat and they were screened for preliminary phytochemicals by using standard protocols. It was revealed that all the accessions were showing presence of important phytochemicals and the total phenolic content ranged from 0.0076 mg/g to 2.98mg/g GAE equivalent. The total flavonoids ranged from 1.12mg/g to 1.59mg/g QE equivalent and the total tannins were found in the range of 0.66mg/g to 1.35mg/g tannic acid equivalent. The antibacterial activity of *Moringa oleifera* accessions was carried out by implying agar well diffusion assay and it was found that MONV, MOVR and MOAN showed potent inhibitory activity against all the test bacteria in except *Vibrio cholerae*. Hence, these accessions could be further explored for *in vivo* studies with pure form of extracts for enhanced applications in pharmaceutical industries.

KEY WORDS: MORINGA OLEIFERA ACCESSIONS, PHYTOCHEMICALS, BIOACTIVITY, MIC.

INTRODUCTION

Moringa oleifera (Moringaceae family) is native to the Indian subcontinent and Africa and is also known as the Miracle tree. It is multipurpose tree species with multifarious pharmacological and nutraceutical property (Thurber and Fahey, 2009). Each and every part of *Moringa oleifera* possesses potential phytopharmacological properties. Apart from its use as a food product it also possesses Medicinal and Industrial application (Moyo et al., 2011). The leaf material of this plant is well-known for its high mineral content as it is rich in essential amino acid, vitamins and minerals (Tahiliani and Kar, 2000; Amabye and Tadesse, 2016). Owing to the multipurpose properties of *M. oleifera*, this plant is also rich in important antioxidants that can quench free radicals (Rockwood et al., 2013). It was also reported that *M. oleifera* is rich in important phytocompounds such as phenolics, flavonoids, tannins, and saponins with superior bioactivity (Mishra et al.,2011; Patel et al., 2014; Zainab et al., 2020).

The incidence of UTI (Urinary tract Infections) infections and Staphylococcal infection is still high in many developing countries and this is because of the lack of information and knowledge regarding multi-drug

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resistance (Arumugam et al., 2011). To overcome the problems associated with synthetic drugs, researchers are now focusing on plant-based materials as they are readily available with no known side effects (Ncube et al., 2008; Al_husnan and Alkahtani,2016). Various portions of this plant including gum, pod extract, flowers and roots, have shown potential effects as a source of indigenous medicine (Odebiyi and Sofowora, 1978; Anwar et al., 2007; Sandeep et al., 2019). Therefore, the objective of this study is to evaluate the antibacterial efficacy and to screen *Moringa oleifera*'s substantial phytochemicals to imply a natural plant-based system as an alternative to the synthetic drug (medicine) system.

MATERIAL AND METHODS

Moringa oleifera accessions were collected from different regions of Gujarat such as Navsari, Anand, Bardoli and Vadodara (Table 1) and were maintained at study farm of Uka Tarsadia University, Bardoli, Gujarat, India. The voucher specimens were deposited at herbarium of C.G.Bhakta Institute of Biotechnology, Uka Tarsadia University. Disease free and healthy *Moringa oleifera* leaves of all the accessions were thoroughly washed under running tap water and dried separately at room temperature for three weeks until completely dried. The powdered samples (5g) were extracted by double distilled water (150 ml) using soxhlet apparatus and were dried and preserved at 4 °C prior to further use (Patel et al., 2020). Preliminary phytochemical screening was carried out by following standard protocols as defined by (Harborne, 1984; Patel et al., 2020).

Table 1. *Moringa oleifera* **accessions collected from different regions of Gujarat (Tables were prepared by us according to the area of collection).**

Sr. no	Accession code	Area of Collection
1	MONV	Navsari
2	MOBL	Bardoli
3	MOVR	Vadodara
4	MOAN	Anand

Table 2. Standard bacterial strains used in the study

Sr.no	Bacterial strain	NCIM Accession no
1	*Escherichia coli*	NCIM 2931
2	*Vibrio cholerae*	NCIM 5316
3	*Bacillus subtilis*	NCIM 2921
4	*Staphylococcus aureus*	NCIM 5345

The quantitative estimation of phenolic and flavonoid contents of *Moringa oleifera* accessions was determined by following Folin–Ciocalteu and Aluminum chloride colorimetric method respectively as reported by (Patel et al., 2020). The presence of total tannins was determined by using the method reported by (Patel et al., 2019). The bacterial strains used in the study were procured from National Collection of Industrial Microorganisms (NCIM), Pune, India (Table 2). The standard bacterial strains were maintained on nutrient agar medium at 37°C prior to further use (Balouiri et al., 2016; Patel et al., 2019).

Agar well diffusion method was employed for monitoring the antibacterial activity of *Moringa oleifera* extracts where streptomycin (10µg/ml) was used as positive control and zone of inhibition was recorded in mm (Balouiri et al., 2016). Broth Macro-dilution method was employed in investigating the Minimum inhibitory concentration (MIC) of *Moringa oleifera* extracts (Rahman et al., 2009; Adamczak, et al., 2020).

RESULTS AND DISCUSSION

The present study was carried out to analyze the preliminary phytochemicals present in *Moringa oleifera* accessions collected from different regions of Gujarat. Preliminary phytochemical screening revealed the presence of Alkaloids, Phenols, Flavonoids, Tannins, Carbohydrates and Amino acids in all the accessions except alkaloids, it was absent in the accession collected from Bardoli and Valsad (Table 3). The quantitative analysis of phenolic, flavonoids and tannin content are as shown in (Table 4). The total phenolic content ranged from 0.0076 mg/g to 2.98mg/g GAE equivalent in the order of MONV>MOAN>MOVR>MOBL. The total flavonoids ranged from 1.12mg/g to 1.59mg/g QE equivalent in the order of MONV>MOAN>MOVR>MOBL. Total tannins were found in the range of 0.66mg/g to 1.35mg/g tannic acid equivalent in the order of MOBL>MOVR>MOAN>MONV.

Table 3. Preliminary Phytochemical Screening of Aqueous extract of *Moringa oleifera* **accessions + indicates present; - indicates Absent**

Phytochemical Constituent	Accessions			
	MONV	MOBL	MOVR	MOAN
Alkaloid	+	-	-	+
Phenol	+	+	+	+
Flavonoids	+	+	+	+
Tannins	+	+	+	+
Carbohydrates	+	+	+	+
Amino acid	+	+	+	+

Antibacterial activity of *Moringa oleifera* accession MONV, MOVR and MOAN showed potent inhibitory activity against all the test bacteria in dose dependent manner except Vibrio cholerae (Table 5 and Table 6). Previous studies also reported good antibacterial activity of *Moringa oleifera* leaf extracts against various human pathogens (Rahman et al., 2009; Amabye and Tadesse, 2016).

The antibacterial property attributed to any plant material is because of the presence of important

phytochemicals such as Phenols, Flavonoids, Alkaloids, Triterpenoids and Saponins (Chandra et al., 2014; Patel et al., 2020). Phytochemicals play a major role in preventing various diseases. They possess immunomodulating and cardioprotective properties. Several researchers have reported that flavonoids and phenols play a major role in showing inhibitory activity by modifying or preparing complex with bacterial cell wall (Olowosulu and Ibrahim, 2006). In a study conducted on antibacterial activity of different extracts of *Moringa oleifera* leaf against pyogenic bacteria the ethanolic extract showed highest bioactivity when compared to hot water extract (Fouad et al., 2019).

Table 4. Quantitative Phytochemical Analysis of Aqueous extract of *Moringa oleifera* accessions n=3, Value indicates Mean ±SEM

Phytochemical Constituent mg/g	Accessions			
	MONV	MOBL	MOVR	MOAN
Phenol	2.98±0.02	0.0076±0.003	0.14±0.031	0.35±0.09
Flavonoids	1.59±0.29	1.12±0.088	1.43±0.18	1.54±0.29
Tannins	0.66±0.008	1.35±0.16	0.97±0.013	0.74±0.03

Table 5. Antibacterial activity of *Moringa oleifera* accessions against test organism at the concentration of 50 mg/ml n=3, Value indicates Mean ±SEM

Test Organisms	Accessions (zone of inhibition in mm) Concentration (50mg/ml)				Streptomycin
	MONV	MOBL	MOVR	MOAN	
E.coli	0.66±0.33	-	-	4.66±0.33	8.6±0.33
S.aureus	7.66±0.33	-	-	-	10.66±0.33
V. cholerae	-	-	-	-	7.25±0.08
B. subtilis	1.66±0.33	-	4.66±0.33	-	9.34±1.3

Table 6. Antibacterial activity of *Moringa oleifera* accessions against test organism at the concentration of 100 mg/ml n=3, Value indicates Mean ±SEM

Test Organisms	Accessions (zone of inhibition in mm) Concentration (100mg/ml)				Streptomycin
	MONV	MOBL	MOVR	MOAN	
E.coli	*4.33±0.33*	-	-	*10.33±0.33*	*8.6±0.33*
S.aureus	*12.33±0.33*	-	-	-	*10.66±0.33*
V. cholerae	-	-	-	-	*7.25±0.08*
B. subtilis	*3.33±0.66*	-	*9.33±0.33*	-	*9.34±1.3*

Table 7. Minimum Inhibitory Concentration against test organism

Test Organisms	Accessions		
	MONV	MOVR	MOAN
E.coli	37.5mg/ml	-	75mg/ml
S.aureus	18.75mg/ml	-	-
B. subtilis	9.37mg/ml	18.75mg/ml	-

However, the present study showed good antibacterial activity of aqueous extract of *Moringa oleifera* against the pathogenic bacteria. The minimum inhibitory activity of the extracts ranged from 9.37mg/ml to 75mg/ml against *E. coli, S. aureus* and *B. subtilis* (Table 7). Thus, in the present study it was revealed that there is a varietal response in bioactivity of *Moringa oleifera* accessions because of broad spectrum of antibiotics present in these extracts. Hence, *Moringa oleifera* can serve as good and natural immunobooster to treat various ailments (Fouad et al., 2019).

CONCLUSION

Owing to the issue of drug resistance caused by microbial mutation over the years, certain antibiotics have become almost ineffective. Thus, it can be inferred from the present analysis that there is a wide variability in phytochemical constituents of *Moringa oleifera* accessions collected from different regions of Gujarat and in order to establish the relationship between the MIC's obtained in this study and the active doses at which the herbs can be used in conventional practice these accessions could be further explored for in vivo studies with pure extracts for enhanced applications in pharmaceutical as well as herbal industries.

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Conflict of Interest: The authors have no conflict of interest to declare.

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Parametric Optimization of Oil Extraction and Lipase Catalyzed Biodiesel Production from Rice Bran

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ABSTRACT

Biodiesel is an environmental friendly, renewable and biodegradable fuel and a potential substitute to conventional diesel, and can be produced using vegetable oil with short chains of alcohol. Due to the high cost of produced biodiesel, it is required to explore inexpensive feedstock with high value-added by products. Rice bran is a by-product of rice milling which is mainly used as animal feed and oil produced is used for industrial applications. Rice bran oil (RBO) can be used as a low-cost feedstock for biodiesel production as compared to traditional oils derived from cereal or seed sources. In this study, rice bran was taken as feedstock and oil was extracted from it which was further converted to Biodiesel. Optimization of oil extraction process from rice bran was done taking into account the affecting factors like solvent used for extraction, solvent to solid ratio and extraction time. Biodiesel was produced from extracted rice bran oil using lipase as catalyst. Process variables like molar ratio of methanol to oil and catalyst concentration were studied for maximum ester yield. The optimized conditions for oil yield were found to be hexane as best solvent, 10:1 solvent to solid ratio and 8 h extraction time. Further, the produced RBO was utilized for lipase enzyme catalyzed biodiesel production. The process parameters affecting ester yield were optimized and were found to be 6:1 methanol to oil molar ratio and 4% (w/w_{oil}) lipase enzyme concentration. The RBO biodiesel was characterized and found to fulfill the requirements of ASTM and DIN international standards for biodiesel.

KEY WORDS: BIODIESEL; RICE BRAN; OPTIMIZATION; OIL EXTRACTION; LIPASE ENZYME.

INTRODUCTION

The increase in industrialization and population worldwide have created a huge demand for fuels and at the same time increased air pollution and decrease in fossil fuels deposits. Hence, there is a requirement of developing new forms of renewable and eco-friendly fuels (Venkanna and Venkataramana, 2009; Dayang et al., 2019; Febrian et al., 2020). One of the approaches is waste-to-energy technologies, where waste matter is converted into renewable energy. This technology can solve both problems: waste and energy. Waste can be treated and reused to be converted into the various forms of fuel which can be used for energy generation. The conversion of waste into biofuel has become more attractive due to it being low-carbon, locally available, safe, and sustainable for the economy (Rengasamy et al., 2018; Goga et al., 2019).

Energy generation from biofuel in various approaches has been explored in order to generate a suitable quality of bioethanol, biodiesel, biogas, and biohydrogen. Biofuel can be produced from three main sources: vegetable oil and animal fat, nonfood crops and algae (Dayang et al., 2019; Anh et al., 2020). There are various types of reaction that can be applied to the production of biofuels, such as fermentation, transesterification, and pyrolysis of biomass and industrial and domestic waste.

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Transesterification is a simple and efficient method for biofuel production. Transesterification involves catalytic (acid, base and enzymes) reaction between alcohol and triglycerides of fatty acids to form esters and glycerol. Enzymatic transesterification is better than chemical transesterification, due to low energy requirements, easy glycerol recovery and transesterification of high free fatty acid content glycerides (Xiulian et al., 2012; Bani et al., 2018; Goga et al., 2019, Dharmaraja et al., 2019; Febrian et al., 2020).

Biodiesel is a biofuel that is produced from the transesterification reaction of vegetable oils, animal fats, or grease. Biodiesel can be used as a substitute for petro diesel because it can be used in any diesel engine without modification (Dayang et al., 2019). Biodiesel is nontoxic, biodegradable, has a high flash point, and reduces emissions of unburned matter and particulate matter (Dayang et al., 2019; Anh et al., 2020). Initially the production of biodiesel was focused on edible oils such as vegetable oil (soybean oil, sunflower oil, and cottonseed oil) and animal fat. Agricultural industries are the main contributor of raw material in producing biodiesel (Nguyen et al., 2019).

India being the largest producer of rice (*Oryza sativa*) has a capability to manufacture about 1 million tonnes of RBO per year. Rice bran containing 15-23% oil is a byproduct of rice milling (Syed et al., 2016; Bani et al., 2018). Rice bran oil contains naturally occurring bioactive and antioxidant compounds. De-oiled rice bran is used as poultry and cattle feed due to its high protein content and vitamins (Fajriyati et al., 2019; Anh et al., 2020). Presently, the industry is utilizing rice bran of around 3.5 million tonnes to produce around 0.65 million tonnes RBO. This unused non-edible oil can be used as an alternative energy source to reduce the biodiesel production cost. The objective of this study was to extract oil from rice bran and produce biodiesel from it. The effect of different process parameters like solvent type, solvent to solid ratio, reaction time affecting the oil yield and variables like alcohol to oil molar ratio, concentration of catalyst affecting the biodiesel yield were studied. Characterization of the produced biodiesel was also done in this study.

MATERIAL AND METHODS

For the materials, rice bran was obtained from local rice mill, Hubballi. Laboratory grade hexane, petroleum ether and methanol were procured from SRL Pvt Ltd. Lipase enzyme was procured from Sigma Aldrich Company. For the extraction of oil from rice bran, twenty grams of powdered rice bran husk was taken in a Soxhlet extractor. 200 ml of hexane / petroleum ether was used in the extraction process. Soxhlet extractor was operated for a reaction time of 8 h and the extract was subjected to filtration using Whatman filter paper to remove the suspended particles. Then it was subjected to rotary evaporator for oil solvent separation. The percentage of oil yield was determined by the ratio of amount of oil extracted by the amount of rice bran husk taken multiply by 100.

For the process parameter optimization for rice bran oil extraction, the effect of three main parameters on oil extraction was studied. The solvent type, extraction time and solvent to solid ratio were studied and optimized the operating conditions for maximum oil yield. Twenty grams of meal (rice bran husk) was subjected to two different solvents namely petroleum ether and hexane, solvent to solid ratio between 6:1 to 10:1 v/w and reaction time between 1 h to 8 h. The optimum conditions were determined by varying one factor at a time. For the process parameter optimization for RBO biodiesel production, transesterification involves catalyzed reaction between triglycerides and an alcohol yielding esters and glycerol. To optimize the operating conditions for RBO biodiesel production, one factor at a time (OFAT) was used. The effect of lipase concentration (1%, 2%, 3%, 4% w/w_{oil}), and molar ratio of methanol to oil (2:1, 4:1, and 6:1) on biodiesel production was studied. Methanol and lipase catalyst were mixed and then added to RBO in the conical flask. The conical flask was plugged to avoid evaporation of methanol and then placed on a shaker for 24 h at 150 rpm at room temperature.

The mixture was kept in the separating funnel overnight where glycerol was separated by gravity separation. The biodiesel produced was washed with warm water several times to remove catalyst, glycerol residuals and methanol. The crude biodiesel was then heated at 100 oC in an open pan to remove all the water particles. The % biodiesel yield was found by the equation: [weight of Biodiesel produced/ weight of oil taken] * 100. For the produced biodiesel physical properties such as carbon residue, density, flash point and viscosity were found. For the characterization of rice bran oil biodiesel, the characterization of RBO biodiesel was done using standard test procedures. Density was determined by ASTM D1298, Flash point by ASTM D93, Kinematic viscosity by ASTM D445 and Carbon residue by ASTM D4530.

RESULTS AND DISCUSSION

Rice bran oil extraction:

Figure 1: a) Powdered Rice bran husk and b) Rice bran oil

Process parameter optimization for RBO extraction: Effect of solvent type on oil yield: Two different solvents petroleum ether and hexane were used to determine their

influence on oil yield. Fig. 2 shows the oil yields by the solvent's petroleum ether and hexane. The oil yield with hexane (6.25%) was observed to be more than that of petroleum ether (4.16%) by 2.09% as evaluated at the end of all experiments conducted, by varying the other two parameters. Therefore, hexane was considered best solvent for RBO extraction. It is similar to the results obtained by (Tamilarasan and Sahadevan, 2012; Syed et al., 2016; Shukla and Pratap, 2017; Majid et al., 2019).

Effect of solvent to solid ratio on oil extraction: Fig. 3, 4, 5 and 6 show the percent of oil extracted by hexane and petroleum ether at different solvent to solid ratios (6:1, 8:1 and 10:1 v/w) and different processing time (1, 3, 6 and 8 h). By increasing the ratio from 6:1 to 10:1 and processing time from 1 h to 8 h, the oil yield using hexane increased from 1.5% to 11.5% and for petroleum ether it increased from 1% to 7%. For both solvents used, the oil yield was observed to increase with the increase in solvent to solid ratio and processing time, which indicated good mass transfer due to the concentration difference between the solid and the liquid phase (Balaji et al., 2012; Majid et al., 2019). Therefore, solvent to solid ratio of 10:1 and processing time of 8 h showed maximum oil yield. Similar results were observed by (Oliveira et al., 2012; Syed et al., 2016; Shukla and Pratap, 2017; Pandey and Shrivastava, 2018).

Figure 2: Effect of different solvents on oil yield

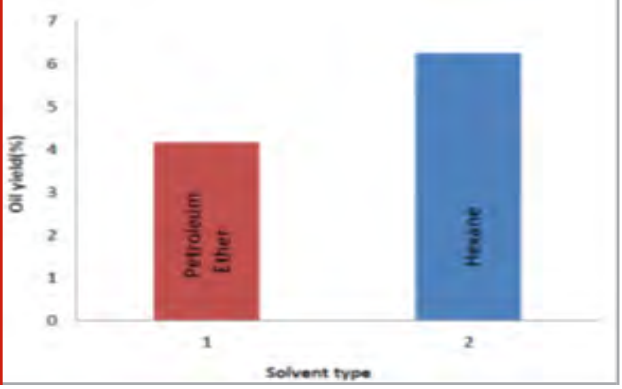


Figure 3: Effect of solvent to solid ratio on oil yield for a reaction time of 1 h.

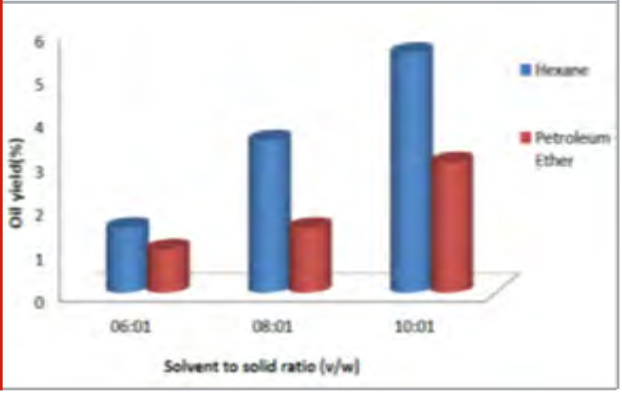


Figure 4: Effect of solvent to solid ratio on oil yield for a reaction time of 3 h

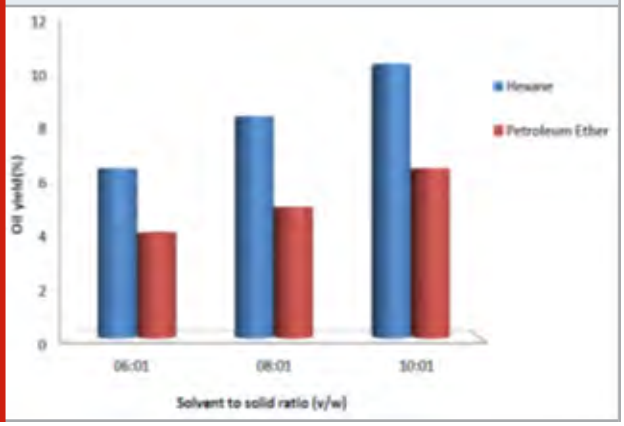


Figure 5: Effect of solvent to solid ratio on oil yield for a reaction time of 6 h.

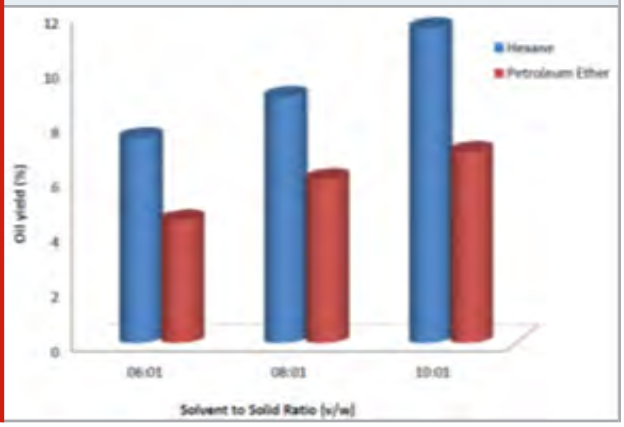
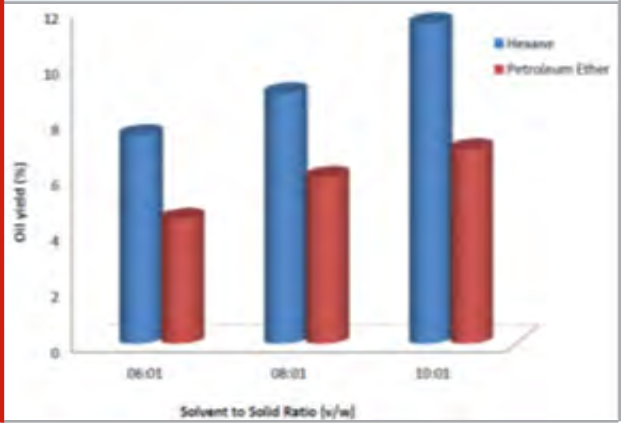


Figure 6: Effect of solvent to solid ratio on oil yield for a reaction time of 8 h.



Effect of alcohol to oil molar ratio on the biodiesel yield: Different molar ratio of methanol to oil ranging from 2:1 to 6:1 was used to determine the effect on ester yield. The transesterification reaction was conducted with varying concentrations of the catalyst. From the Fig. 7, it can be observed that with the increase in the methanol to oil molar ratio the ester yields increased. The maximum ester

yield of 99.25% was observed for molar ratio of 6:1 and catalyst concentration of 4%. The similar trend in the results were obtained by other researchers (Sanjay et al., 2011; Anil et al., 2012a; Anil et al., 2012b; Joshua, 2013; Jayaprabakar et al., 2019; Febrian et al., 2020).

Figure 8: Effect of catalyst concentration on biodiesel yield for 2:1 molar ratio of methanol to oil

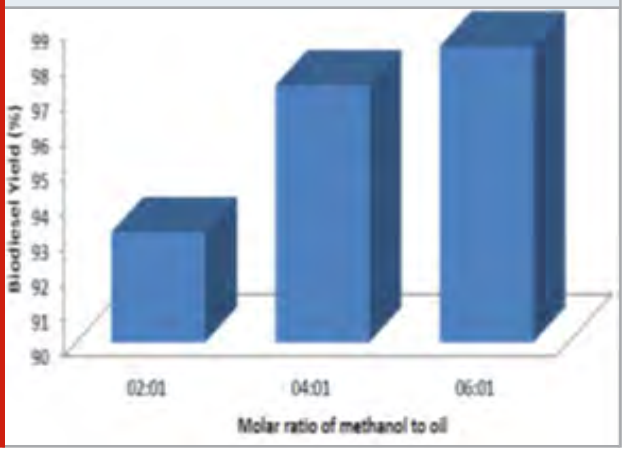


Figure 9: Effect of catalyst concentration on biodiesel yield for 4:1 molar ratio of methanol to oil

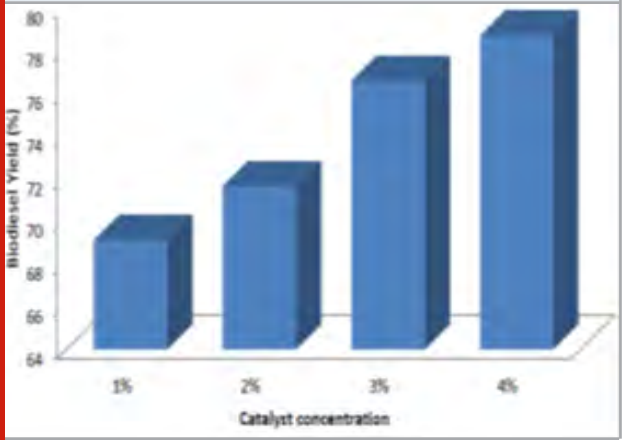
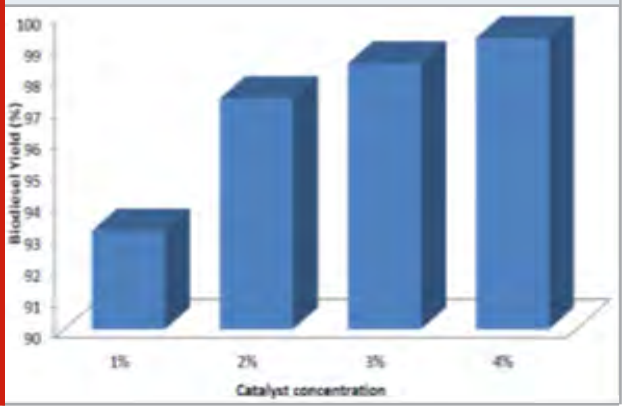


Figure 10: Effect of catalyst concentration on biodiesel yield for 6:1 molar ratio of methanol to oil



Effect of catalyst concentration on the biodiesel yield: Different catalyst concentration ranging from 1% to 4% (w/w_{oil}) was used in the study. The processing time and temperature was maintained at 24 h and room temperature respectively. From Fig. 8, 9, 10, it was observed that for different molar ratio of alcohol to oil, the ester yield increased with the increase in catalyst concentration. Therefore 4% catalyst concentration gave maximum ester yield of 99.25%. The Similar results were obtained by (Edward et al., 2001; Xiaohu and Feng, 2010; Arumugam and Ponnusami, 2017; Jayaprabakar et al., 2019; Febrian et al., 2020).

Characterization of RBO biodiesel: The characterization of RBO biodiesel was done as per standard test procedures. All the properties of biodiesel were determined at department of biotechnology and mechanical engineering, KLE Technological University, Hubballi. The flash point and fire point were observed to be 180 oC and 210 oC respectively. Density was found to be 890 Kg/m^3. Viscosity of RBO biodiesel was found to be 3.6 mm^2/s and carbon residue was found to be 0.24 %w/w. Similar results were obtained by (Joshua, 2013; Nguyen et al., 2019; Veeranna et al., 2020). These results fulfilled ASTM D6751-02 and DIN V51606 biodiesel standards.

Table 1. Characterization of RBO Biodiesel

Property	Unit	RBO Biodiesel	Biodiesel standards	
			ASTM D 6751-02	DIN V 51606
Density	Kg/m^3	890	--	875-900
Viscosity	mm^2/s	3.6	1.9-6.0	3.5-5.0
Flash point	°C	180	>130	>120
Carbon residue	%w/w	0.24	--	<0.3

CONCLUSION

The present study involved rice bran oil extraction by solvent extraction process and enzymatic biodiesel production by transesterification. The optimum conditions obtained were 10:1 solvent to solid ratio, 8 h reaction time for maximum oil yield of 12%. Hexane gave good oil yield when compared to petroleum ether. Different factors like alcohol to oil molar ratio and concentration of catalyst affecting biodiesel production was studied. The optimum values obtained were 6:1 alcohol to oil molar ratio and 4% concentration of catalyst for maximum biodiesel yield of 98.5%. The produced RBO biodiesel was characterized for carbon residue, viscosity, density and flash point. All the values obtained were as per ASTM and DIN international standards. The results showed that the rice bran oil is a potential raw material for biodiesel production. Results indicated that the Rice bran oil can be used for biodiesel production.

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DNA Barcoding of Some Avian Species Killed Due to Road Vehicle Collision in Vidharbha Region, India

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ABSTRACT

DNA Barcoding is considering a novel tool for the identification of species and for discovering the new species by using molecular methods. The mitochondrial cytochrome C oxidase subunit I (COI) serve as fast and accurate method for species identification by molecular methods. The present study was carried out on the three state highways of Vidharbha region, passing through Amravati District which includes Amravati - Chandur Railway state highway (passing through Pohra – Malkhed reserve forest, Amravati- Paratwada state highway passing through agricultural landscape and Paratwada - Semadoh state highway passing through Melghat Tiger reserve. The pectorial muscle tissue samples of the birds killed due to vehicle collision were collected during the study period from 2015 to 2017. Samples on collection immediately dipped in 95 % ethanol, labelled it and brought to the laboratory where they were stored at -20°C for DNA isolation and barcoding analysis. The generated DNA barcodes has been submitted to gene bank and accession numbers for were received. In this study the DNA barcodes has been generated for 17 road killed avian species. The present study, probably for the first time is reporting the barcode sequences of 6 different birds species from India, as there were no other sequences for these bird's species found in NCBI database from India. The 6 bird species are Prinia *inornata* (Plain Prinia), Prinia socialis (Ashy Prinia), *Streptopelia senegalensis* (Laughing Dove), *Otus bakkamoena* (Indian Scops Owl), *Coturnix backgammon* (Common Quail) and *Caprimulgus indicus* (Indian Nightjar). The present study also demonstrated that the DNA barcoding technique can be accurately applied to the identification of road-killed avian carcasses.

KEY WORDS: DNA BARCODING, BIRDS, ROAD KILLS, VIDHARBHA, INDIA.

INTRODUCTION

In Vidarbha, a total of 417 bird species has been reported including 394 bird species of Amravati District, of which the Melghat Tiger Reserve in Amravati District shows the greatest diversity of avian fauna with recorded 265 bird species (WECS 2009; Wadatkar et al., 2016).

DNA Barcoding is considering a novel tool for the identification of species and for discovering the new species by using molecular methods. The mitochondrial cytochrome C oxidase subunit I (COI) serve as fast and accurate method for species identification by molecular methods. Mitochondrial DNA (mt DNA) used extensively in phylogenetic studies of animals as it evolves much more rapidly than nuclear DNA results in accumulation of differences between closely related species (Brown et al., 1979; Savolainen et al., 2005; Hebert et al., 2004, 2010). Many researchers have suggested that the 648 bp region of mitochondria for mitochondrial cytochrome C oxidase subunit I (COI) serves as the most accurate molecular marker for species identification in animals due to its high interspecific variation, low intraspecific

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variation, and relatively universal primers for taxonomic groups at the level of orders and even classes (Hebert et al., 2004; Ward et al., 2005; Hajibabaei et al., 2006; Johnsen et al., 2010; Wadatkar et al., 2016).

Considerable amount of work and a large number of publications in this field has lead to the formation of Consortium for the Barcode of Life (CBOL, http://barcoding.si.edu), with the objective of obtaining DNA barcodes from all species on the planet (Yoo et al., 2006). By using this approach various animal groups have been studied such as Neotropical bats, North American birds, New Zealand birds, Australian fishes, and tropical Lepidoptera (Ward et al., 2005; Hajibabaei et al., 2006; Clare et al., 2007; Kerr et al., 2007; Tizard et al., 2019).

Species identification through DNA barcoding has many practical utilities such as in conservation biology, food security control, and bird strike identification (Neigel et al., 2007; Dove, 2008; Ward et al., 2008; Wong and Hanner, 2008). Tizard et al. (2019) studied the COI sequence data of New Zealand birds and found that DNA barcoding accurately identified most New Zealand bird species. DNA barcoding provides the rapid method for screening the biodiversity of the particular region for identification of species and also serves as a promising tool for differentiation of two species which have similar or identical phenotypes. It could also be a novel tool for identification of small and immature organisms which are difficult to identify morphologically (Tizard et al., 2019).

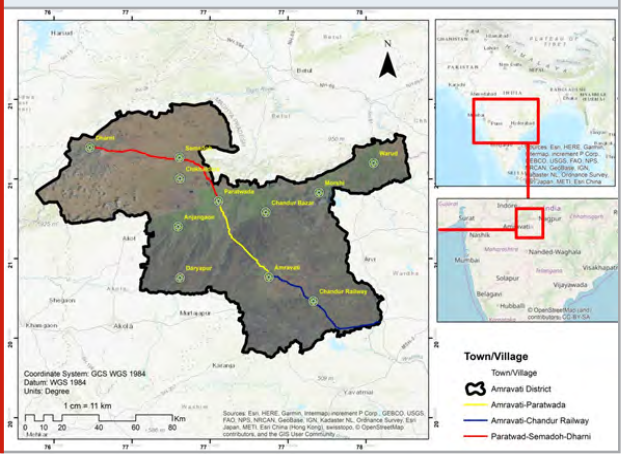
Birds are the most studied and taxonomically variable class of animals and hence it is very much useful for testing the efficacy of DNA barcoding in species identification. Birds are routinely killed in the road accident by various vehicles in the forest roadside and sometimes it is very difficult to identify the species morphologically hence attempt has been made to develop the DNA barcode for the road killed avian species of Amravati district of Vidharbha region, (Tizard et al., 2019).

MATERIAL AND METHODS

The pectorial muscle tissue samples of the birds killed due to vehicle collision were collected during the study period from 2015 to 2017. The tissues were collected during road vehicle collision study of avian fauna, on the three roads of Amravati District viz., 1. Road passing through reserve forest (Pohra – Malkhed) from Amravati to Chandur Railway (State Highway number -243; 30 Km), 2. Road passing through the agricultural landscape (from Amravati to Paratwada) (State Highway Number -6; 50 km), 3.A road from Paratwada to Semadoh passing through Melghat Tiger reserve (State Highway number -6; 46 Km). Fig No. 1 shows the study area and all the three studies highways.

Samples on collection immediately dipped in 95 % ethanol, labelled it and brought to the laboratory where they were stored at -20°C for DNA isolation and barcoding analysis. These samples were used for COI gene sequence analysis. Feather sample was used to generate DNA barcode sequence for Indian Peafowl. The feather sample was collected from the Agricultural land. The process of DNA isolation, Polymerase chain reaction, purification of Polymerase chain reaction product and DNA sequencing were carried out at GenOmBiotechnologies Pvt Ltd. Pune. The blast analysis and phylogenetic analysis were carried out in the research laboratory of the Department of Zoology, Shri Shivaji Science College Amravati.

Figure 1: Map of Amravati District (Study Area) (Sources: Esri, HERE, Garmin, Intermap, increment P Corp., GEBCO, USGS, FAO, NPS, NRCAN, GeoBase, IGN, Kadaster NL, Ordnance Survey, Esri Japan, METI, Esri China (Hong Kong), swisstopo, ©OpenStreetMap contributors, and the GIS User Community; Map Prepared by Shubham Wagh)



DNA was isolated using QIAGEN kit, QIA amp DNA FFPE Tissue (CAT.NO. 56404) as per manufacturer's instructions. DNA was eluted in 20.0 µl of elution buffer. Two types of primers were used for the amplification of COI genes: COI gene specific forward and reverse universal primers (Folmers Primer) (Used for birds which include Greater Coucal, Barn Owl, Indian Scops Owl, Spotted Owlet, Ashy Prinia, Indian Nightjar, Plain Prinia, Indian Roller, Red Wattle Lapwing, Cattle Egret, Asian Koel, Common Tailor Bird, Rufous Treepie).Birds specifics primers – BirdF and BirdR (Used for birds which includes Red Vented Bulbul, Laughing Dove, Indian Peafowl and Common Quail). COI gene specific forward and reverse universal primers were used for then amplification is:LCO- 1490 5'- GGTCAACAAATCATAAAGATATTGG- 3' and HCO –2198 5'- TAAACTTCAGGGTGACCAAAAAATCA - 3'

Primer Name	Primer Sequences
BirdF	5'-TTCTCCAACCACAAAG ACATTGGCAC-3'
BirdR	5'-ACGTGGGAGATAATTC CAAATCCTG-3'

PCR reaction mix was prepared for DNA samples. Final volume of each reaction was 25.0 µl. Thermal cycling

program for PCR was used as below: Initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 40 °C for 1 min, Extension at 72 °C for 1.30 min and final extension at 72 °C for 7 min and hold at 4 o c until use.

1. Birds specifics primers – BirdF and BirdR: COI gene specific forward and reverse primers were used for the amplification are:

PCR reaction mix was prepared for DNA samples. Final volume of each reaction was 25.0 µl. Thermal cycling program for PCR was used as below: Initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 0.45 Sec, annealing at 58 °C for 0.45 Sec, Extension at 72 °C for 1.00 min and final extension at 72 °C for 7 min and hold at 4 o c until use. Agarose gel electrophoresis of the PCR products was performed using 2% (w/v) agarose gel using standard 0.5X TBE gel electrophoresis buffer. Sizes of the amplicons generated by this primer pair is 708 bp and is also evident from the gel. The purification of all amplicons was performed using Purelink PCR product purification kit from Life technologies as per the manufacturer's instructions. The purified PCR products were again checked on 2% Agarose gel to confirm that the amplicons are not lost during purification.

DNA sequencing of PCR product was performed using both the primers using Applied Biosystems BigDye Terminator V3.1 Cycle sequencing kit. The sequencing products were loaded on Applied Biosystems 3130 Genetic Analyzer – automated DNA sequencing instrument. Sequences were analyzed using Sequencing Analysis 5.1 software available in the sequencing machine. These sequences were further copied and analyzed using ChromasPro v 1.34. Forward and reverse sequences were aligned to form the contig with best sequence calls, hence for each sample using two sequences (Forward and Reverse), one contig was generated. Contig sequence was further subjected to BLAST analysis using http://blast.ncbi.nlm.nih.gov/Blast.cgi tool available at NCBI website.

RESULTS AND DISCUSSION

In the present study, the DNA barcoding of 17 bird species, which were killed due to road vehicle collision has been done. The DNA Barcode sequence of all the 17 species was submitted to the GenBank and the accession numbers were collected. The present study demonstrated that the DNA barcoding technique can be accurately applied to the identification of road-killed avian carcasses. DNA barcoding method was found significant for the identification of road-killed birds' species.

Table 1. The species for which DNA Barcodes have been generated along with their GenBank Accession Number

Sr. No	Common Name	Scientific Name	Accession No.
1	Indian Nightjar	*Caprimulgus indicus*	KT240050
2	Common Tailor Bird	*Orthotomus sutorius*	KT240051
3	Plain Prinia	*Prinia inornata*	KT240052
4	Indian Roller	*Coracias benghalensis*	KT240053
5	Red Wattle Lapwing	*Vanellus indicus*	KT240054
6	Cattle Egret	*Bubulcus ibis*	KT240055
7	Asian Koel	*Eudynamys scolopaceus*	KT240056
8	Greater Coucal	*Centropus sinensis*	KT240057
9	Ashy Prinia	*Prinia socialis*	KT240058
10	Rufous Treepie	*Dendrocitta vagabunda*	KT240059
11	Red Vented Bulbul	*Pycnonotus cafer*	KT240060
12	Laughing Dove	*Streptopelia senegalensis*	KT240061
13	Barn Owl	*Tyto alba*	KR779892
14	Indian Scops Owl	*Otus bakkamoena*	KR779893
15	Spotted Owlet	*Athene brama*	KR779894
16	Indian Peacock	*Pavo cristatus*	MN206039
17	Common Quail.	*Coturnix cristates*	MN206040

The present study, probably for the first time has reported the barcode sequences of 6 different bird species from India, as there were no other sequences for these bird species found in NCBI database from India. The 6 birds' species are Prinia inornata (Plain Prinia), Prinia socialis (Ashy Prinia), Streptopelia senegalensis (Laughing Dove), Otus bakkamoena (Indian Scops Owl), Coturnix cristates (Common Quail) and Caprimulgus indicus (Indian Nightjar). The species for which DNA Barcodes have been generated along with their GenBank Accession Number are shown in table No.1.

Large number of bird species have been studied through DNA barcoding worldwide, such as DNA Barcoding of

Korean Birds, Scandinavian birds, critically endangered bird species Asian Houbara Bustard, Netherlands birds, feather mite studies, to identify bird species involved in bird-aircraft collision (Yoo et al., 2006; Dove, 2008; Johnsen et al., 2010; Arif et al., 2012; Aliabadian et al., 2013; Dona et al., 2015; Gaikwad et al., 2016). Tizard et al. (2019) studied DNA Barcoding in New Zealand birds and demonstrates that DNA barcoding can identify the majority of New Zealand birds to the species level. Many of the times birds are get killed by the road vehicle collision. Goncalves et al. (2015) report a case in which DNA Barcoding technique help criminal investigations and to design species-specific anti-poaching strategies, and also demonstrate how DNA sequence analysis in the identification of bird species is a powerful conservation tool (Goncalves et al., 2015; Tizard et al., 2019).

Dimitriou et al. (2017) studied DNA barcoding of the large majority of bird species resident in Cyprus plus several migrants that were illegally captured. Their study was carried out to support local authorities in their anti-poaching actions. Gaikwad et al. (2016) studied the utility of DNA barcoding for the identification of bird-strike samples from India. They have evaluated the utility of DNA barcoding for species identification of birds involved in bird-strike incidences and concluded that DNA barcoding offers a fast and reliable technique for species identification compared to traditional methods because if blood spots or damaged tissues are provided, traditional methods very often failed to identify the species. In agreement with the above studies, the present study also demonstrated that the DNA barcoding technique can be accurately applied to the identification of road-killed avian carcasses (Gaikwad et al., 2016; Dimitriou et al., 2017).

DNA barcoding method was found significant for the identification of road-killed birds' species (Gaikwad et al., 2016; Dimitriou et al., 2017). As mentioned above, the DNA Barcoding offers a range of applications in various situations to identify the species. Birds are routinely killed in the road accident by various vehicles in the forest roadside and sometimes it is very difficult to identify the species morphologically due to complete pressing of the animal, hence attempt has been made to develop the DNA barcode for the road killed avian species of Amravati district (Dimitriou et al., 2017).

CONCLUSION

In this study the DNA barcodes has been generated for 17 road vehicle collision killed avian species. The study uses the road killed bird specimens for tissue collection. Total 17 bird's DNA Barcode sequences were generated and submitted to Genbank. The present study, probably for the first time reporting the barcode sequences of 6 different birds' species from India, as there were no other sequences for these bird's species found in NCBI database from India. The present study also demonstrated that the DNA barcoding technique can be accurately applied to the identification of road-killed avian carcasses.

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Conflict of Interest: The authors declare that they have no Conflict of interests.

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Isolation and Screening of Actinomycetes from Umm Jirsan Cave, Saudi Arabia for their Antibacterial Activity

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ABSTRACT

Chemical fertilisers have been used intensively in recent years leading to the degradation in the quality of the soil. Diversity of microorganisms is important, as their unique features can be utilized for crop production and environment. Microorganisms are usually inhabited in all parts of the plant from the roots to the shoot and internal regions of the plants. Rhizosphere microbial variety conveys an assortment of microorganisms which offer advantageous properties to the plant environments. In the present study, an attempt has been made for the screening of bacteria for plant growth-promoting activities such as nitrogen fixation, phosphate solubilization and indole acetic acid production. Soil samples were collected from thirty-four different places of districts Junagadh, Gir Somnath, Amreli, Diu, Dwarka and Jamnagar. Twenty soil samples were from forest region and fourteen soil samples were from the coastal region of Saurashtra. The nitrogen-fixing capability of the isolates was evaluated using Ashby's media containing bromothymol blue. Total 57 nitrogen-fixing bacteria based on their colony morphology were isolated, of which 49 bacterial isolates were able to solubilize phosphate and 27 were able to produce indole acetic acid. Of 57 bacterial isolates, 23 isolates showed positive results for nitrogen fixation, phosphate solubilization and indole acetic acid production. Nitrogen and phosphorus are one of the major essential macronutrients for plant growth and development. Indole acetic acid serves as one on the plant hormone for growth of plants. The present study indicates 23 bacterial isolates can have the potential for plant growth-promoting bacteria and as a greater number of isolates were from forest region which also indicates the fertility of the soil.

KEY WORDS: BACTERIA, PLANT-GROWTH PROMOTING, NITROGEN FIXATION, PHOSPHATE SOLUBILIZATION, INDOLE ACETIC ACID.

INTRODUCTION

Farmers are currently using chemical fertilisers to intensively supplement the basic nutrients of the soil-based plant system. The advantage of accessibility and intensive use create environmental issues of chemical fertilizers today's agriculture. The use of chemical fertilizers does however have their advantages and drawbacks in agriculture. Hence, there's an increasing demand for various ways to support the crop production and to maintain the nutrient within the soil environment for ecological equilibrium in an agroecosystem. The engagement of microorganism as inoculants or for plant growth-promotion is promising and are widely accepted practices which are been employed in agriculture for the agricultural produce. Symbiotic / non-symbiotic soil bacterium that colonizes root rhizosphere of plant and promotes the expansion in terms of crop yields, (Gouda et al., 2018; Santos et al., 2019, Lebrazi et al., 2020).

Diversity of microorganisms is important, as their unique features can be utilized for crop production and environment (Costa et al., 2018). Variety of microorganism

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assists with developing the biological system comprises of an organism, soil, and plant. The working of this environment is significantly represented by microbial elements. Microorganisms are usually inhabited in all parts of the plant from the roots to the shoot and internal regions of the plants (Harman and Uphoff, 2019). In all structures, most of these microorganisms help and raise the plant to live emphatically and offer significant great conditions to the plants. In all structures, the greater part of these organisms helps and elevate the plant to live soundly and offer useful focal points to the plants. Among all these plant growth-promoting bacteria undertake a significant job and are a focal situation in quality and the quantity of yield. Rhizosphere microbial variety conveys an assortment of microorganisms which offer advantageous properties to the plant environments (Thakur et al., 2020).

Plant growth-promoting effect of the PGPB is usually explained by the discharge of metabolites which directly promote the plant growth (Rilling et al., 2019). There are several ways to elucidate the activities of PGPB benefit to the host plant. PGPB have potential to supply plant growth regulators like cytokinins, indole acetic acid (IAA) and gibberellins, enhancing organic process, promote solubilization of inorganic and organic phosphate. The inoculation with PGPB strain like *Azotobacter* could help to scale back the utilization of nitrogen-based chemical fertilizer (Sharma et al., 2016; Roriz et al., 2020).

Plant growth-promoting bacteria (PGPB) have been studied as a sustainable alternative to the use of chemical fertilizers to increase crop yields, and effective PGPB have been isolated from diverse plants and soil compartments. Naturally occurring bacteria, commonly found in the soil associated with the roots of plants, positively affect the growth of plants in a number of different ways (Rilling et al., 2019; Glick, 2020). This includes increases in plant yield, nutritional content, tolerance to various abiotic and biotic stresses, and the production of useful secondary metabolites.

Due to its topographic state, the Saurashtra region, Gujarat India has a wide variation. The region has a range that ranges from both forest and coastal areas to wetlands. Saurashtra region has shallow, medium black, calcareous soils with a rainfall range of 400mm to 700mm and dry sub-humid climate. Groundnut, cotton, sesamum, sugarcane, rice, pulses, jowar and bajra are major crops produced in the Saurashtra region (Gondaliya et al., 2017; Ravi and Fulekar, 2018). In the present study, an effort has been made to screen for free-living plant growth-promoting bacteria from the forest and coastal region of Saurashtra.

MATERIAL AND METHODS

Soil samples from Gir forest and Coastal areas of Saurashtra region, Gujarat were collected. Soil samples were collected from thirty-four different places of districts Junagadh, Gir Somnath, Amreli, Diu, Dwarka and Jamnagar from Saurashtra region, Gujarat India. Of the thirty-four soil samples, 20 were from forest region and 14 were from the coastal region of Saurashtra. The sampling area for the soil was dug to a depth of about 25-30 cm and then collected and transferred to sterile polyethene bags. The soil samples for further use were stored in a refrigerator at low temperatures. For the isolation and screening of nitrogen-fixing bacteria from soil, serial dilution technique and spread plate method using Jenson agar medium was used (Sahoo et al., 2014).

In case of soil samples from coastal regions Jenson agar medium with varying salt concentration of 0.5%, 2%, 4%, 6%, 8% and 10% respectively. Different components of Jenson agar medium were weighed, dissolved in an appropriate amount of water, pH was adjusted and autoclaved at 1210C (15 psi) for 15 minutes. Ten gram of soil sample was suspended in 90 ml of sterilized distilled water blank and kept on a rotary shaker for 30 minutes so that microorganism adhered to the soil particles get dispersed uniformly into the water. Using serial dilution technique serial dilution were made up to 10-7. From dilution of 10-5 to 10-7, 100 µl was spread on Jenson agar medium plates in triplicates. The spreaded plates were incubated at 30±1°C till the visible colonies appeared. Individual colonies of different bacterial isolates showing different morphological features were picked up, purified by streaking on solidified Jenson agar medium plates.

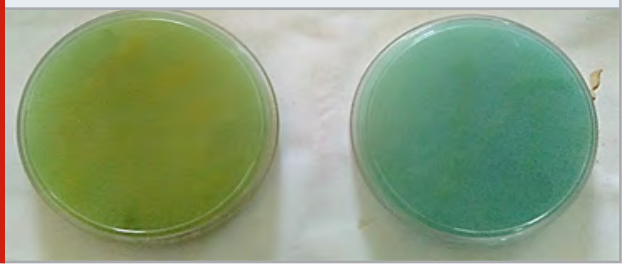
The isolated colony of each isolate, colony characters were described according to Microbiology: A Laboratory Manual (Cappuccino and Welsh, 2017). Individual isolated pure colonies were picked up and maintained on Jenson agar slants for further use. They were streaked on freshly prepared nutrient agar plates and incubated for 3 days at 30±1 °C. Gram's staining of isolates was done according to the procedure given by (Brown and Heidi, 2015). Cell shape was also recorded. Nitrogen-fixing capability of the isolates was evaluated using Ashby's media containing bromothymol blue (Hingole and Pathak, 2016).

Plates containing medium were prepared and streaked with different isolates in triplicates. Plates were incubated at 30±1°C. Isolates fixing nitrogen showed growth on the medium with a change in colour from green to blue. Uninoculated plates in triplicates served as control. Different bacterial isolates were screened for their phosphate solubilizing ability by growing them on Pikovskaya agar medium (Gupta and Pandey, 2019). Fifty microlitres of two days old culture suspension of selected isolates were spotted on the solidified agar medium plates incubated at 30±2°C for 5-6 days. The plates were examined for the production of a clear zone around the bacterial growth. As a result of acid production, isolates which used tricalcium phosphate developed a clear zone around the colony (Gupta and Pandey, 2019).

The bacterial isolates were screened for their ability to produce IAA, in the absence and presence of tryptophan. The bacterial isolates were inoculated in 5 ml Jensen's

liquid medium incubated at 30±2°C. Cultures were centrifuged at 3000 rpm for 30 minutes. Two ml of Salkowski's reagent and two drops of ortho-phosphoric acid was mixed with 2 ml of supernatant. The presence of the pink colour indicated the production of IAA. Further study was performed for ammonia production and other biochemical properties, such as capsule staining, indole analysis, oxidase test and catalase test, in isolates that have shown positive results for nitrogen fixation, phosphate solubilization and indole acetic acid production (James Cappuccino and Welsh, 2017; Gupta and Pandey, 2019).

Figure 1: Green coloured plate (negative control) and Blue coloured plate which indicated the production of ammonia and nitrogen fixation.



RESULTS AND DISCUSSION

Screening of nitrogen-fixing bacteria from soil: The accession number given to the isolates were GFS for the isolates obtained from the soil of forest region of Saurashtra and SCS for the coastal region of Saurashtra. Different isolates were isolated based on colony characteristics like morphology, size, and shape. From all the soil samples, about 57 bacterial isolates have been isolated. Twenty-seven isolates were from the forest region and thirty were from the coastal region of Saurashtra. It was observed that out of 57 isolates 34 were Gram-negative coccobacilli,6 were Gram-negative bacilli, 10 were Gram-positive bacilli, 6 were Gram-positive coccobacilli and one was Gram-positive cocci. They were grouped based on Gram reaction and shape of the bacterial cell (Table1). Upon capsule stain of 57 isolates, 30 were capsulated and 27 were non-capsulated.

Table 1. Gram's Stain, Morphology, and presence of capsule of all bacterial Isolates.

Sr.	Accession no.	Gram's stain	Morphology	Capsule	Sr.	Accession no.	Gram's stain	Morphology	Cap-sule
1	GFS01C1	Negative	Cocco Bacilli	Yes	30	SCS01C3	Negative	Bacilli	Yes
2	GFS01C2	Negative	Cocco Bacilli	No	31	SCS02C1	Negative	Bacilli	Yes
3	GFS02C1	Negative	Cocco Bacilli	No	32	SCS03C1	Positive	Cocco Bacilli	Yes
4	GFS03C1	Negative	Cocco Bacilli	Yes	33	SCS03C2	Negative	Cocco Bacilli	No
5	GFS04C1	Negative	Bacilli	Yes	34	SCS04C1	Positive	Bacilli	No
6	GFS05C2	Negative	Cocco Bacilli	Yes	35	SCS05C1	Positive	Cocco Bacilli	No
7	GFS05C1	Negative	Cocco Bacilli	No	36	SCS05C2	Positive	Cocco Bacilli	No
8	GFS06C1	Negative	Cocco Bacilli	Yes	37	SCS06C1	Negative	Cocco Bacilli	No
9	GFS07C1	Positive	Bacilli	Yes	38	SCS07C1	Positive	Bacilli	No
10	GFS07C2	Negative	Cocco Bacilli	No	39	SCS07C2	Negative	Cocco Bacilli	Yes
11	GFS08C1	Negative	Cocco Bacilli	Yes	40	SCS07C3	Negative	Cocco Bacilli	No
12	GFS10C1	Negative	Cocco Bacilli	Yes	41	SCS08C1	Negative	Cocco Bacilli	No
13	GFS11C1	Positive	Bacilli	Yes	42	SCS09C1	Negative	Cocco Bacilli	Yes
14	GFS12C1	Negative	Cocco Bacilli	No	43	SCS10C1	Positive	Bacilli	Yes
15	GFS13C1	Positive	Cocci	No	44	SCS11C1	Negative	Cocco Bacilli	Yes
16	GFS13C2	Negative	Cocco Bacilli	No	45	SCS11C2	Negative	Bacilli	No
17	GFS14C1	Negative	Cocco Bacilli	No	46	SCS12C1	Positive	Bacilli	No
18	GFS15C1	Positive	Bacilli	Yes	47	SCS12C2	Negative	Cocco Bacilli	No
19	GFS15C2	Negative	Cocco Bacilli	Yes	48	SCS12C3	Positive	Bacilli	No
20	GFS16C1	Positive	Bacilli	Yes	49	SCS12C4	Negative	Cocco Bacilli	No
21	GFS16C2	Negative	Cocco Bacilli	Yes	50	SCS12C5	Positive	Cocco Bacilli	Yes
22	GFS17C1	Negative	Cocco Bacilli	No	51	SCS13C1	Negative	Bacilli	No
23	GFS18C1	Negative	Cocco Bacilli	No	52	SCS13C2	Negative	Bacilli	Yes
24	GFS18C2	Negative	Cocco Bacilli	Yes	53	SCS13C3	Positive	Bacilli	Yes
25	GFS19C1	Negative	Cocco Bacilli	Yes	54	SCS13C4`	Negative	Cocco Bacilli	Yes
26	GFS19C2	Negative	Cocco Bacilli	No	55	SCS13C1	Negative	Cocco Bacilli	No
27	GFS20C1	Negative	Cocco Bacilli	Yes	56	SCS13C2	Negative	Cocco Bacilli	No
28	SCS01C1	Negative	Cocco Bacilli	Yes	57	SCS13C3	Positive	Cocco Bacilli	Yes
29	SCS01C2	Positive	Cocco Bacilli	Yes					

Nitrogen fixation: Nitrogen is required for the synthesis of amino acids, chlorophyll, nucleic acids, and ATP which are required for the growth and survival of plants (Chakraborty and Tribedi, 2019). All the 57 isolates indicated by growth on Ashby's medium and turned the greenish colour of the medium to blue (Table 2). The development of blue colour was due to the production of ammonia in the medium making it alkaline (Figure 1). It has been previously observed, that Azospirillum possess high nitrogenase activity allowing for the possibility of using this bacterium as a biofertilizer to improve soil fertility for improved and efficient farming (Richard et al., 2018). However, the confirmatory test of nitrogenase activity using acetylene reduction assay (ARA) needs to be performed to establish their nitrogen-fixing capability (El-Khaled et al., 2020).

Table 2. Plant Growth Promoting activities of the bacterial isolates.

Sr.	Accession no.	Nitrogen fixation	Phosphate Solubilization	Indole Acetic Acid	Sr.	Accession no.	Nitrogen fixation	Phosphate Solubilization	Indole Acetic Acid
1	GFS01C1	Positive	Positive	Positive	30	SCS01C3	Positive	Positive	Negative
2	GFS01C2	Positive	Positive	Negative	31	SCS02C1	Positive	Positive	Negative
3	GFS02C1	Positive	Negative	Negative	32	SCS03C1	Positive	Positive	Positive
4	GFS03C1	Positive	Positive	Positive	33	SCS03C2	Positive	Positive	Positive
5	GFS04C1	Positive	Positive	Positive	34	SCS04C1	Positive	Positive	Positive
6	GFS05C2	Positive	Positive	Positive	35	SCS05C1	Positive	Positive	Negative
7	GFS05C1	Positive	Positive	Negative	36	SCS05C2	Positive	Positive	Negative
8	GFS06C1	Positive	Negative	Negative	37	SCS06C1	Positive	Positive	Negative
9	GFS07C1	Positive	Positive	Positive	38	SCS07C1	Positive	Negative	Negative
10	GFS07C2	Positive	Negative	Positive	39	SCS07C2	Positive	Positive	Positive
11	GFS08C1	Positive	Positive	Positive	40	SCS07C3	Positive	Positive	Positive
12	GFS10C1	Positive	Positive	Positive	41	SCS08C1	Positive	Negative	Negative
13	GFS11C1	Positive	Positive	Positive	42	SCS09C1	Positive	Positive	Negative
14	GFS12C1	Positive	Positive	Positive	43	SCS10C1	Positive	Positive	Negative
15	GFS13C1	Positive	Positive	Positive	44	SCS11C1	Positive	Positive	Negative
16	GFS13C2	Positive	Negative	Negative	45	SCS11C2	Positive	Positive	Negative
17	GFS14C1	Positive	Negative	Negative	46	SCS12C1	Positive	Positive	Positive
18	GFS15C1	Positive	Negative	Positive	47	SCS12C2	Positive	Positive	Positive
19	GFS15C2	Positive	Positive	Positive	48	SCS12C3	Positive	Negative	Positive
20	GFS16C1	Positive	Positive	Positive	49	SCS12C4	Positive	Negative	Positive
21	GFS16C2	Positive	Positive	Positive	50	SCS12C5	Positive	Positive	Positive
22	GFS17C1	Positive	Positive	Negative	51	SCS13C1	Positive	Negative	Negative
23	GFS18C1	Positive	Positive	Negative	52	SCS13C2	Positive	Positive	Negative
24	GFS18C2	Positive	Negative	Negative	53	SCS13C3	Positive	Positive	Negative
25	GFS19C1	Positive	Negative	Negative	54	SCS13C4	Positive	Positive	Negative
26	GFS19C2	Positive	Positive	Positive	55	SCS13C1	Positive	Positive	Negative
27	GFS20C1	Positive	Positive	Negative	56	SCS13C2	Positive	Positive	Negative
28	SCS01C1	Positive	Positive	Positive	57	SCS13C3	Positive	Negative	Negative
29	SCS01C2	Positive	Positive	Negative					

Figure 2: Solubilization of phosphate as seen around the bacterial isolate.

Phosphate solubilization: Phosphorus is one of the major essential macronutrients for plant growth and development. However, the concentration of soluble P in the soil is very low (Zhu et al., 2011). The use of phosphate solubilizer bacteria as inoculants will increase P intake by plant and cultivation at the same time (Olanrewaju et al., 2017). Of the 57 bacterial isolates, 49 isolates solubilized tri-calcium phosphate as indicated by the production of clearance zone around the bacterial colony on Pikovskaya's agar medium plates (Table 2). Solubilization of tri-calcium phosphate requires either acid production or chelate formation by the bacterium in the medium. Probably other isolates did not produce acid insufficient amount or chelate to solubilize tri-

calcium phosphate in the medium (Figure 2). Several studies showed that PGP bacteria were responsible for solubilizing the insoluble P. It was also reported that excretion of organic acids was one of the most important factors in phosphate solubilization (Hemambika et al., 2013; Alori et al., 2017; Pérez-Rodriguez et al., 2020).

Production of Indole Acetic Acid: Out of 57 bacterial isolates 27 produced IAA from tryptophan (Table 2). These broth cultures containing tryptophan showed red colouration on the addition of Salkowski reagent. Indole acetic acid production is characteristic of the production of plant growth promoters.Bacterial IAA contributes to the growth of the lateral and adventitious root lead and triggers the bacterial proliferation of roots by exuding the root in order to increase their absorption of minerals and nutrients (Glick, 2010). In previous studies it has been indicated that IAA-producing rhizobacteria could be harnessed to improve plant growth (Das et al., 2019; Lebrazi et al., 2020).

Table 3. List of bacterial isolates which showed plant promoting properties nitrogen fixation, phosphate solubilization & indole acetic acid production.

Sr.	Accession no.	Nitrogen fixation	Phosphate Solubilization	Indole Acetic Acid	Sr.	Accession no.	Nitrogen fixation	Phosphate Solubilization	Indole Acetic Acid
1	GFS01C1	Positive	Positive	Positive	13	GFS16C2	Positive	Positive	Positive
2	GFS03C1	Positive	Positive	Positive	14	GFS19C2	Positive	Positive	Positive
3	GFS04C1	Positive	Positive	Positive	15	SCS01C1	Positive	Positive	Positive
4	GFS05C2	Positive	Positive	Positive	16	SCS03C1	Positive	Positive	Positive
5	GFS07C1	Positive	Positive	Positive	17	SCS03C2	Positive	Positive	Positive
6	GFS08C1	Positive	Positive	Positive	18	SCS04C1	Positive	Positive	Positive
7	GFS10C1	Positive	Positive	Positive	19	SCS07C2	Positive	Positive	Positive
8	GFS11C1	Positive	Positive	Positive	20	SCS07C3	Positive	Positive	Positive
9	GFS12C1	Positive	Positive	Positive	21	SCS12C1	Positive	Positive	Positive
10	GFS13C1	Positive	Positive	Positive	22	SCS12C2	Positive	Positive	Positive
11	GFS15C2	Positive	Positive	Positive	23	SCS12C5	Positive	Positive	Positive
12	GFS16C1	Positive	Positive	Positive					

Biochemical tests: All the 57 isolates were able to produce ammonia, oxidase and catalase. Isolates tabulated in the Table 3 can act as potential plant growth promoting bacteria. Of the 23 isolates tabulated in Table 3, 14 are from forest region and 9 were from coastal region of the Saurashtra region. These isolates have potential for biofertilizers which can be useful in agricultural practices.

CONCLUSION

In conclusion the present study attempt was made to isolate plant growth promoting bacteria which could be harnessed to improve plant growth. Nitrogen fixing bacteria, and phosphate solubilizing bacteria and Indole acetic acid producing bacteria were isolated. So it can be stated that presence of growth promoting bacteria are responsible for the beneficial effects on plant growth and they can be used as potential biofertilizers. However quantitative analysis of the above parameters can help us to better understand the efficiency of the bacterial isolates.

Conflict of Interest: The authors declare no conflict of interest among themselves.

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Leisure-Time, Socializing with Peers: Digital Technology as a Mediator or Distemper for Net-Generation

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ABSTRACT

Leisure-time is a free or spare time, which provides someone to do something of their interest apart from their normal working/ educational life. During pandemic COVID-19, as classes are off, it is observed that adolescents like to spend most of their leisure time in non-scholastic pursuits, for which they opted digital technology; they specifically spend most of their time on online social networks sites (SNS) rather than using time in productive works. The importance of the study is to explore the untouched facts related to digital technology as a mode of leisure activities. Two-stage stratified sampling was used; firstly on a geographical lbasis and secondly on a demographic profile. The proposed study had selected, 460 adolescents, studying in Grade XII in the age-group of 16-19 years; who were defined as 'Net Generation' from India and Bhutan. Leisure Interest Measure (LIM) and Structure questionnaire called Smart Phone Usage Pattern (SPUP) had been used for data collection. The study used SPSS21, AMOS 23, and other related statistics for analysing the data. The results revealed the fact that adolescents boys were more involved in online activities rather than girls and Indian adolescents were facing severe health problems in comparison to Bhutanese as they were less involved in online activities. The study suggested that there should be a balance in activities performs in daily life by screenagers otherwise digital technology as a mediator becomes distemper and it will hamper their life. The study will help the academicians to secure their attention towards the planning of leisure-time activities of the adolescents so that it can be utilised in a productive manner which will help in improving the results of the institutions.

KEY WORDS: ADOLESCENT, DIGITAL TECHNOLOGY, MEDIATOR, NET GENERATION, SNS.

INTRODUCTION

Leisure is perceived as an antidote to all types of psychological and physiological problems, but if we used it in an improper way this pill will create problems for us. Most adolescents around the world are online and involved in social networking this has becomes popular activity among adolescents during their leisure time, in the past 10 years. Previous studies have found that 70–90 percent of adolescents use SNSs, most commonly Facebook (Madden et al., 2013). The average time spent with screen media among 08-18-year-olds is more than twice the average amount of time spent in school every year (Kaiser Family Foundation Study, 2010 and National Center for Education Statistics, 2007–2008).

It is quite observed that during leisure time adolescents are using digital gadgets for their enjoyment, a study by, Pew Research Center (2015) found that 93 percent of smartphone owners aged18–29 years, use the device to avoid boredom. John Coleman and Leo Hendry's focal theory linked age-graded relational concerns with leisure transitions during adolescence. In late adolescence, casual leisure gives way to commercialized leisure (e.g.,

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visiting pubs, discotheques, sports clubs, or wellness centers), which adolescents use to affirm their maturity and independence from parents (Maria and Rainer, 2015 Gupta 2020).

Now from 22nd March 2020 onwards due to pandemic COVID-19 schools were shut down and regular classes are not going on. Classes are conducting through online mode for senior secondary classes, due to which the usage of electronic gadgets like smartphones has been increased among adolescents and now they are getting more leisure-time. Even in some cases, it is observed that students are bunking classes and are involved in online games, chatting, and other activities besides studying. Public health expert and executive director of Population Foundation of India (PFI), Poonam Muttreja says, "In this digital day and age, being stuck at home during lockdown also means increased and sometimes unfettered access to television and social media, which can negatively impact their mental health". "At 243 million, India has the largest adolescent population in the world who are greatly at risk today" (Muttreja, 2020).

Socialising in context to leisure activities implies social interaction (Kelly, 1981), consequently, when there is little or no interaction during an activity, the meaning changes and leisure is converted into entertainment (Rojek, 2006). The connection to a network of people communicating via machines, defined by Allen (2010) as social connectivity, differs from face-to-face interaction because digital technologies multiply the opportunities for social interaction (Allen, 2010). Technologies used for socialisation can be shown with the help of the given Table1. Participation in virtual environments (online games, social media, etc.) develops skills that facilitate interpersonal relationships (Schroeder, 2010).

The current generation of adolescents has been variously conceptualized with theoretical categories including "screenagers", "digital native", and as "Net Generation" by (Rushkoff, 1999; Tapscott, 2008; Palfrey and Gasser, 2008). Social networking sites such as Facebook, MySpace, Google+, and Twitter are a regular part of leisure in everyday life among people of various age groups, genders, and racial/ethnic backgrounds (Iryna and Monika, 2015). A survey of young British people aged 11–16 found that participants chose different SNS platforms to 'manage' different types of friendship relationships. For example, Facebook was mainly used to communicate with 'friends', whereas Snapchat and Instagram were mainly used to communicate with 'close friends' (Wang and Edwards, 2016).

There are currently around 1.28 billion monthly active users (MAUs) of Facebook worldwide, with a yearly increase of 15% (Facebook, 2014). For adolescents, SNSs are now "a primary way of communicating with and acquiring information about others in their social network", including family and friends (Engelberg and Sjöberg, 2004; Spies and Margolin, 2014). Bargh and McKenna (2004) maintained that this is because SNS's possess unique features for sharing personal information including photos, "likes", and reflections via "wall posts" and "status updates" (Bargh and McKenna, 2004).

Table 1. Popular Communication-Orientated Internet Technologies

Social network sites (SNSs)	Facebook(2006), Bebo (2005), My Space (2003)
Mobile phone applications	Viber (2010), What's App (2009)
Blogs	Blogger (2003), Wordpress (2003)
Microblogs	Twitter (2006)
Video sharing	YouTube (2005)
Photo sharing	Instagram (2010)
Massively multiplayer online computer games (MMOG)	World of Warcraft (1994)
Virtual worlds	Club Penguin (2005), Moshi Monster (2007) Online simulated 3D environments where users construct avatars, assume fantasy roles and interact with other players.

Source: Kennedy and Lynch, 2016, p.3

SNS activities have become a core part of teen culture, redefining the meaning of the word friend in this global digital community and it provides a platform to enjoy their leisure time by using digital technology. SNSs may, therefore, provide an advantage in understanding adolescents' inner lives and in building relationships with them (Subrahmanyam et al., 2009). Social activities with friends continue to be identified by many adolescents as the most important and valued leisure activities (Abbott-Chapman, 2001).

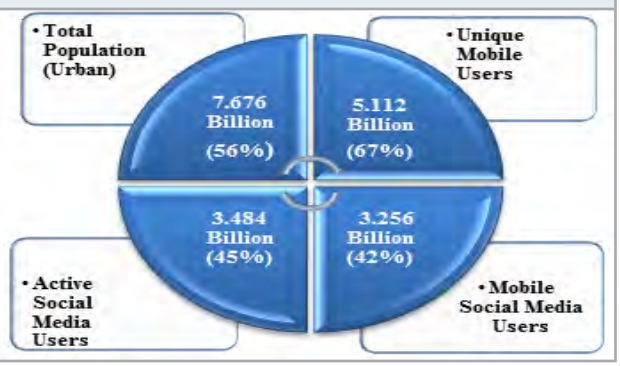
Technology and adolescent's worlds were identified in 2009 as an important area for development (Fok et al., 2009). Digital technologies for adolescents are essentially and primarily "tools for leisure and sociability" (Sánchez-Navarro and Aranda, 2013). Individuals' engagements

with these technologies are increasingly being considered forms of "technologically mediated leisure", or "digital leisure" (Parry and Penny Light, 2014; Sintas et al., 2015; Spracklen, 2015; Valtchanov et al., 2016). Digital leisure activities, including social interaction, have been transformed into the traditional way of planning and performing face-to-face leisure activities, it's all due to digital technologies. Digital leisure significantly expands the "sphere of sociability" in which individuals can come together online (Parry and 2013; Laura et al., 2016).

Digital leisure helps in receiving support, form friendships, and explore mutual interests and identities (Drotner, 2008). Adolescents' digital leisure takes many forms and is frequently done simultaneously (Tapscott, 2008), including texting friends, downloading music, uploading videos, sharing photos, perusing Facebook profiles, and updating Twitter comments, to name a few common practices (Bronwen and Parry, 2017). Social media usage and Asia's present position along with India's are highlighted through, published reports, there were 3.5 billion social media users worldwide and this number is continuously growing (Emarsys, 2019).

Social media usage is one of the most popular online activities, in 2018, around 2.65 billion people were using social media worldwide, a number projected to be increased to almost 3.1 billion in 2021 and the number of social media users in India were 326.1 million in 2018 and expected to be almost 448 million in 2023 (Clement, 2019). Global Digital Year Book, the report showed that the average amount of time per day spent by Indians on the internet; surfing via mobile phone 03:43 hours and through computers (desktops, laptops or tablet) 04:03 hours whereas social media usage via any device, 02:32 hours. The report, highlighted the facts related to digital technology shown in the given Fig.1 (Kemp, 2019).

Figure 1: Digital Technology and its Users



The world's most visited websites as per the report by Hootsuite; Google stands first, as far as social networking sites were concerned Facebook stands third, Twitter, seventh, and Instagram, tenth. Mobile social media growth rankings are concerned India ranked second with an absolute increase of +60 million users and growth rate of +26 percent. According to 2018-19, monthly mobile data use by North-East Asia is 12.1 billion gigabytes which are highest than other regions of the world, whereas Global Mobile App rankings by monthly active users; Facebook ranks first, WhatsApp Messenger second, Facebook Messenger third and WeChat ranks fourth place (Global Digital Year Book, 2019).

The above discussion covers all the aspects related to digital technology, emphasizing social networking sites and the active user worldwide. It is seen that adolescents nowadays engaged in online activities, during leisure time for socialising with peers and making new relationships, The need and significance of the proposed study, to explore the facts related to digital leisure, adolescence, and social activities. The study will help in creating awareness amongst adolescents, parents, school teachers, and administrators about the negative impacts of social media and provide suggestions to channelise the potential of adolescents in the right direction. The study supports, Family Media Use Plan, minimise unhealthy habits and behaviours due to the use of traditional and new media that can negatively affect health, wellness, social and personal development, and academic performance and success (Reid-Chassiakos, 2016).

The term 'social network sites' is relatively new, there has been a growing body of literature focused on their impact on psychological health (Lemola et al., 2015; Moreau et al., 2015). Some of these studies have found a relationship between depression and the amount of time spent on SNSs (Pantic et al., 2012; Wright et al., 2013). However, others have reported no direct relationship between the amount of time spent on SNSs and depressive symptoms (Datu and 2012; Jelenchick and 2013; Simoncic et al., 2014).

The above reports reflected that due to excessive use of technology and engagement on SNS, makes our youth 'digitoholic'. It will affect their academic achievement, mental health, physiological problems, disturbed family, social relations , and various other problems, being a researchers we must find the best plausible solution for this. The understudy follows the instructions and moves ahead in this direction, to study the indulgement of an adolescent in online activities and later suggested remedial measures for it. Research is needed on how parents can supervise and guide their children's media use (Reid-Chassiakos, 2016).

Objectives of the Study: To check the mediating effect of Digital leisure in the relation of independent variable LIM to dependent variable Adolescent health problems, to study the social activities of boys and girls students, to study the Digital Leisure (DL) of science and non-science students and to study the Digital Leisure (DL) of boys and girls students. Hypotheses of the Study:i) Ho: Digital Leisure mediates the effect of Leisure Interest Measure on Adolescent Health Problems ii) Ho: There will be statistically no significant difference between boys and girls social activities. iii) Ho: There will be statistically no significant difference between science and non-science student's digital leisure, iv) Ho: There will be statistically no significant difference between boys and girls digital leisure.

MATERIAL AND METHODS

The study was a descriptive survey-based, conducted on adolescents studying in Grade XII between (16-19 years) in CBSE affiliated schools from India and BHSEC governed schools in Bhutan. Two-stage stratified sampling was used; firstly based on geographical locations and secondly based on the demographic stat.

The study had selected, 460 adolescents, studying in senior secondary classes in science and non-science streams, from three regions of India; north, central and north-eastern parts as well two schools randomly selected from Bhutan.
The schematic representation of the selected sample has been shown in tabular form:

Table 2. Selected Sample for Study

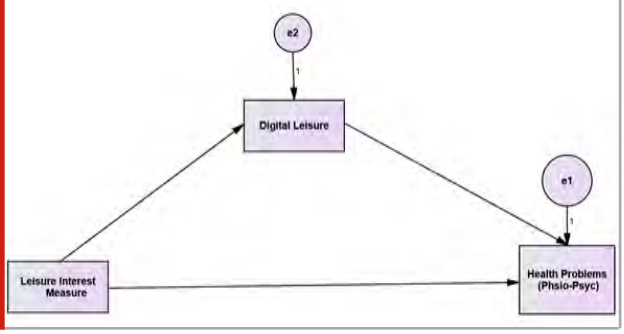
S. No.	Region	School Location	Science Students	Non-Science Students	Total Students
1.	Central India	Sagar (M.P.	30	26	56
		Gwalior (M.P.)	33	55	88
2.	North India	Agra (U.P.)	58	62	120
		Bhimtal (U.K.)	-	31	31
3.	North-East India	Guwahati (Assam)	35	27	62
4.	Bhutan	Two Schools Selected	59	44	103
		GRAND TOTAL	215	245	460

The study had used two questionnaires; Leisure Interest Measure (LIM), 29 items; 17 items had been selected from reading, physical, outdoor, artistic and social sections. A standardised questionnaire, designed by the investigator; Smart Phone Usage Pattern (SPUP), was used for studying the digital activities during leisure time (Beard and Ragheb, 1980). The study tried to understand the socialisation activities amongst adolescents using digital technology. Dependent Variable- LIM, Social Activities, Anxiety Level, Time Spent _SNS, Health Problems, Independent Variable- Boys and Girls, Science and Non-Science Stream and Mediating Variable- Leisure using Digital Technology (Digt_Leis). SPSS-21 and AMOS- 23 were used for testing the research model and hypotheses. Apart from it, qualitative analyses were used to explore other additional facts. Model fit criteria proposed by Bagozzi and Yi (1988) and regarding cut-off values for the indices (Beard and Ragheb, 1980; Bagozzi and Yi, 1988; Hair et al., 2006).

RESULTS AND DISCUSSION

I. Ho: Digital Leisure mediates the effect of Leisure Interest Measure on Adolescent Health Problems

Figure 2: Mediation Effect



Mediation was a type of multiple regressions; here LIM as an independent variable (IV), Health Problems as a dependent variable (DV) and DL as mediating variable also an independent variable (IV). The methodology was, first of all, to check the direct effect of IV on DV, and then the indirect effect of IV on DV in the presence of a mediator. Baron and Kenny, (1986) discussed three types of mediation, partial, full, and indirect mediation, later on, Prof. Hayes (2009) criticised that there are only two types of mediation; partial and full (Baron and Kenny, 1986).

Step A. To study whether there any direct relation between LIM and Health Problems
The value of R2 of the dependent variable (Hlth_ Prb) was 0.000 (0%) whereas the value of (Effect Size) was 0.03. Now to check whether this relation, significant or not

Table I.1 Regression Weights: (Group number 1 - Default model)

	Estimate	S.E.	C.R.	P	Label
Hlth_Prb <- LIM	.004	.006	.689	.491	

The relation between IV to DV, since the p-value 0.491 (greater than 0.05) relation was non-significant. Therefore the direct relation between LIM and Health problems was non-significant, so it reflects that there will be no mediation between them.

Step B. To study the indirect effect of independent variable LIM on dependent variable Health Problems in the presence of mediator DL; significant or not.
Prof. Hayes said that two direct relations together never make indirect so now we will check the indirect effect between them.

Table I.2. Regression Weights: (Group number 1 - Default model)

	Estimate	S.E.	C.R.	P	Label
Digt_Leis <--- LIM	.017	.044	.382	.702	
Hlth_Prb <--- LIM	.002	.003	.754	.451	
Hlth_Prb <--- Digt_Leis	.124	.003	40.804	***	

Only the relation between Digt_Leis to Hlth-Prb was significant while the other two relations were non-significant. The total effect on Hlth-Prb was 0.004 now R2 increased and becomes 0.78 (78%), out of which the direct effect was 0.002 whereas the indirect effect was also 0.002. The advisable was Standardise Indirect Effect which was 0.016, this was significant we had to check this.

Table I.3 Standardized Indirect Effects - Two-Tailed Significance (BC) (Group number 1 - Default model)

	LIM	Digt_Leis
Digt_Leis	...	...
Hlth_Prb	.729	

The p-value of 0.729 (greater than 0.05) represented that this effect was non-significant it reflected that there was no mediation effect. It means that if there was an indirect effect then there will be mediation if it didn't exist then there will be no mediation effect and if there was no initial direct relation between independent and dependent variables then there will be no mediation effect. Now the question arises if there was any mediation than what will be its type, it depends on in the presence of mediator what was the relationship between independent (LIM) and dependent variable (Hlth_Prb), initially the relation was not significant. Now in the presence of a mediator, if this relation changed, it will affect the mediation type.

Table I.4 Regression Weights: (Group number 1 - Default model)

	Estimate	S.E.	C.R.	P	Label
Digt_Leis<---LIM	.017	.044	.382	.702	
Hlth_Prb<---LIM	.002	.003	.754	.451	
Hlth_Prb<---Digt_Leis	.124	.003	40.804	***	

Now in the presence of mediator Digt_Leis, the relationship between LIM to Hlth_Prb was 0.451 which was still insignificant whereas the relationship between mediator to independent variable Digt_Leis to Hlth_Prb was significant which shows there was only one relationship exist which was between Digt_Leis and Hlth_Prb, it means neither full nor partial mediation worked. Thus the result showed that initially when we studied the direct relation in absence of a mediator between independent (LIM) and dependent variable (Hlth_Prb) existed a non-significant effect. In the presence of mediation, the standardised indirect effect of .016, sig. value 0.729 (greater than 0.05) indicates there was no mediation effect and later when we check LIM to Hlth_Prb it was0.451 again showed a non-significant result, which reflected that there was no mediation effect. There was only one relationship that existed which was a direct relationship between Digt_Leis to Hlth_Prb which had no importance. Therefore the null hypothesis was rejected; it reflected that there was no mediation effect exists.

II. Ho: There will be statistically no significant difference between boys and girls social activities

Table II.1 Group Statistics

	Gender	N	Mean	Std. Deviation	Std. Error Mean
Social Activities During Leisure Time	BOY	240	9.9792	1.94366	.12546
	GIRL	220	9.7864	2.16337	.14585

Table II. 2 Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means					
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference
									Lower / Upper
Social Activities During Leisure Time	Equal variances assumed	4.970	.026	1.007	458	.315	.19280	.19150	-.18352 / .56913
	Equal variances not assumed			1.002	441.499	.317	.19280	.19239	-.18531 / .57092

The first table showed the descriptive statistics the mean score for Boy and Girl were almost approximately equal to 10, which falls under Active_ Social Activist. Nearly 286 adolescents fall under this category of active social activists (10-12), 158 (65.83%) boys and 128 (58.18%) girls are active in social activities. It reflects that boys are more active than girls in social activities during leisure time. The first table reflects that social activities of boys and girls were almost the same during leisure time. The second table showed inferential statistics with, t value=1.007 which was smaller than CV=1.96, which means that mean values were not different, p value=0.315(greater than 0.05), which reflected that test was not significant, it, means that test scores didn't differ significantly between the groups. The 95% confidence interval of the difference Lower (-0.18352) and Upper (0. 56913), the confidence interval includes 0. Therefore Ho was accepted at t value=1.007, with df= 458 and p-value = .315(greater than 0.05), because it satisfied all the three conditions.

III. Ho: There will be statistically no significant difference between science and non-science student's digital leisure.

The Group Statistics table showed the descriptive statistics, the mean score for Science and Non-science student's digital leisure differed; Science mean score was 9 whereas for Non-science mean score was 10, both fall under Moderate_ DLA. Nearly 209 students fall under this category of moderate digital leisure (8-12), 99 (47.37%) science, and 110 (52.63%) non-science were moderate digital leisure active. It reflects that non-science students were more active in digital leisure activities than science students during leisure time.

The first table reflected that digital leisure of science differed from non-science students during leisure time. Independence Samples Test table showed inferential statistics with t value= -2.083 which was greater than CV= +_ 1.96, reflected that mean values were different, p value= 0.038 (smaller than 0.05), it reflected that test was significant, it means that test scores differed significantly between the groups. The 95% confidence interval of the difference between Lower (-1.32106) and Upper (-0.03841), the confidence interval didn't include 0. Therefore Ho was rejected at t value= -2.083, with df= 458 and p value= 0.038 (smaller than 0.05), it didn't satisfied all three conditions.

Table III.1 Group Statistics

	Students Opted Science and Non- Science Stream	N	Mean	Std. Deviation	Std. Error Mean
Digital Leisure	Science Group	215	8.9488	3.49261	.23819
	Non-Science Group	245	9.6286	3.49191	.22309

IV. Ho: There will be statistically no significant difference between boys and girls digital leisure.

The Group Statistics table showed the descriptive statistics, the mean score for Boys and Girls student's digital leisure differed. Boy's mean score was 10 whereas Girl's mean score was 9, both fall under Moderate_ DLA. Nearly 209 students fall under this category of moderate digital leisure (8-12), 118 (56.45%) boys and 91 (43.54%) girls were moderate digital leisure active. The first table reflected that digital leisure of boys differed from girls students during leisure time. Independent Samples Test table showed inferential statistics with t value= 2.312 which was greater than CV= 1.96, it reflected that means values were different, p value= 0.021 (smaller than 0.05), therefore the test was significant, it highlighted that test scores differ significantly between the groups. The 95% confidence interval of the difference between Lower (0.11278) and Upper (1.39252), the confidence interval didn't include zero. Therefore Ho was rejected at t value= 2.312, with df= 458 and p value= 0.021 (smaller than 0.05), it didn't satisfied all three conditions.

Table III.2. Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Digital Leisure	Equal variances assumed	.678	.411	-2.083	458	.038	-.67973	.32635	-1.32106	-.03841
	Equal variances not assumed			-2.083	450.250	.038	-.67973	.32635	-1.32110	-.03837

It was found that girls were generally involved in household activities apart from their studies whereas boys were generally free from household or other activities. The results revealed that adolescents especially boys and non-science students involved in digital activities than girls and science students. Adolescents were active on social networking sites; 286 nearly 65% were active most of their time on Facebook and Twitter, they were spending on an average 06-08 hours daily on online activities. The study highlighted the health facts while discussing with adolescents, most of them claimed that they were facing headache and eye problems, whereas quite a few were facing sleeping problems and psychological problems as anger, fear and depression.

Indian adolescents were facing severe health problems in comparison to Bhutanese students as they were on an average 3.5 hours less time engaged in online activities It was found that the risks associated with overuse of SNS by adolescents; depression, anxiety, sleeping disorder, decreased self-esteem and the suicidal tendency was quite common. It has been asserted that adolescents may reveal their emotions and thoughts on SNSs (Subrahmanyam et al., 2009; Memon et al., 2018).

Adolescents are of greater risk side, risks from abusers within, or outside the family, their close friends or acquaintance in this digital sphere. With the rapidly growing number of social media users globally, suicidality and self-harm behavior become a more complex issue depression and suicidality (Luxton and 2012; Memon et al., 2018). Despite, divergent views and the so-called generation gap, parents should try to bridge the gap with their children by regularly talking and discussing their scholastic, non-scholastic, and leisure time activities; friends, and other social interaction activities in a friendly manner, so that children can share their activities with them. Parents should discuss the drawbacks of such technology with adolescents and suggest them to use it for a specified period; otherwise, it will affect them in all spheres right from their academic to health issues. It should be seriously taken, as it was observed that, heavy parent use of mobile devices is associated with fewer verbal and nonverbal interactions between parents and children and may be associated with more parent–child conflict (Radesky et al., 2014; Radesky et al., 2015; Singh and Sharma, 2019).

Table IV. 1 Group Statistics

	Gender	N	Mean	Std. Deviation	Std. Error Mean
Digital Leisure	BOY	240	9.6708	3.55294	.22934
	GIRL	220	8.9182	3.41667	.23035

Table IV. 2. Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Digital Leisure	Equal variances assumed	.544	.461	2.312	458	.021	.75265	.32561	.11278	1.39252
	Equal variances not assumed			2.315	456.942	.021	.75265	.32505	.11387	1.39144

CONCLUSION

Education and Health accelerate the process of human development backed up by sound financial position. The findings of the study revealed that adolescents are using digital technologies in most of their leisure- time, especially boys in comparison to girls due to which they are facing health problems. Now the question arises how we overcome the problem of digital dependency, and 'screen addiction', among adolescents? The study suggested that the ministry of education should run programs in schools for adolescents, which creates awareness, how young people use SNS? Even the parents and teachers must keep eye on the activities of students regarding the usage of technology in school and at home and discussed with them the adverse effects of this technology. The study concluded that we should help adolescents in realising that digital technology assists them in shaping their future but it should be used in a proper way otherwise they will mislead from their path and their dreams will be shattered. We should tame the technology as well as treat it as our mate, but there will be a mete between both roles, it is in our hand how to handle the technology adequately.

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Conflict of Interests: Authors declare that the work and data present in the study are our original and innovative research, carried out by us. The study is not published earlier and we have not misused the facts and evidence.

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Survey Based Analysis of Tele-Rehabilitation in the Field of Speech Language Pathology During COVID 19 Outbreak in Kerala

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ABSTRACT

Tele-rehabilitation is the application of telecommunications technology for the delivery of speech language pathology and audiology services at a distance by linking clinician to client or clinician to clinician for assessment, intervention, and/or consultation. It is an emerging field, but due to the lack of trained professionals, the number of professionals providing tele-rehabilitation in Kerala is few in number. The outbreak of the pandemic COVID 19 has forced the speech language pathologists (SLPs) to shift from the traditional face to face therapy to tele-rehabilitation which was a new experience for most of the speech language pathologists. The present study aimed to understand the challenges faced by the speech language pathologists to provide tele-rehabilitation services to the clients during the outbreak of pandemic COVID-19 and how they overcome those barriers using a self- rated questionnaire developed. The questionnaire was sent to speech language pathologists through mail and WhatsApp. 105 speech language pathologists responded. Among them, 77 speech language pathologists provided tele-rehabilitation and served clients of all ages and different disorders with language disorder being the most common and dysphagia and apraxia being the least served client population. Tele-rehabilitation was found to be a viable form of service delivery in the field of speech language Pathology. All possibilities of Information and Communication Technology (ICT) were utilized by the SLPs to provide the best services despite the lack of training and non-availability of resources. This survey depicts the need for publishing standard guidelines for providing tele-rehabilitation services and also it emphasizes the need for improved infrastructure and training to professionals to ensure quality services to their clients.

KEY WORDS: COVID 19, KERALA, SPEECH LANGUAGE PATHOLOGY, TELE-REHABILITATION.

INTRODUCTION

COVID-19 is a disease caused by a new strain of coronavirus. 'CO' stands for Formerly, this disease was referred to as '2019 novel coronavirus' or '2019-nCoV.'With the outbreak of pandemic COVID 19, social distancing is practiced all over the world to prevent the spread of the disease. This has led to the exploration of the possibilities of technology in almost every aspect of

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life. Tele-rehabilitation refers to the use of Information and Communication Technologies (ICT) to provide rehabilitation services to people remotely in their homes or other environments (Brennan et al., 2009; World Health Organization, 2020).

Telepractice in speech language pathology is the application of telecommunications technology to the delivery of speech language pathology and audiology professional services at a distance by linking clinician to client or clinician to clinician for assessment, intervention, and/or consultation (ASHA, 2005a; World Health Organization, 2020). The Special Group Interest (SIG) 18 of American Speech and Hearing Association (ASHA) is Telepractice (Brown, 2014). The aim of SIG 18 is to provide education, leadership, and advocacy for issues in telepractice for audiology and speech-language pathology. More than 1,000 speech-language pathologists and audiologists joined SIG 18 within a span of four years. In India, telepractice in the field of speech language pathology is only a decade old (Brown, 2014).

The launch of a specialised centre for rehabilitation and education through distance mode in 2009, by the All India Institute of Speech and Hearing, Mysore may be considered as a formal beginning of teleservices (Rao and Yashaswini, 2018). There are only a few speech language pathologists in India providing telerehabilitation in the field of speech language pathology. The number of participants in the reported studies from India indicates this (Mohan et al., 2017; Rao and Yashaswini, 2018). The first published article on telepractice in India was a case report of a person with Broca's Aphasia. The results showed significant improvement in the domains of expression, repetition, naming and memory (Goswami, Bhutada and Jayachandran, 2012; Rao and Yashaswini, 2018). The authors concluded that telepractice is effective in the Indian context and is an upcoming area in the field of speech language pathology (Goswami, Bhutada and Jayachandran, 2012; Rao and Yashaswini, 2018). The first ever survey on telepractice in Speech Language Pathology and Audiology in India was carried out by Mohan et al. (2017).

The questionnaire was emailed to the members of ISHA (Indian Speech and Hearing Association). There were 205 respondents out of which only 25 respondents reported using telepractice to deliver clinical services. The results showed that telepractice services were provided to clients throughout the lifespan. The service provided includes screening, assessment, management, follow-up or monitoring/ guidance and/or professional consultation. Disorders managed via telepractice include various child and adult speech language disorders (Mohan et al., 2017; Rao and Yashaswini, 2018). Among the disorders, speech sound disorders in children ranked first and motor speech disorders were ranked least. Positive feedback from clients about telepractice services was received by fifty-six percent of the tele practitioners. Lack of training was a reported drawback and a short-term training certification course in telepractice was suggested by telepractioners (Rao and Yashaswini, 2018).

The service providers in India had learned to implement telerehabilitation through personal experience rather than formal training. 92% of the tele-practitioners reported that they have not authored a publication on telepractice. This suggests a dearth of research on telerehabilitation in speech-language pathology and audiology in India (Mohan et al., 2017). The shortage of professionals in India to deliver clinical services can be met via the use of telerehabilitation. In a study on tele-speech language pathology and audiology in India, the majority of the participants reported technical issues as barriers for telerehabilitation. Other challenges reported in the delivery of telerehabilitation were concerns about client and clinician confidentiality, lack of direct feedback and environmental distractions at client end. The benefits identified were improved access of services to clients with linguistic and cultural diversity; increased ease of collaboration among multi-disciplinary team members; and saves travel time with cost benefits for clients (Mohan et al., 2017).

India is well equipped to fully develop telerehabilitation to overcome the barriers of distance and amplify the availability of speech language pathology, audiology and other healthcare services. The extensive use of telerehabilitation throughout India would require an improved infrastructure (e.g., to uphold privacy and security); training for professionals; and telerehabilitation policies (Rao and Yashaswini, 2018). The outbreak of the pandemic COVID 19 has forced the speech language pathologists to shift from the traditional face to face therapy to telerehabilitation which was a new experience for most of the speech language pathologists. The American Speech-Language-Hearing Association (ASHA) has mentioned that telepractice is a viable process for delivering SLP services during COVID-19 pandemic and that both evaluation and treatment were possible through telepractice. Sarsak (2020) emphasized the need of speech language and hearing associations to promote telerehabilitation during the outbreak of COVID-19 (Sarsak 2020).

Courses on telerehabilitation can be conducted by these associations in various countries to increase the awareness of speech therapists on telerehabilitation and also research on this issue is prioritized. It will also improve the attitude of therapists toward telepractice and update their knowledge and skills. These associations must also pursue legal efforts to make these services legitimate (Sarsak 2020). Overall, it is suggested that further measures can be taken by the professional associations to eliminate barriers in the path of therapists and promote telepractice facilitators so that this type of care be used more extensively by SLPs (Tohidast et al., 2020).

The present study aimed to understand the challenges faced by the speech language pathologists to provide

telerehabilitation services to the clients during the outbreak of pandemic covid 19, how they overcome those barriers, and benefits of telerehabilitation. Information gathered from this survey results can be used to upgrade the professional performance of speech language pathologists during this COVID 19 pandemic, develop the quality of telerehabilitation delivered to patients, mend existing deficiencies of the services provided through telerehabilitation and create awareness about various aspects of telerehabilitation (Tohidast et al., 2020).

There is a dearth of published literature on telerehabilitation in India and there are no published studies on telerehabilitation in Kerala to the best of the authors' knowledge. Hence the objectives of the present study were to Report the status of telerehabilitation in speech-language pathology in Kerala Compare the opinions of Speech-Language pathologists about telerehabilitation in Kerala.Report the challenges faced by Speech Language Pathologist during telerehabilitation and the strategies practiced to overcome them. Identify the benefits of telerehabilitation in speech-language pathology.

MATERIAL AND METHODS

The study was conducted in three phases; development of the questionnaire, administration of the questionnaire and the analyses of the responses. In phase 1, a questionnaire was developed to gather responses from Speech Language Pathologists practicing in the state of Kerala and it consisted of 25 questions. The questionnaire contained questions to collect the demographic details of the participants and questions to elicit the information about service delivery through telerehabilitation. Twenty-one closed ended questions were used to gather the opinions of SLPs about telerehabilitation. The given questions in the questionnaire addressed the opinions of Speech-Language pathologists about telerehabilitation, the challenges faced by Speech Language Pathologist during telerehabilitation, the strategies practiced to overcome them and the benefits of telerehabilitation.

Most of the questions required a response selected from multiple options and open-ended response options were given for the questions where the participants could provide their comments. The participants could select more than one response from multiple options for some of the questions and some of the questions were answered with either yes or no. The clinicians who have not provided telerehabilitation services could submit the questionnaire after filling the 8th question. Content validity of the questionnaire was done by 5 speech language pathologists and the questionnaire was modified according to the suggestions given data.

In the second phase, the developed questionnaire was transformed into a google form. The google form was sent to speech language pathologists through mail and WhatsApp and 105 speech language pathologists working across the state of Kerala responded to the questionnaire. Informed consent was taken from all the participants. Analysis of the responses was done in the third phase. Descriptive statistics is used to analyse the responses from the participants. Responses were analysed by calculating the percentage values of the questions. This was done separately for each participant and also for the overall responses.

Table 1. Demographic details of the participants

	Demographic details	Number	Percentage
Gender	Male	4	3.8
	Female	100	96.2
Years of experience	-	-	6 months -30 years
Educational qualification	MASLP	54	51.9
	BASLP	28	26.9
	MSc SLP	12	11.5
	MSc Audiology	4	3.8
	PhD	4	3.8
	MSc Speech and Hearing	1	1
	MSc Deglutology	1	1
Work set up	Academic institute	35	33.7
	Clinics	22	21.2
	Govt hospitals	11	10.6
	Private hospitals	14	13.5
	Private practice	9	8.7
	Special school	3	2.9
	Block resource center	2	1.9
	Rehabilitation centers	6	5.8
	Urban resource centers	1	1
	NGO	1	1

RESULTS AND DISCUSSION

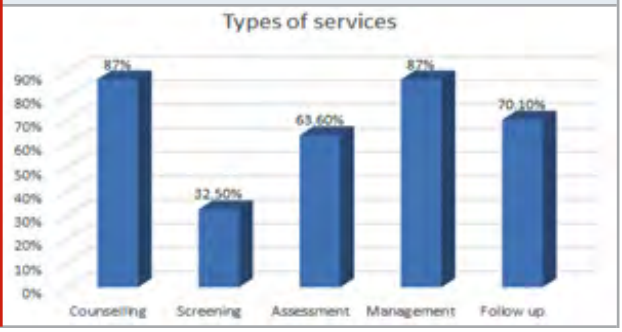
The results of this study give an outline about the status of telerehabilitation in the field of Speech Language Pathology in Kerala and the results were analysed using descriptive statistics. The demographic details are given in Table 1. There were 104 respondents and years of experience ranged for 6 months to 30 years. Majority of the respondents were post graduates and were working in academic institutions, clinics and hospitals. The gender distribution was skewed towards females with 96.2% of the respondents being females. This gender disparity in the field of speech and hearing has already been established (Rowden-Racette, 2013).

The tele-rehabilitation services were provided by 74% (N=77) of the participants and 26% (N=27) of the participants did not provide telerehabilitation services during the pandemic COVID 19. The most common reasons for not providing telerehabilitation are given in Table 2. In the survey conducted by Mohan et al. (2017), there were only 25 SLPs doing telepractice in India among the 205 respondents. However, in the current survey in Kerala, 77 SLPs are providing tele-rehabilitation services among 104 participants. The lockdown followed by the pandemic Covid 19 has imposed the SLP's to shift from the traditional face to face therapy to telerehabilitation which is the best method to provide the required services during Covid 19 (Mohan et al., 2017; Tohidast et al., 2020).

Table 2. Common reasons for not providing telerehabilitation

Reason	Percentage of respondents
No insistence from clinics or institutes	51
Client population is difficult to handle	25.9
Not confident in providing telerehabilitation	11.1
Clients or parents are not interested.	14.8

Figure 1: Percentage of respondents who delivered various services



Majority of the SLPs (79.2%) had no experience in telerehabilitation before the outbreak of Covid 19 and 20.8% (N=16) had experience in delivering telerehabilitation. Their experience in providing telerehabilitation ranged from one month to three years. Among the speech language pathologists who provided telerehabilitation, 76.6% people agree that tele rehabilitation is a viable form of service delivery, 15.6% are not sure and 7.8% did not find telerehabilitation as a viable form of service delivery. This finding is in agreement to the earlier survey conducted by Mohan et.al in 2017. Figure 1 shows the percentage of respondents providing various telerehabilitation services. Various services provided through telerehabilitation include counselling, assessment, screening, management and follow up sessions. Similar results were observed by Mohan et al. (2017).

Figure 2: Percentage of users of various online platforms

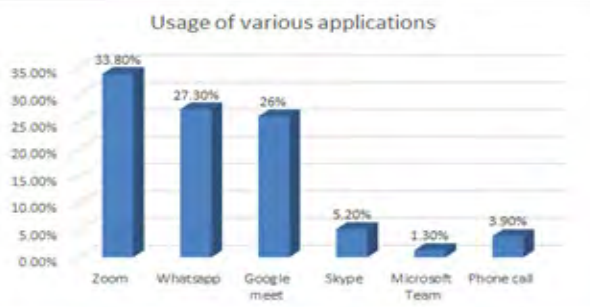
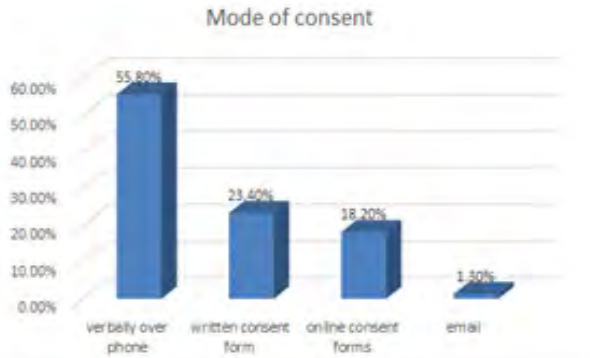


Figure 3: Percentage of clinicians who used different modes of consent.



The various online platforms used by SLPs for delivering telerehabilitation included zoom, whatsapp, google meet, phone call, skype and microsoft teams. Percentage of use of each online platform are given in Figure 2. Two participants reported that they may use any of the above mentioned online platforms depending on the comfortability of their clients. The most reported reasons for using the specific apps were familiarity of the clients with the app (64.9%), familiarity of the clinician with the app (36.45%), better video quality (27.3%) and reduced data usage (14.3%).

Initially in India, Skype was used for telerehabilitation. Over the time, many video conferencing applications were developed which were user friendly. Custom made applications were also developed by private centers for their own use, but none of the respondents in the present survey mentioned that (Goswami et al., 2012). In the present study, all were using the free video conferencing applications which are available over the internet. First research paper on telerehabilitation from India reported the extensive use of the skype application for telerehabilitation (Goswami et al., 2012; Mohan et al., 2017).

Figure 4: Percentage of clinicians providing telerehabilitation for clients across various age groups.

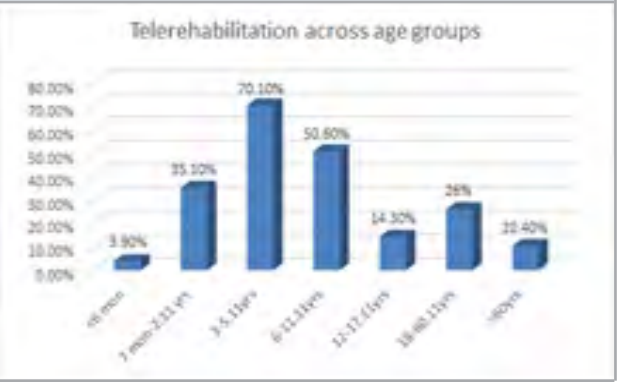


Table 3. Percentage of clinicians who served various disorders served through telerehabilitation

Disorder/ client population served	Percentage of respondents
Language Disorder	53.2
Autism spectrum Disorders	46.8
ADHD	15.6
Learning disabilities	24.7
Cerebral Palsy	13
Global developmental delay	23.4
Speech Sound Disorders	41.6
Fluency disorders	39
Aphasia	23.4
Dysarthria	14.3
Voice and resonance disorders	15.6
Cognitive communicative disorders	10.4
Hearing loss	24.7
Clients attending auditory verbal therapy	10.4
Clients using AAC	9.1
Dysphagia	1.3
Verbal apraxia of speech	1.3

Taking consent before the commencement of telerehabilitation is very important. Lack of ethical guidelines, issues related to privacy and confidentiality on e-platforms, data protection were issues which required immediate attention in telepractice. A well written informed consent may resolve the issue to an extent. Even though the mode of getting consent was different, all SLPs except one had taken consent from the clients (Rao and Yashaswini, 2018). This shows the awareness regarding this issue among the SLPs. Percentage of clinicians who used different modes of consent is summarised in Figure 3. The guidelines issued by ISHA clearly states the need for getting informed consent prior to the commencement of telepractice. Clients should be well informed regarding the modality of service delivery, its benefits and limitation, their rights and responsibilities including the process for communicating complaints or feedback (ISHA, 2020).

Telerehabilitation services were provided to clients of all ages. Figure 4 depicts the percentage of clinicians providing telerehabilitation for clients across various age groups. Even though telerehabilitation was provided to clients of all age groups, the paediatric population were served the most. Similar results were observed in the survey done by Mohan et al. (2017). Through telerehabilitation, clients with various speech and language disorders were served which is summarised in Table 3. Results revealed that language disorders were the most common client population served followed by autism spectrum disorder and speech sound disorder. Dysphagia and apraxia were the least served (Mohan et al., 2017).

Table 4. Difficult to manage client population

Difficult to handle population	Percentage of respondents
Language Disorder	6.5
Autism spectrum Disorders	66.2
ADHD	51.9
Learning disabilities	1.3
Cerebral Palsy	20.8
Global developmental delay	22.1
Speech Sound Disorders	9.1
Fluency disorders	6.5
Aphasia	9.1
Dysarthria	6.5
Voice and resonance disorders	11.7
Cognitive communicative disorders	13
Clients attending auditory verbal therapy	23.4
Clients using AAC	16.9
Dysphagia	2.6

Table 4 summarizes the responses of the SLPs regarding the client population which are difficult to manage. Even though the majority of the participants have provided services to children with autism spectrum disorder and ADHD, they reported that these children were the most difficult to manage client population while providing telerehabilitation. This could be due to their hyperactivity and inattention which makes it difficult to sit in front of the screen and follow clinician's instructions.

The difficulty to manage dysphagia through telerehabilitation was reported by only one participant and this can be due to the smaller number of SLPs providing telerehabilitation for dysphagia clients. Managing dysphagia through tele mode may involve many risks. But through proper planning and training of both client and the caregiver, we can successfully treat dysphagia through telerehabilitation. Considerations for the management of dysphagia through telerehabilitation is given by Miles et al., (2020). But both research and clinical practice in this area show that the use of telehealth for dysphagia management can be safe, feasible, and reliable, but several safeguards and considerations need to be in place (Miles et al., 2020).

Table 5. Client/ clinician related challenges while providing telerehabilitation

Challenges/ Barriers faced	Percentage of respondents
Lack of training	22.1
Lack of online resources	46.8
Lack of confidence	5.2
Parents or patients are not willing to attend	44.2
Difficult to manage children through online mode	76.6
Non availability of caretaker or parent	18.2
Non cooperative parents	27.3
Children with attention issues	22.1
Others	7.8

The sudden trend in telerehabilitation has led SLPs to face various challenges / barriers. Majority of the respondents did not have any previous experience in telerehabilitation and hence had to face many challenges while implementing telerehabilitation. Table 5 summarizes the client/ clinician related challenges while providing telerehabilitation. Difficult to manage children through online mode was the one of the biggest challenges faced by SLPs followed by scarcity of online resources about telerehabilitation and non-willingness of parents or patients to shift from traditional face to face therapy to telerehabilitation (Miles et al., 2020).

Table 6 represents the technical challenges faced by SLPs while implementing telerehabilitation. Among the technical challenges faced, internet connectivity issues stand first followed by inadequate knowledge of clients to use tele-service applications and non-availability of smartphones or computers. SLPs learned through personal experiences rather than formal training. Webinars have become a trend in the Covid season and webinars on telepractice is the only formal kind of information gaining that has happened. Similar results including insufficient resources (i.e., structural framework, technical support, resource materials) to provide appropriate tele-speech-language pathology services and the lack of formal training in India were the major concerns reported by Mohan et al. (2017).

Table 6. Technical challenges

Technical challenges	Percentage of respondents
Internet connectivity issues	94.8
No internet connection	7.8
Non availability of smart phone / computer	39
Clients not good at using applications	58.4
Non availability of interactive software	1.3

Even though the participants reported of various challenges faced while providing telerehabilitation, evident effort has also been taken to overcome these challenges which is given in Table 7. The reported solutions tried to overcome the challenges were discussing with the SLPs who have experience in telerehabilitation, trial and error method, attending webinars and reading articles on improving telerehabilitation. Development of professional skills for telepractice, validation of digital resources in the different languages of India; empirical studies on mode of service delivery in telepractice (face-to-face, virtual or hybrid); mechanisms to protect client's privacy on e-platforms; and revision of code of ethics for speech-language pathologists and audiologists who are using telepractice were the immediate concerns as reported in the report on tele speech language pathology and audiology in India and these concerns still remain the same (Rao and Yashaswini 2018). But due to the sudden boom in telerehabilitation, we expect a sudden growth in these areas and in the near future, we will be able to overcome the barriers faced. Despite these challenges, telerehabilitation has a critical role during the infectious pandemics and it will reduce the risk of spreading the infection which are transmitted by person-to-person contact (Smith et al., 2020).

Table 7. Steps taken to overcome the challenges

Steps taken	Percentage of respondents
Discussed with SLPs who have experience in telerehabilitation	71.4
Read articles on telerehabilitation	49.4
Attended webinars on telerehabilitation	61
Trial and error method	71.4
Educating parents	1.3
Watched youtube videos on telerehabilitation	1.3

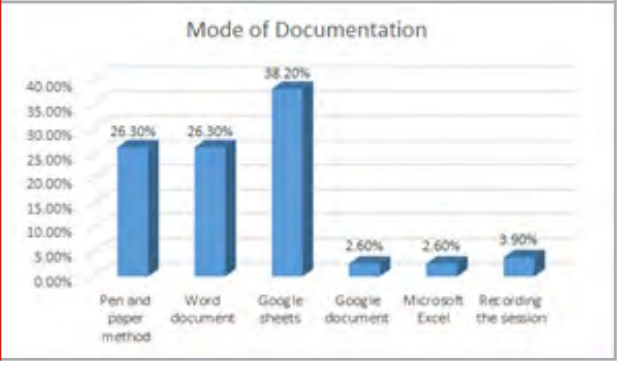
There were many benefits experienced through telerehabilitation. Table 8 shows the various benefits of telerehabilitation identified by participants. Eliminating long distance travelling thus avoiding the risk of Covid 19 was the benefit cited by majority of the SLPs

(92.2%). Other benefits include comfortability of clients in performing activities at the home set-up, increased involvement of parents in therapy, regularity in taking sessions, increased patient reach or access, easy to follow up, easy to perform creative screen-based activities and patient satisfaction (ISHA, 2020). The benefits of telerehabilitation makes it a viable form of providing services to clients with speech and language impairments and the majority of clinicians could achieve their goals through telerehabilitation. The technological advancements and the benefits can make it a regular form of service even after the COVID 19 season. The scarcity of resources and other issues such as lack of legal guidelines and policies for safe and secure service delivery should be addressed by the concerned authorities at the earliest. Recently Indian Speech and Hearing Association (ISHA) has compiled and published the telepractice guidelines for audiology and speech language pathology services in India including operational and ethical aspects (ISHA, 2020).

Table 8. Benefits identified through telerehabilitation

Benefits	Percentage of respondents
Regularity in taking sessions	54.5
Increased patient access or reach	53.2
Eliminating long distance travel and 19 thus avoiding the risk of Covid	92.2
Parents could involve more in therapy	61
Patient satisfaction	22.1
Easy follow up/ monitoring	46.8
Clients are comfortable in performing activities at the home set-up.	84.5
Easy to perform creative screen-based activities	1.3

Figure 5: Methods of documentation used by SLPs



Different methods of documentation used by SLPs were depicted in figure 5. As noted from the chart, the various methods include traditional pen and paper method, word document, google sheets, excel sheets, google docs and recording of sessions were reported. The clinical record is an overall indicator of clinical and service quality, and serves as a basis for planning care and for service continuity (Sutherland, 2006). Documentation style may vary among professionals or organizations but should adhere to specific facility standards.

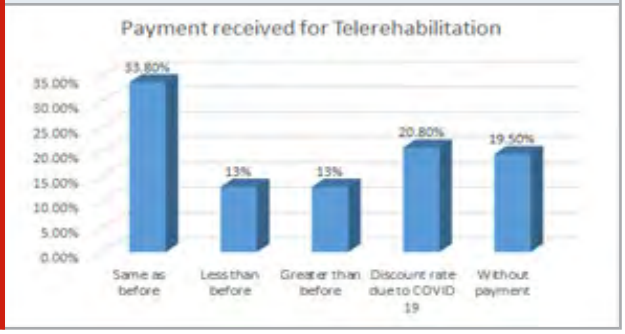
Whatever be the style, clinical records should be consistent in format and style and should use appropriate terminology, approved abbreviations, and correct diagnosis and procedure codes. Majority of the respondents in the survey use various electronic documentation methods. E- records give the flexibility of accessing it anywhere, anytime. Indian Speech Hearing Association had recommended storing all the client related reports and records of the telepractice session using the unique patient identification number in a confidential manner (ISHA, 2020).

Majority of the tele practitioners could achieve the targeted goals through telerehabilitation. 74% (N= 57) of the tele practitioners reported that they could achieve the goals through telerehabilitation. 22.1 % (N=17) were not sure and 3.9 % (N=3) reported that they could not achieve the goals. A small percentage who could not achieve their goals would be those tele-practioners who serve the difficult to handle population such as clients with dysphagia, autism spectrum disorder or ADHD (ISHA, 2020). The feedback from the clients regarding telerehabilitation as reported by the SLPs is shown in Figure 6 and only one SLP among the participants reported that the clients were not satisfied with telerehabilitation (ISHA, 2020).

Figure 6: Feedback from clients

The payment received for telerehabilitation services were differing among SLP's as depicted in Figure 7. To the best of authors' knowledge, telerehabilitation services are charged more than the traditional face to face therapy due to the use of high-speed internet data and the increased time required for planning and preparation. But in the present study, only a small percentage (13%) of SLPs have charged more than the charge paid for traditional face to face therapy. Majority (33.8%) has charged the same fees as that of pre COVID face to face therapy. Some provided telerehabilitation services at a discounted rate (20.80%) due to the COVID 19 and some had provided services free of cost (19.50%) (Smith et al., 2020).

Figure 7: Payment received for telerehabilitation



Free telerehabilitation services were provided by SLPs working in government services including hospitals and institutes. To the best of authors' knowledge, none of the previous studies had mentioned the fees charged for telerehabilitation. Regardless of the fees charged, telerehabilitation is the best method to provide the required services during Covid 19 (Tohidast et al., 2020). To fulfil the need for continuous therapy sessions for children and adults with speech-language disorders, the implementation of telepractice in the field of Speech Language Pathology is necessary which will also help to prevent the transmission of COVID-19, and thereby guaranteeing the health of SLPs and patients (Smith et al., 2020).

CONCLUSION

The present survey was conducted using a self-rated questionnaire and reported the status of telerehabilitation in the field of speech-language pathology in Kerala, the challenges faced by Speech Language Pathologist during telerehabilitation, the strategies practiced to overcome them and the benefits of telerehabilitation in speech-language pathology. Even though all of the participants were practicing traditional face to face therapy before the outbreak of COVID 19 pandemic, majority of them easily shifted to a tele-mode of providing services during the COVID 19 pandemic outbreak.

This survey depicted the need for publishing standard guidelines for providing telerehabilitation services. At the time of data collection, there were no published standard guidelines in India for providing telerehabilitation. But in November 2020, Indian Speech and Hearing Association (ISHA) came forward with the guidelines for telepractice which will help the telerehabilitation service providers to provide better services to their clients. It also emphasizes the need for improved infrastructure and training to professionals to ensure quality services to their clients.

Conflict of Interest Statement: There is no conflict of interest to disclose.

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Conflict of Interest: Authors declares no conflicts of interests to disclose.

Ethical Clearance Statement: The Current Research Work Was Ethically Approved by the Institutional Review Board (IRB) of National Institute of Speech and Hearing, NISH Road, Aakkulam, Trivandrum, Kerala, India.

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In-Silico Screening of T-Cell Epitopes as Vaccine Candidate from Proteome of H9N2 Virus

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ABSTRACT

The H9N2 virus outbreak has increased worldwide in last decade due to the zoonotic potential of these viruses. The H9N2 virus cause low pathogenicity but when co-infected with other pathogen it causes high mortality. Since 1998, H9N2 infection caused one death & more than 59 cases reported worldwide in animals including humans. There are currently no clear methods to control the pandemic potential of H9N2 virus globally, so there is urgent need for vaccine designing against these viruses. In this study, screening of T-Cell epitopes from H9N2 virus proteins viz nuclear export protein, nonstructural protein 1, matrix protein 2, matrix protein 1, neuraminidase, nucleocapsid, hemagglutinin, polymerase PA, PB1-F2 protein & polymerase PB2 protein followed by highest binding affinity of selected T-cell epitopes with their corresponding HLA alleles has been done. The server ProPred1 & ProPred facilitates the binding prediction of HLA class I & class II allele with specific epitopes from the antigenic protein sequences of H9N2 virus. PEPstrMOD server was used structure modeling of the screened epitopes. We docked the selected T-cell epitopes with their corresponding HLA allele structures using the HPEPDOCK Server. Toxicity & immunogenicity of epitopes were analyzed by Toxin Pred and IEDB tools, respectively. The screened T-cell epitopes viz FQGRGVFEL, AEIEDLIFL, IIEGRDRTL, RRVDINPGH, YIGVKSLKL, LVMKRKRDS, VVLVMKRKR, LVRKTRFLP are anticipated to be valuable in designing comprehensive epitope-based vaccines against H9N2 virus after further *in-vivo* studies. This analysis hopes to be a credible milestone for researchers around the globe helping them with finding optimal results for their analysis.

KEY WORDS: H9N2 VIRUS, T-CELL EPITOPE, HLA ALLELES, VACCINE DESIGNING..

INTRODUCTION

H9N2 viruses cause worldwide infections and the majority of confirmed cases were young children. Different combination of hemagglutinin and neuraminidase, surface proteins of Influenza A viruses, give rise to subtypes viz H1N1, H5N6, or H9N2. Different studies showed that the primary routes of transmission of H9N2 virus is respiratory and direct contact. Aerosol, droplet particles, oral-facial route & direct touch are the rout of transmission for this virus (Killingley et al., 2013). H9N2 virus infection in humans observed in Hong Kong, India, Bangladesh, Pakistan, Oman, Egypt & China (Butt et al., 2003; Shanmuganatham et al., 2013; Pan et al., 2018; Ali et al., 2019; Potdar et al., 2019). H9N2 outbreaks in commercial chickens from Asia, Middle East and African countries reported recently (Li et al., 2020).

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A recent report of H9N2 virus (strain A/India/TCM2581/2019) infection observed in a 17-month-old boy residing at Melghat District, Maharashtra, India showed a threat to human infection in India & there is an urgent need for the treatment of this emerging virus (Potdar et al., 2019). Important implication of this study is to screen promiscuous T-cell epitopes from H9N2 virus proteins viz nuclear export protein, nonstructural protein 1, matrix protein 2, matrix protein 1, neuraminidase, nucleocapsid, hemagglutinin, polymerase PA, PB1-F2 protein & polymerase PB2 protein. The screened selected T-cell epitopes may be the promising targets for epitope-based vaccine design for H9N2 virus (Li et al., 2020).

MATERIAL AND METHODS

Complete genome sequence of H9N2 virus study strain (A/India/TCM2581/2019/(H9N2) was taken in this work (Potdar et al., 2019). The amino acid sequence of nuclear export protein, nonstructural protein 1, matrix protein 2, matrix protein 1, neuraminidase, nucleocapsid, hemagglutinin, polymerase PA, PB1-F2 protein & polymerase PB2 protein of H9N2 virus were retrieved from protein sequence database from NCBI (http://www.ncbi.nlm.nih.gov/protein) and their accession number were shown in Table 4. A proteomics server, ExPASy ProtParam (www.expasy.org) was used to analyze the primary structure of the target protein. Several parameters given by ProtParam tool for example estimated half-life, amino acid composition, theoretical pI, molecular weight, extinction coefficient, atomic composition, aliphatic index, grand average of hydropathicity (GRAVY) and instability index were examined.

SOPMA server used to check the secondary structure (alpha helix, beta plated sheets, turns and coils) of the proteins, its aim to predict solvent accessibility, transmembrane helices, coiled-coil regions, globular regions and ultimately determines the stability and function of proteins (Geourjon and Deleage, 1995). To predict the protective antigens as vaccines, the sequence was then analyzed by VaxiJen.VaxiJen server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) with default parameters to find out the antigenicity. All the antigenic proteins with their respective predicted score were computed. The prediction of potential HLA class I & class II binding nanomer epitopes completed by using Propred I & Propred respectively. Threshold percentage of highest scoring peptides is taken at 3%. Top four binders for different HLA allele are taken into consideration. Immunoproteosome site & Proteasome site filters were put in 'on' mode with threshold of 4% for each filter (Singh and Raghava, 2001; Doytchinova and Flower, 2007).

Table 1. Primary structure analysis using ProtParam

Name of Protein	No. of amino acids	Molecular weight	Theoretical PI	Total no. of negatively charged residues (asp-Glu)	Total no. of positively charged residues (asp-lys)	Extinction coefficient	Estimated half-life	Instability index	Aliphatic index	Grand average of hydropathicity
Nonstructural protein 1	237	26966.89	5.51	36	31	34615	30	58.37	86.41	-0.354
matrix protein 2 matrix	97	11268.83	4.92	16	11	15595	30	57.86	92.37	-0.242
protein 1 Neuramini	252	27763.07	9.28	24	30	13075	30	36.32	85.99	-0.218
Dase Nucleo	469	51417.82	6.02	47	42	93610	30	33.69	75.22	-0.280
capsid	498	56137.54	9.47	58	70	52745	30	44.25	70.76	-0.561

ToxinPred (http://crdd.osdd.net/raghava/toxinpred/) was used to predict toxicity of predicted T- cell epitopes (Gupta et al., 2013). ToxinPred is an in-silico tool to predict the selected epitope as toxic or non-toxic. ToxinPred was run with default parameters and only non-toxic T-cell epitopes were selected for further study. The PEPstrMOD method performed to find out the tertiary structure of selected nanomer epitopes. The PEPstrMOD tool prediction strategy utilizes the secondary structure data & β-turns data anticipated by PSIPRED and BetaTurns respectively. The amino acid sequences of HLA alleles were retrieved from IMGT/HLA database (http://www.ebi.ac.uk/ipd/imgt/hla/intro.html) and homology model of alleles was constructed using program HPEPDOCK (http://huanglab.phys.hust.edu.cn/hpepdock/) Server (Robinson et al., 2012; Singh et al., 2015; Zhou et al., 2018).

HPEPDOCK Server has been used to perform docking of epitopes with alleles models. Docking studies was performed to study the interaction of epitopes with alleles. For such interaction studies, the most important requirement was the proper orientation and conformation of epitope, which fit to the binding site

of the allele appropriately and form the epitope-allele complex. The obtained docking scores was tabulated and analysed. Immunogenicity of the selected T-cell epitopes was predicted by using IEDB (Immune Epitope Database and Analysis Resource) (http://tools.iedb.org/immunogenicity/). This tool predicts the relative ability of an epitope-HLA complex to elicit an immune response. Amino acid properties & their position within the epitope are utilized by this tool to predict the immunogenicity of a class I epitope-HLA complex (Calis et al., 2013).

RESULTS AND DISCUSSION

H9N2 viruses are emerging zoonotic infectious viruses that cause fatal diseases in both animals and humans (Pusch and Suarez, 2018). New efficient vaccines against H9N2 virus infection are urgently needed to control the disease and its proliferation. In the present study, prediction and modeling of T cell epitopes of H9N2 virus antigenic proteins followed by docking studies of predicted highest binding scores with their corresponding HLA class I and class II alleles have been performed (Pusch and Suarez, 2018).

Primary and secondary structure analysis: Primary structure analysis viz theoretical isoelectric point (PI), molecular weight, total number of positively charged residues (Arg+Lys) and negatively charged residues (Asp+Glu), estimated half-life (in vitro) in mammalian reticulocytes and instability index (II) are shown in table 1 while secondary structure analysis viz alpha helix, extended strand, beta turn & random coil are shown in table 2 (Kamthania and Sharma, 2015; Pusch and Suarez, 2018; Kamthania et al., 2019).

Table 2. The secondary structure analysis using SOPMA

Protein	Alpha helix	Extended strand	Beta turn	Random coil
Nonstructural protein 1	132(55.70%)	30(12.66%)	7(2.95%)	68(28.69%)
matrix protein 2	49(50.52%)	14(14.43%)	4(4.12%)	30(30.93%)
matrix protein 1	153(60.71%)	24(9.52%)	16(6.35%)	59(23.41%)
Neuraminidase	32(6.82%)	162(34.54%)	29(6.18%)	246(52.45%)
Nucleocapsid protein	218(43.78%)	60(12.05%)	31(6.22%)	189(37.95%)
Hemagglutinin	192(34.29%)	117(20.89%)	42(7.50%)	209(37.32%)
polymerase PA	388(54.19%)	79(11.03%)	30(4.19%)	219(30.59%)
polymerase PB2	289(38.08%)	147(19.37%)	44(5.80%)	279(36.76%)

Table 3. VaxiJen result of antigenicity

S.No.	Protein	Overall Antigen Prediction
1	nuclear export protein	0.3441 (Probable NON-ANTIGEN)
2	nonstructural protein 1	0.4290 (Probable ANTIGEN)
3	matrix protein 2	0.5641 (Probable ANTIGEN).
4	matrix protein 1	0.4805 (Probable ANTIGEN)
5	Neuraminidase	0.5513 (Probable ANTIGEN)
6	nucleocapsid protein	0.5208 (Probable ANTIGEN)
7	Hemagglutinin	0.4322 (Probable ANTIGEN)
8	polymerase PA	0.5273 (Probable ANTIGEN)
9	PB1-F2 protein	0.1654 (Probable NON-ANTIGEN)
10	polymerase PB2	0.5291 (Probable ANTIGEN).

Protein antigenicity determination: Amino acid sequences of proteins viz nuclear export protein, nonstructural protein 1, matrix protein 2, matrix protein 1, neuraminidase, nucleocapsid, hemagglutinin, polymerase PA, PB1-F2 protein & polymerase PB2 were screened by VaxiJen. All the proteins were found antigenic except nuclear export protein & PB1-F2 protein which were non-antigenic at threshold value of 0.4 (default threshold for viral proteins) (Table 3). Antigenic proteins selected for further analysis (Pusch and Suarez, 2018).

Prediction and analysis of HLA Class I & Class II binding peptides: H9N2 virus proteins were subjected to Propred1 & Propred for selection of HLA Class I & HLA Class II specific T- cell epitopes binders respectively. Epitopes showing highest score with the maximum number of HLA alleles binders were selected at a threshold value of 3% (Table 4) (Pusch and Suarez, 2018).

Toxicity prediction: ToxinPred (Gupta et al., 2013) used for toxicity prediction of selected T- cell epitopes. ToxinPred tool is a unique in-silico method based on

Support Vector Machine (SVM) in predicting toxicity of peptides along with important physico-chemical properties viz Charge, Hydrophobicity, Hydropathicity, Hydrophilicity and Molecular weight. The selected epitopes were subjected to ToxinPred and only non-toxic T-cell epitopes were selected for further studies (Table 5) (Kamthania and Sharma, 2015; Pusch and Suarez, 2018; Kamthania et al., 2019).

Table 4. ProPred1 & ProPred predicted T-cell epitopes for HLA Class I & Class II with binding scores

Protein name	Amino acid length	Accession no.	Position	Epitopes	HLA class alleles	Propred (% of highest score)
nucleocapsid protein	498	QBP33428.1	457-465	FQGRGVFEL	HLA-B*0705	3000.000
nucleocapsid protein	498	QBP33428.1	250-258	AEIEDLIFL	HLA-B*2705	3000.000
polymerase PA	716	QBP33426.1	77-85	IIEGRDRTL	HLA-B*5101	387.200
polymerase PB2	759	QBP33423.1	142-150	RRVDINPGH	HLA-B*2705	9000.000
hemagglutinin	560	QBP33427.1	316-324	YIGVKSLKL	DRB1-0703	72.41
polymerase PB2	759	QBP33423.1	732-740	LVMKRKRDS	DRB1-1301	89.77
polymerase PB2	759	QBP33423.1	730-738	VVLVMKRKR	DRB1-1328	59.09
polymerase PB2	759	QBP33423.1	210-218	LVRKTRFLP	DRB1-1327	55.68

Table 5. Toxicity prediction of the peptides by ToxinPred

PEPTIDE SEQUENCE	SVM SCORE	PREDICTION	HYDRO PHOBICITY	HYDRO PATHICITY	HYDRO PHILICITY	CHARGE	MOL WT
FQGRGVFEL	-1.37	NON-TOXIN	-0.05	0.14	-0.23	0.00	1052.33
AEIEDLIFL	-0.94	NON-TOXIN	0.16	1.19	-0.13	-3.00	1062.36
IIEGRDRTL	-0.94	NON-TOXIN	-0.32	-0.48	0.69	0.00	1072.36
RRVDINPGH	-0.91	NON-TOXIN	-0.44	-0.39	0.60	1.50	1063.31
YIGVKSLKL	-1.14	NON-TOXIN	0.01	0.67	-0.32	2.00	1020.42
LVMKRKRDS	-0.84	NON-TOXIN	-0.60	-1.24	1.19	3.00	1132.51
VVLVMKRKR	-0.71	NON-TOXIN	-0.37	0.17	0.49	4.00	1128.62
LVRKTRFLP	-0.65	NON-TOXIN	-0.30	-0.07	0.11	3.00	1129.54

Table 6. Template PDB ID for modeling of selected HLA alleles

S.No.	Allele	Template of model	Sequence Identity
1	HLA-B*2705	6AT5 A	92.8%
2	DRB1*1328	6ATF B	92.1%
3	HLA-B*0705	6AT5 A	99.4%
4	HLA-B*5101	6AT5 A	90.9%,
5	DRB1*0703	4H25 B	84.3%
6	DRB1*1301	6PX6 B	66.3%
7	DRB1*1327	6PX6 B	66.3%,

Molecular Docking: 3D structures of selected epitopes were predicted by PEPstrMOD while HPEPDOCK Server was employed to generate homology model of alleles. Template PDB ID (protein data bank) formed by server was used for alleles model (table 6). HPEPDOCK Server has been utilized to perform docking study of epitopes with alleles models (Figure 1-8). The best conformation of docked complex was chosen on the basis of minimum docking score (table 7) (Kamthania and Sharma, 2015; Pusch and Suarez, 2018; Kamthania et al., 2019).

Figure 1: Docked complex of Nucleocapsid protein epitope AEIEDLIFL& HLA-B*2705 allele

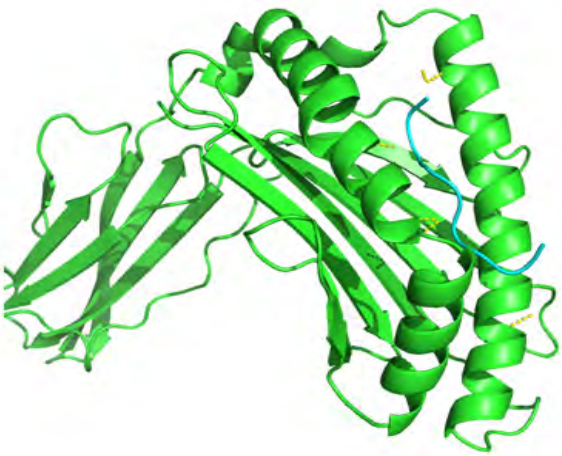


Figure 2: Docked complex of Nucleocapsid protein epitope FQGRGVFEL& HLA-B*0705allele

Figure 3. Docked complex of polymerase PAprotein epitope IIEGRDRTL & HLA-B*5101allele

Figure 4: Docked complex of polymerase PB2 protein epitope LVMKRKRDS & DRB1-1301 allele

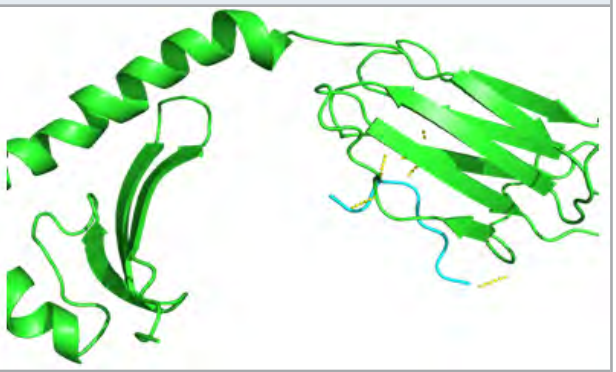


Figure 5: Docked complex of polymerase PB2 protein epitope LVRKTRFLP & DRB1-1327 allele

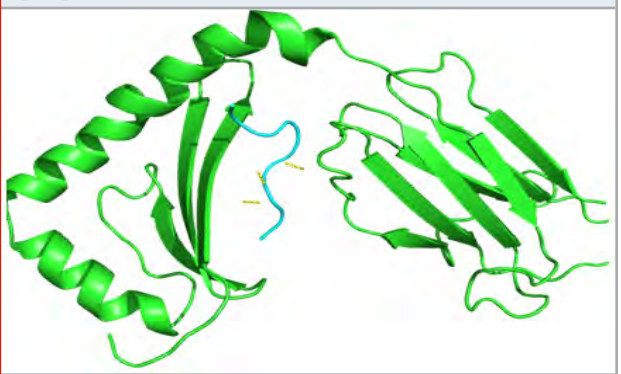


Figure 6: Docked complex of polymerase PB2 protein epitope RRVDINPGH & HLA-B*2705 allele

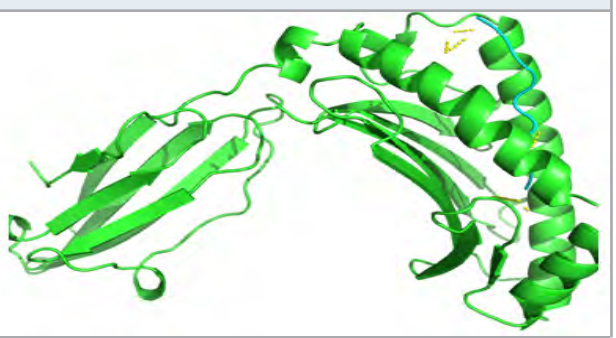


Figure 7: Docked complex of polymerase PB2 protein epitope VVLVMKRKR &DRB1-1328 allele

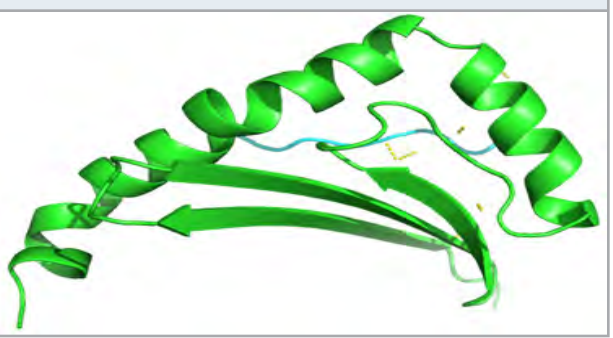
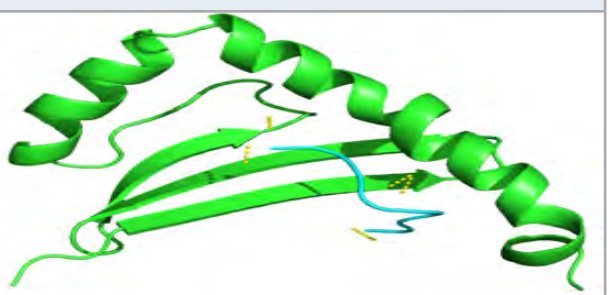


Figure 8: Docked complex of hemagglutininprotein epitope YIGVKSLKL&DRB1-0703 allele



Docking of selected nanomer T-cell epitopes FQGRGVFEL, AEIEDLIFL, IIEGRDRTL, RRVDINPGH, YIGVKSLKL, LVMKRKRDS, VVLVMKRKR, LVRKTRFLP with their corresponding allele HLA-B*0705, HLA-B*2705, HLA-B*5101, HLA-B*2705, DRB1-0703, DRB1-1301, DRB1-1328, DRB1-1327 respectively showed stable HLA–peptide complexes with docking score -229.708, -184.637, -206.640, -197.206, -176.581, -159.444, -177.159, -227.036 respectively (Table 7) (Kamthania and Sharma, 2015; Pusch and Suarez, 2018; Kamthania et al., 2019).

Table 7. Docking result of selected T-cell epitopes with allele structures.

S.No.	Protein name	Epitopes	HLA class alleles	Docking Score
1	nucleocapsid protein	FQGRGVFEL	HLA-B*0705	-229.708
2	nucleocapsid protein	AEIEDLIFL	HLA-B*2705	-184.637
3	polymerase PA	IIEGRDRTL	HLA-B*5101	-206.640
4	polymerase PB2	RRVDINPGH	HLA-B*2705	-197.206
5	hemagglutinin	YIGVKSLKL	DRB1-0703	-176.581
6	polymerase PB2	LVMKRKRDS	DRB1-1301	-159.444
7	polymerase PB2	VVLVMKRKR	DRB1-1328	-177.159
8	polymerase PB2	LVRKTRFLP	DRB1-1327	-227.036

Epitope antigenicity determination: VaxiJen is used with default parameters to predict the antigenicity of epitopes as vaccines candidate. All the antigenic epitopes with their respective predicted score (value greater than 01) were selected (table 8 & 9) (Kamthania and Sharma, 2015; Pusch and Suarez, 2018; Kamthania et al., 2019).

Table 8. Vaxijen for HLA class-I epitopes

S. No.	SEQUENCE	VAXIJEN RESULT
1	FQGRGVFEL	1.2783 (PROBABLE ANTIGEN)
2	AEIEDLIFL	1.0317 (PROBABLE ANTIGEN)
3	IIEGRDRTL	1.2600 (PROBABLE ANTIGEN)
4	RRVDINPGH	2.5888 (PROBABLE ANTIGEN)

Table 10. Immunogenicity of HLA class I epitopes

S.NO	SEQUENCE	EPITOPE LENGTH	IMMUNOGENIGITY SCORE
1	FQGRGVFEL	9	0.29224
2	AEIEDLIFL	9	0.33583
3	IIEGRDRTL	9	0.20424
4	RRVDINPGH	9	0.16967

Immunogenicity prediction of selected epitopes: Immunogenicity of nanomer HLA class I selected epitopes were analysed by IEDB. The selected epitopes with positive value showed high immunogenicity (Table 10) (Kamthania and Sharma, 2015; Pusch and Suarez, 2018; Kamthania et al., 2019).

The selected T-cell epitopes FQGRGVFEL, AEIEDLIFL, IIEGRDRTL, RRVDINPGH, YIGVKSLKL, LVMKRKRDS, VVLVMKRKR, LVRKTRFLP also show positive values of antigenicity & immunogenicity (in case of HLA class I) as shown in table 8-10. We have previously published similar work for HLA class I alleles for Nipah & HLA class II alleles for Hendra viruses (Kamthania and Sharma, 2015; Kamthania et al., 2019).

Table 9. Vaxijen for HLA class-II epitopes

S. No.	SEQUENCE	VEXIJEN RESULT
1	YIGVKSLKL	1.9299 (PROBABLE ANTIGEN)
2	LVMKRKRDS	1.9837 (PROBABLE ANTIGEN)
3	VVLVMKRKR	2.4990 (PROBABLE ANTIGEN)
4	LVRKTRFLP	1.6827 (PROBABLE ANTIGEN)

CONCLUSION

In this study, we have identified the potential nanomer T-Cell epitopes as vaccine candidate against H9N2 virus. The results confirming high binding affinity of selected epitopes with HLA alleles, stable complex formation tendency with HLA allele and tendency to induce high and specific immunogenic response makes the selected nanomer T-Cell epitopes to be a potential candidate for epitope-based vaccine development against H9N2 virus infection. Hence reported nanomer epitopes may undergo further in-vivo trials to develop vaccine against H9N2 virus infection.

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A Descriptive Analysis of the Psychosocial Status of Breast Cancer Survivors from Chennai, India

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ABSTRACT

Globally, the incidence of breast cancer is increasing day by day. In India, it is statistically maintaining an inclination status. The illness's discomfort initiates stress factors causing emotional instability, aggression, and periods of ups and downs. The impact of coping and social support facilitates quality of life among breast cancer survivors. This descriptive cross-sectional study aimed to explore and analyze the level of stress, coping, and quality of life among 150 breast cancer survivors in Chennai. Socio-demographic and clinical variables were used to assess the baseline data. Stress, coping, and quality of life (QOL) were assessed using a standardized tool like the Perceived stress scale, Hamby, Grych, and Banyard coping scale, and EORTC QLQ – C30 quality life scale, respectively. The mean age of the participants in this study was 51±9.51years. 77.33% of the participants had a high level of stress. The Coping levels among Breast cancer survivors were 58.67 % (Moderate), and 13.33% (Good). It was reported that 66% of them had a moderate quality of life, and whereas only 20% had a good quality of life. There is a significant, positive, moderate correlation between stress score and coping score (r= 0.56 P=0.001). There was a fair correlation between Stress and Coping with Quality of life. The results revealed that the psychosocial problems among Breast cancer patients were alarming. Necessary screening protocols and health education regarding the management stress, coping strategies are required for the breast cancer survivors, which will help to manage their psychosocial Problems and improve their quality of life.

KEY WORDS: BREAST CANCER SURVIVORS, COPING, PSYCHOSOCIAL, QUALITY OF LIFE, STRESS.

INTRODUCTION

Globally, breast cancer incidence is increasing in the developing world because of increased life expectancy, increase in urbanization, and adaptation of western lifestyle Breast cancer is the commonest cancer diagnosed among women pointing to 1 in 4 cancer cases. Breast cancer is the second frequent cancer and the leading cause of death among women. In 2018, there were 2.1 million new breast cancer cases and 626,679 deaths from breast cancer, in which a large percentage of patients from the low resource sector (UICC 2020). In India, it

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statistically maintains an inclination status. Moreover, 14% of women, i.e., 1 in 28 females, are affected with breast cancer, and urban women are more affected than rural women (Cytercare 2018, WHO 2021).

Cancer survival is more difficult in higher stages, and 50% of Indian women suffer from the 3rd and fourth stages of cancer. Moreover, new patients' age group also has gradually fallen from < 55years to below 40years of age. Women's survival rates are also markedly low in India due to lack of awareness, fewer screening programs, and diagnosed at advanced stages of cancer (Cytercare 2018). Breast cancer diagnosis along with its treatment can direct to physical, social, and psychological turmoil. These difficulties extent to the patients until post-treatment and recovery. The psychosocial impact of breast cancer for a woman is broader, as the breast is the essence of femininity, motherhood, and sexuality (Cytercare 2018; Iddrisu et al. 2020).

In turn, it adds value to their physical appearance, and disfigurement after the surgery creates a loss of self-esteem. The state of disease, the uncertainty of the recurrence in the future, treatment protocols, drug side-effects all these stress factors may lead them to loss of hope, periods of anxiety, impairment of concentration, sleep disorders, mental and cognitive reservation, sexual dysfunction, infertility and fear of death and even depression (Carreira et al. 2018). Breast cancer is a challenging disease that includes a crisis in psychological balance and is perceived as a disaster in the patient and her family's view. It develops vast impacts both physically and emotionally. Emotional sensibility and excessive irritability are observed in women diagnosed with breast cancer. The stress factors initiated by the disease process causing emotional instability, aggression, feelings of fear, guilt, and desperation affect their coping ability and quality of life (Hajian et al. 2017; Iddrisu et al. 2020).

The implication of coping mechanism, social support aids in upholding the quality of life of breast cancer survivors. Accompanying psychiatric disorders have a significant impact on a patient's quality of life, self-care, adaptability to treatment, and over time, the severity and prognosis of cancer and response to treatment. Hence the diagnosis and treatment of psychiatric disorders help in raising the adherence to therapy and quality of life (Izci et al. 2016). The majority of the study analyses found that 50% of the increased risk for breast cancer is due to stressful life in women. It is found that half of all women with stressful life events are one of the topmost causes of breast cancer in India. The women who experience stress had twice the chance of developing breast cancer than a woman who stays calm and relaxed. That chances of risk double with increased risk factors of breast cancer such as family history, obesity, consuming alcohol, smoking, age of menarche, age of menopause, history of reproductive life, etc (Helgesson 2013; Izci et al. 2016; Iddrisu et al. 2020).

Studies on cellular growth and molecular function stated that due to chronic stress factors, specific pathways originate in cancer growth cells and paves the way for metastasis (Moreno-Smith et al. 2010). Chronic stress creates a perfect storm where pre-cancerous cells can flourish (Parikh et al. 2014). In humans, psychological stress influences the main processes in cancer pathogenesis, such as DNA repair, cellular aging, and alterations in the immune system. The long-lasting biological, psychological, and behavioral changes and have severe effects on health (Krukum et al. 2019). The prolonged impact of psychological stress causes inflammation and several health problems like anxiety, heart problems, gastrointestinal disturbances, cancer, etc., and incidence of cancer rank topmost among these health problems (Dai et al. 2020). Stress and coping mechanisms among breast cancer patients and family caregivers after breast cancer diagnosis were very high. Coping strategies could either be problem-focused or emotion-focused aids in resolving Stress and increase adaptative coping (Mukwato et al. 2010; Benson 2020).

Coping strategies are specific efforts, both behavioral and psychological, that people use to combat stressful events. It helps to deal with the multi sectoral favorable experiences of psychological, social, and spiritual nature. Coping is behavioral efforts adopted to deal with specific external and internal stressors (Mukwato et al. 2010; Khailli et al. 2013). Coping aids in adaptation and adjustment to breast cancer and enhances the quality of life and survival. The effectiveness of coping strategies depends on the degree of illness, individual distress, variations in individual coping, the level of social support available, and extended service of consultation skills and support of health personnel (Benson 2020). Quality of life is a state of well-being and the ability to perform daily activities with satisfaction and functioning with control of the disease. Breast cancer patients experience physical symptoms and psychological dysfunctions that adversely affect their quality of life (Sheila et al. 2019).

Clinicians have accepted that survival without complications is the eminent factor for breast cancer patients to enhance their quality of life. Family members play a vital role in breast cancer lives by managing their roles in the family, financial crisis, emotional support, and remaining throughout illness. Hence family support and health-related quality of life lead to improvements in the status of the individual cancer patients and enhance the wellness of the family caregivers. Therefore, breast cancer is an alarming health problem increasing gradually, the implication of screening protocols and health education among younger women population is essential. Along with a general treatment schedule, standard programs have to be developed to intervene in coping ability to structure their quality of life better, thereby effectively reducing the stress factors (Alexander et al. 2019; Sheila et al. 2019).

MATERIAL AND METHODS

This was a descriptive cross-sectional study conducted among breast cancer survivors. A total of 150 samples

were selected by convenient sampling technique. The participants were selected from those attending the Medical Oncology Department, Tamil Nadu Government Multi Super Specialty Hospital, Chennai – 02. The inclusion criteria were a) diagnosed as Breast cancer patients seeking treatment, b) able to understand and speak Tamil c) patients attending the oncology Outpatient and Inpatient department. The study participants were informed about the study in their known language; both oral and written consents were obtained. The data collection was done with a structured questionnaire consisting of socio-demographic and clinical variables; the perceived stress scale was used to assess the level of stress, coping ability by Hamby, Grych, and Banyard Coping quality of life was assessed using EORTC QLQ -C30 scale. About 20 minutes were spent for each participant to complete the tool. Ethical approval was granted by the Institutional Ethics Committee of Tamil Nadu Govt. Multi-Specialty Hospital vide ref. no. 1577/P&D-I/TNGMSSH/2017/PMS/ 003/07/2020 (Sheila et al. 2019).

The study was also registered with the clinical trial registry of India no. CTRI/2020/08/027291. SPSS software was used for analyzing descriptive and inferential statistics. Descriptive statistics were used in demographic and clinical variables in which categories were given in frequencies with their percentage. Association between demographic variables, coping, and quality of life was interpreted by non - parametric Mann Whitney U test and Kruskal-Wallis H- test.

RESULTS AND DISCUSSION

The study investigated three important psychosocial aspects: stress level, coping ability, and Quality of life of survivors with breast cancer. The socio-demographic variables resulted that the mean age of the participants was 51.57 ±9.51 years (Figure 1), majority of participants were overweight (56.67%), the incidence of breast cancer was high among participants who had primary level education (59.33%), percentage of incidence is high among married women (88%), and participants from the semi-urban region also had higher rate incidence (62.67%). The clinical variables reported that participants who have breast cancer for the past five years were 93.33%, 61.33% were living with III stages of cancer, and 76% slept for 4 to 8 hours.

The study results reveal that none of the participants scored a low level of Stress, 22.67% of the participants scored moderate level, and 77.33% were demonstrated a high level of stress score (Table 1). A similar result reported a significant prevalence of Stress among breast cancer survivors and stated that stress factors contribute a significant proportion in increasing the disease process's severity and reducing the coping skills and Quality of life of breast cancer survivors (Chiriac et al. 2020).

In our study, the association between the socio-demographic profile and Stress reported that Hindu religion participants (P=0.001***), Semi-urban / rural residents (P=0.02*), those who belongs to the nuclear family (P=0.01**), whose cohabitation status were with two and more person (P=0.02*) and those who sleeps less than four hours (P=0.02*) had significant high level of Stress than others. It was also consistent with the study that Stress was commonly prevalent among breast cancer patients among rural residents and those having trouble sleeping and understanding about management aids in the quicker ailment of the patient (Alagizy et al. 2021).

Figure 1: Histogram with Normal Curve Shows the Age Distribution of Breast Cancer Patients

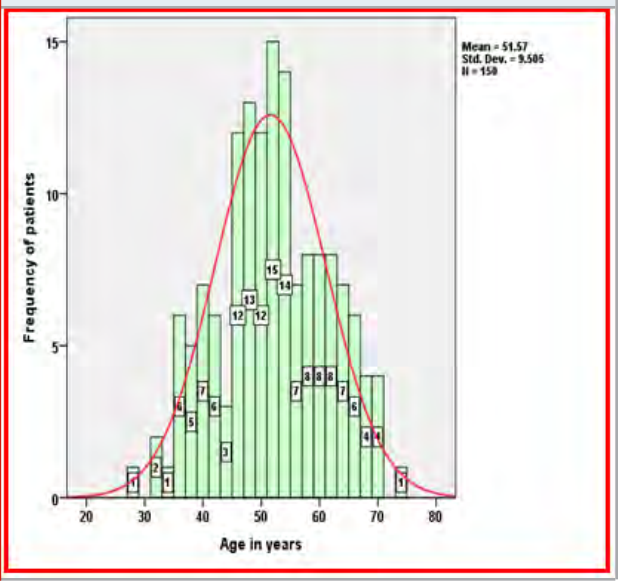


Table 1. Level of Perceived Stress Scale Score

LEVEL OF SCORE	NO. OF PATIENTS	%
Low	0	0.00%
Moderate	34	22.67%
High	116	77.33%
Total	150	100.00%

The coping ability among Breast cancer survivors was found to be 20% (Good), 58.67% (moderate), and 28 % (Poor) (Figure 2). Participants with a family income of Rs 10,000 (p = 0.05), staying with five or more other persons (p = 0.02), those who were having a recurrence of the breast cancer (p=0.01**), who were partially dependent on others (p=0.01*) and those were sleeping for less than four hours (p=0.01**) gained significant coping score. Similar study also stated that women who perceived proper family functioning were seven more times likely to use active coping strategies (Lopez et al.2018). It is consistent with another study, which reported that family composition and sleeping troubles contribute to the survivors' coping status (Benson et al. 2019).

About Quality of life, it was found that 66% of the patients had moderate QOL, 20% of the patients were having good QOL, and 14% of the patients were having poor QOL (Table 2). The association between socio-demographic variables with Quality of life of participants demonstrated a significant association with participants who attained education up to graduation level (p = 0.05*), the participant on II stage of breast cancer (p = 0.001***) and sleeping 4 to 8 hours/day (p=0.001***) were having more QOL score. The factors affecting the Quality of life in breast cancer patients stress that age, educational status, cancer stage, and status of the disease significantly affect the Quality of life among breast cancer survivors (Sharma et al. 2017). Another study suggested that stages of cancer and educational level were significant predictors of breast cancer survivors' Quality of life. The tertiary level of education of women had a significant association with Quality of life, and at the early stages of cancer, it is possible to reduce the complications and enhance the Quality of life (Chen et al. 2018).

Figure 2: Level of Coping Among Breast Cancer Survivors

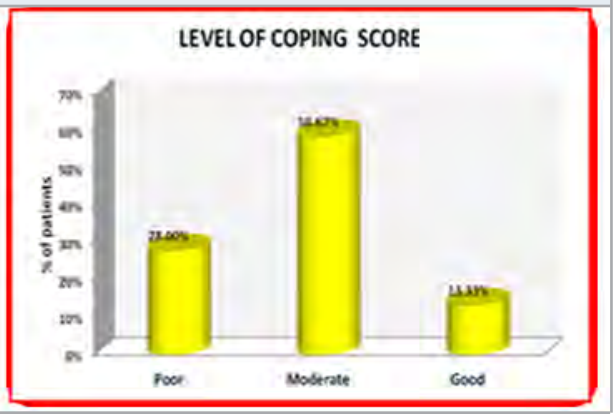


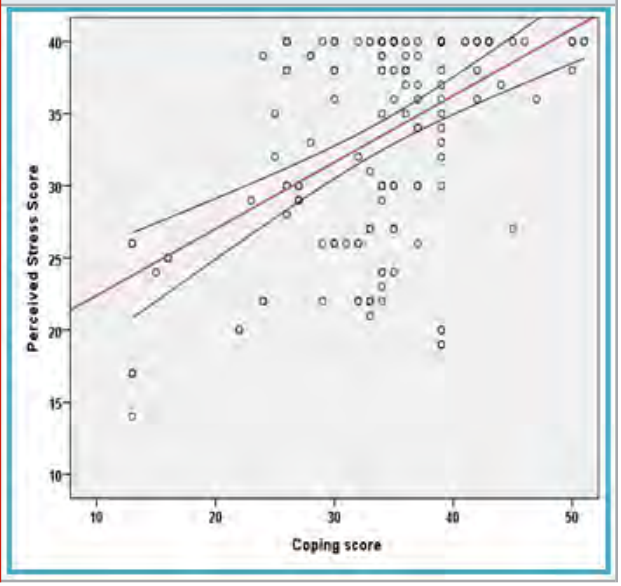
Table 2. Level Of Quality-Of-Life Score

Level of QOL score	NO. OF PATIENTS	%
Poor	21	14.00%
Moderate	99	66.00%
Good	30	20.00%
Total	150	100.00%

It was found that there was a positive fair correlation between Stress and coping ability (P≤0.001) (Figure 3). Similar study supported this study, which assesses Stress and coping strategies among women diagnosed with breast cancer. It reported that in situations of Stress aggravated by breast cancer at earlier stages, the patient could adapt and cope with it (Alves et al. 2019).

This study conveys the message that psychosocial problems are prevalent among breast cancer survivors, which reflects on the quality of life and recovery. Nurses play a significant role in tackling breast cancer survivors' psychosocial problems and prolonging the life of the breast cancer survivors in a better way.

Figure 3: Scatter Diagram between Stress Score and Coping Score among Breast Cancer Patients.



CONCLUSION

Breast cancer is the commonest cancer among women worldwide, with an increasing incidence day by day; poverty and illiteracy are the prompt symptoms responsible for delay in seeking medical services. The breast cancer survivors' psychosocial needs are highly prevalent among them, which need utmost attention at an early stage. Primary prevention aids in the detection of breast cancer survivors at an earlier stage. It also minimizes the severity of illness, enhances the prognosis of health, and reduces the stress factors. In turn, all this develops faith and coping skill in an individual and aids in a better quality of life for cancer breast survivors.

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A Comprehensive Review on the Uptake by and Accumulation of Some Heavy Metals in Fresh Water Fishes

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ABSTRACT

An alarming threat for environment, pollution is undoubtedly posing different adverse impacts on the living organisms. Aquatic environment is not the expectation. Among all the sources of aquatic pollution, heavy metal induced hazards better have been found to be most important for both freshwater and marine ecosystems. Heavy metals can prove to be deleterious for aquatic organism when exposed for short term (acute) as well as long term (chronic) period. Fishes are best known model for determining the degree of aquatic pollution. Thus, it is very essential to find a consolidated research article describing the accumulation pathway of heavy metals in freshwater fishes. In this review, attempts have been made to compile all the available scientific data related to the uptake and accumulation of different heavy metals (As, Hg, Cd, Cu, Cr and Pb) and the general histopathological changes due to chronic exposure to sublethal concentrations. Data obtained from the previous researches are meticulously chosen in order to avoid ambiguous presentation here. The focal objective of the scientific review is to offer an imminent guideline for the students, scientific community, and public officials involved in environmental health risk assessment and management ensuring a better future environmental condition. During the review process, we have found out that entry routes of different heavy metal is mainly GI tract, gill and/or skin. Most of the heavy metal may be present in more than one form and have specific way of accumulation in tissues. This review also provides the accumulated data of heavy metal contamination in Indian rivers, factors related to metal uptake in fishes and scientific information about the source and bioaccumulation of selected heavy metals.

KEY WORDS: HEAVY METALS, FRESHWATER FISH, UPTAKE, BIOACCUMULATION, HISTOPATHOLOGY.

INTRODUCTION

Nowadays, pollution, especially in aquatic ecosystem, due to the contamination of heavy metal becomes a significant issue of concern to the researchers of environmental sciences. It is evident that wide spreading of industries, rapid urbanization and population explosion impose deleterious impact on the hydro-biological quality of both lentic and lotic ecosystems viz., ponds, lakes, and rivers. The consequences become more critical because the small and large-scale industries frequently discharge their wastes containing different heavy metallic contaminants directly into the environment which often go beyond the permissible limit of the environment (Velma et al., 2009; Praveena et al., 2013).

In spite of the development in waste management technologies, the difficulties due to heavy metal release

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are continuously putting immense adverse effect on the biolife of aquatic ecosystems (Bakshi and Panigrahi, 2018). Especially class- B or lithophilic metals are considered to be more deleterious to the ecosystem and fundamental group of aquatic pollutants because of the long persisting nature (or longer half-life), mechanism of bioaccumulation, process of biomagnification and non-biodegradability. Another important reason behind the consideration is their potency to destroy the framework of species diversity in any ecosystem (Vutukuru et al., 2007; Lodhi et al., 2007; Saha and Zamman, 2011; Ahmed et al., 2014).

Thus, entering into the food chain heavy metals often show high toxicity even in minimum concentration providing cumulative injurious effects in an aquatic system (Velma et al., 2009; Velma and Tchounwou, 2009). Heavy metals are thus considered to put ecological, evolutionary, nutritional and environmental impact on the ecosystem (Jaishankar et al., 2014). In the recent days, fresh water ecosystems are mostly polluted by waste waters released from different industries and municipalities. The most frequently available heavy metals in the waste water are Lead, Arsenic, Cadmium, Chromium and Mercury (Bakshi, 2016; Mehana et al., 2020). The main objective of the review is to provide insight of the uptake and accumulation of some heavy metals like lead, cadmium, arsenic, chromium and mercury in fresh water fishes. Accumulation rate of heavy metals is very specific in different fishes (Khan et al, 2020). In this review we have only tried to consolidate the data related to heavy metal accumulation in fresh water fishes though further studies can be done on the heavy metal accumulation in estuarine or marine fishes (Khan et al, 2020).

Table 1. Status of heavy metals concentration in Indian Rivers (CWC, 2014; CWC, 2018; BIS 10500,2012; EPA, 1972)

Metal	Chemical symbol	Sources	Maximum permissible concentration (µg/ l)		Number of the rivers in India with metal concentration beyond permissible limit			
					2014		2018	
			EPA	BIS	Rivers	Maximum Observed Conc. (µg/ l)	Rivers	Maximum Observed Conc. (µg/ l)
Arsenic	As	Pesticide industries, mining, Chemical industries	50	10	0	9.47	0	9.53
Cadmium	Cd	Cd-NI batteries, Nuclear reactors, Television phosphor	10	3	4	4.0	25	70.51
Chromium	Cr	Dyeing, Mines, Electroplating	50	50	11	366.91	21	450.26
Copper	Cu	Electroplating, Pesticide industries	1000	50	68	180.70	10	314.93
Lead	Pb	Paint, Pesticide, Batteries, Crystal glass industries	5	10	30	48.92	69	374.58
Mercury	Hg	Mining, Pesticide industries.	2 (1.44)	1	0	<1	-	-

MATERIAL AND METHODS

An attempt is made to produce an utmost consolidated manuscript on this topic. In order to make the manuscript more comprehensive and relevant for the future study, extensive review has been done compiling and consolidating the maximum number of available scientific data. All data has been collected, from science journals of repute, published reports (particularly from international agencies) and doctoral or postdoctoral theses. Priority has been paid to the reproducible articles which are indexed in science journal database like Copernicus, Scopus, PubMed etc. The scientific articles highlighting ambiguous working methodologies are avoided carefully. Key words have been meticulously selected and searched based on systematic scientific approaches. Our own experimental findings (both laboratory and field) have been encompassed at various parts of the manuscript to improve the essence of the article.

Heavy metal concentration in fresh water: Most of the heavy metals are available in natural water in the form of soluble and/or in particulate form. Water soluble forms of heavy metals are found in labile or non-labile fractions (Jezierska and Witeska, 2001). *Labile metallic* forms are most detrimental to the aquatic organisms, especially fishes. Aquatic ecosystem contains not only the heavy metals but also the essential metals (both major and trace metals). In aquatic environment, trace metals are present in very low amount affecting fresh water fishes detectable in minimum. Amount of metal concentration in fresh water ecosystem is continuously increasing directly through atmospheric deposition and waste water contamination or indirectly through rising solubilisation followed by mobilization from sediments. EPA and BIS recommended permissible limit of various metals in water are often crossed in some rivers in India (EPA,1972; BIS 10500,2012). Central Water Commission conducts surveys to estimate the concentration of the

heavy metals in the Indian river waters (Table 1) (CWC, 2014; CWC, 2018).

Central Water Commission report, 2018 shows that forty-two rivers of India have been found to be polluted due to receive of neurotoxic heavy metals. The report also describes that river Ganga is highly contaminated by chromium, copper, lead, iron and nickel due to receive of run-off mostly from milling, plating, mining and surface finishing industries. Report shows that Cadmium contamination in Indian rivers has increased during the last four years. In the rivers of Godavari basin, most of the rivers contain cadmium, chromium, arsenic, nickel and zinc within the acceptable or permissible limit of Bureau of Indian Standards (BIS 10500, 2012) (CWC, 2018).

Environmental factors affecting metal uptake and accumulation in fish: Fishes in the heavy metal contaminated aquatic system must accumulate the metals in their tissues. The rate of accumulation solely depends on the concentration of the metal, method of uptake and time of exposure. Some extrinsic factors and some intrinsic factors are also important parameters which determine the rate of accumulation (Jezierska and Witeska, 2001). The environmental factors like water temperature, hydrogen ion concentration, hardness etc. influence the uptake, accumulation and depuration of metals in fish. Water temperature is a key environmental factor that influence metal uptake, accumulation and depuration (Jezierska and Witeska, 2001).

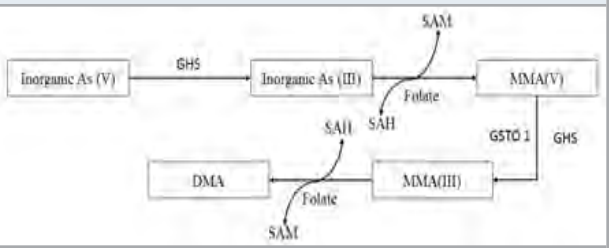
Metal accumulation in fishes is also related with some biological or intrinsic factors like age, size, feeding habits. Except mercury, other heavy metals have shown an inverse relation with age and size (Jezierska and Witeska, 2006). Accumulated metals show different tissue affinity but most of them accumulate especially in gill, liver and kidney. Very small number of metals is found to be accumulated in muscles in most of the fishes. Except mercury, other heavy metals have shown an inverse relation with age and size (Jezierska and Witeska, 2001; Jezierska and Witeska, 2006).

Uptake and accumulation dynamics of different metals in fish: In recent days, pollution (especially water pollution) due to heavy metal contamination has undoubtedly grow into a great issue of concern to the environmental scientists. Extensive industrialization, exploitation and rapid increase of urban communities have measurably forced adversative impact on the hydrobiological quality of lakes, ponds and rivers all over the world (Praveena et al., 2013). Heavy metals especially cadmium (Cd), copper (Cu), Chromium (Cr) and Lead (Pb) are found to be highly available in Indian rivers. Though, contamination of detectable arsenic (As) and mercury (Hg) is not found to be reported in any river of India. In this review, an attempt has been made to focus on the various environmental forms of the heavy metals (As, Hg, Cd, Cu, Cr and Pb), method of uptake, accumulation and dynamics in the fish body (Praveena et al., 2013).

Arsenic: Arsenic is one of the harmful heavy metals (metalloid) in the aquatic bioloife. It has a metalloid property and is predominantly available in the form of oxides (arsenate and arsenite) or sulfides or as a salt of sodium, iron, copper, calcium, etc. (Singh et al., 2007). Excessive use of arsenical pesticides, industrial activities, mining operations and chemical laboratory exhaustion has led to the global occurrence of water-soluble arsenic concentration above the permissible limit (Table 1). Water soluble inorganic arsenic (iAS) are converted to methylated arsenical forms i.e., monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) through enzymatic activities in organism body. These arsenical forms are main end metabolites and biomarker of the long-term arsenic exposure (Jaishankar et al., 2014; Kumari et al., 2016). Arsenic exposure may be waterborne and diet-borne. So, main routes of entry are gill and GI tract. Waterborne arsenic after taking entry through gill significantly accumulated in gill, liver and intestine and manipulates growth of the fish (Tsai and Liao, 2006; Han et al., 2019).

Inorganic arsenic (iAs) may be present in two forms i.e., iAs(III) and iAs(V). According to Kumari et al., (2016), after entry of iAs (V), it converts into iAs(III). Then iAs (III) changes into MMA(V) coupled with SAM –SAH conversion (SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine). After that MMA(V) transforms into most toxic and accumulating form MMA (III) through reduction reaction by the action of MMA(V) reductase or GSTO 1 (glutathione S-transferase omega 1). MMA (III) can also be converted into DMA coupled with a SAM –SAH conversion (SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine). Most of the bio-transformation reactions are taken place in liver (Fig 1) (Han et al., 2019).

Figure 1: Biotransformation of arsenic compound (Source: Modified from Kumari et al., 2016)



When arsenical compounds enter through the dietary route, it basically accumulates into digestive tract. From GI tract it goes to Liver where most of the biotransformation takes place. Then it deposited into the other organs or tissues of the body viz., brain gonads, muscles either directly or via gill circulation and get accumulated (Tsai et al., 2012). Elimination of little amount of metal is also observed through feces during depuration experiments (Kumari et al., 2016). Water dissolved arsenical compounds can also enter through gills and can be deposited directly into the brain, kidney, gonads and other tissue through speciation. Dietary uptake shows accumulation in the digestive tract till

the end of exposure but the concentration gradually decreases during depuration. Though several researches are there but, in most cases, liver is said to be the highest accumulator organ of the metal (Kumari et al., 2016; Han et al., 2019).

Cadmium: Being completely non-essential to all the organisms, cadmium, is considered as a highly toxic heavy metal. With the increase of industrialization, deposition of cadmium in fresh water bodies (lakes, rivers etc.) becomes a major issue of concern to the environmentalists. Cadmium related contamination in the aquatic organism has been reported to be increased in last decade with a high degree of its accumulating property (Okocha and Adedeji, 2011). According to ATSDR (1999) report, the main sources of cadmium in the environment is anthropogenic (90%) and very low amount of cadmium is contributed by natural activities (viz., volcanic eruption, decaying of vegetables, forest fire etc.).

Anthropogenic activities like agricultural uses, electroplating, mining, industrialization etc. are main contributors of the cadmium into the environment (Table 1). Aquatic organism like fish can readily uptake cadmium in its ionic form (Cd II) through the gills (AMAP, 1998; AMAP, 2002). The ions are usually absorbed through carrier mediated transport or passive diffusion over the chloride cells of the gills. It has been reported by many researchers that cadmium enters into the cell through calcium ion channels and interacts with the cytoplasmic components like metabolic enzymes and metallothioneine (Rodriguez et al., 2015). It is believed that high affinity of Cd^{2+} ion for Ca^{2+} binding site in the gill facilitates its entry through the apical side of the chloride cells (Okocha and Adedeji, 2011).

Cadmium can also bind with the active site of Ca^{2+}-ATPases present on the basolateral side of the chloride cell facilitating the translocation of ionic cadmium into the blood circulation (Okocha and Adedeji, 2011). Another route of cadmium entry is through dietary ingestion when cadmium is associated with organic material. Then the ions are absorbed by endocytosis through intestine Cadmium is highly accumulated in liver and kidney causing various deletorious pathological changes (Sumet and Blust, 2001). Cadmium is found to be present in maximum concentration in the kidney of the fishes posing various degree of renal damage (Kumar et al., 2009; Vesey, 2010). A very little amount of cadmium is found to be liberated out through feces (from intestine) and bile (from liver) secretion at the time of depuration (Okocha and Adedeji, 2011).

After renal damage cadmium can also be liberated from kidney of the fishes (Kumar et al., 2009; Vesey, 2010). Gills are said to be the storehouse of the cadmium showing high degree of morphological and biochemical changes after chronic exposure. The prime target of cadmium ion is chloride cells of the gill where it competes with calcium ion for the entry into the cell resulting hypocalcemia in fish (Wong and Wong, 2000). Several workers have reported that cadmium is highly toxic in both acute and chronic exposure causing nephrotoxicity, hepatotoxicity, lamellar degeneration and hypocalcemia in fish which also put some deleterious impact on the human life though food chain (Okocha and Adedeji, 2011; Khan et al., 2020).

Chromium: Chromium is present in three oxidation states viz., Cr^{2+}, Cr^{3+}, Cr^{6+}, among which divalent Chromium is most unsteady. Only, the Cr^{3+} and the Cr^{6+} are the stable chemical state of Chromium available in the environment. Being one of the most common ubiquitous pollutants in the aquatic medium, Chromium and its particulates get contaminated into the aquatic medium through effluents discharged from various industries like electroplating workshops, printing-photographic, tanneries, textiles, ore mining, dyeing, and medical industries (Bakshi and Panigrahi, 2018). Among all the oxidation state, hexavalent chromium can be considered as the most toxic form because it can readily pass the cellular bio-membranes and then reduced to trivalent form.

Then, trivalent chromium reacts with different cellular molecules, and ultimately exposes the mutagenic and toxic properties of chromium industries (Bakshi and Panigrahi, 2018). Chromium enters into fish body either through gastro-intestinal tract and/or respiratory tract (Bakshi, 2016). The amount of the metal inside the fish varies with the form of available chromium time of exposure and its concentration (Mallesh et al., 2015). Bakshi and Panigrahi (2018) reported that chromium (VI) gets associated with the plasma protein and encompasses in transportation after penetrating the plasma membrane through sulphate ion channel. After that, the metal biologically gets accumulated in various internal organs of fish. The general pattern of distribution of Cr^{6+} in fishes is as follows: Gills> Liver> Skin> Muscles (Jaishankar et., al., 2014; Bakshi, 2016).

After getting entry through passages for isoelectric and isostructural anions (such as SO_4^{2-} and HPO_4^{2-}) of cell membrane, the hexavalent chromium undergoes metabolic reduction within the cell. During these metabolic reactions, different reactive intermediates are released which are reported to be detrimental to ensuring the stability of DNA helix, causing fatal effects in the affected individual (Wang et al., 1997; Jaishankar et., al., 2014). The same authors have also reported that migration of various intermediate chromium metabolites to nuclei and interaction with DNA are evident during this process causing the final negative effect (Vutukuru, 2005; Velma et al., 2009).

The primary storage and detoxification site for chromium is said to be liver in experimental condition. Higher concentration of metals is evident in bile of the experimental organism (*Clarias batrachus*) being exposed to metal contaminated food and environment (Bakshi, 2016; Bakshi and Panigrahi, 2018). It is reported that this storage is stabilized mainly by protein linkage or small peptide linkage such as glutathione linkage. In case of fishes the main elimination route of chromium or its

compounds is through feces (Bakshi, 2016; Bakshi and Panigrahi, 2018).

Lead: Lead is considered to be a toxic metal which do not have any importance in the physiological processes of any living organisms. The metal is highly toxic in aquatic environment as it easily accumulates in fish tissues like gill, liver, kidney, bones and scales. It also can cross the blood-brain barrier causing neurotoxicity in fish (Rabitto et al., 2005; Ju-Wook et. al., 2019). Lead has now becoming a ubiquitous metal with various source in the environment. The sources are mainly of industrial, agricultural and domestic origin.

Gasoline and house paints also contribute lead in the environment, furthermore, lead bullets, plumbing pipes, storage batteries, pewter pitchers, faucets and toys are also helping in lead contamination (Sharma and Agarwal, 2005; Jaishankar et al., 2014). Automobile exhaust and smoking also contaminate lead into the air. Lead can enter through the gill, altering the morphological character of gill when gets attached to the mucus (Mobarak and Sharaf, 2011). Then it enters into the blood stream and accumulates in liver. Liver is the main organ for detoxification in fish. The metal can enter also through gastrointestinal pathway if lead contaminated diet is consumed or through skin (Łuszczek-Trojnar et al., 2013; Ju-Wook et. al., 2019).

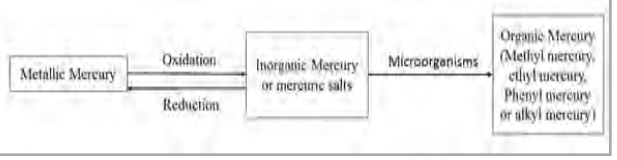
The divalent lead can compete with the divalent calcium ion for entry through the gills (Ju-Wook et al., 2019). After getting entry through the metal traverses the basal membrane and enters into the blood flow from where it readily accumulates in liver. Dietary entry of lead also leads to the accumulation in liver though very little amount of the metal is defecated out. Then the metal accumulated into the kidney, it makes a huge damage to the organ. As the metal can cross blood brain barrier, it shows high degree of neurological damage. Several researchers reported about the acculamation of this metal into bones and scales also (Rabitto et al., 2005; Ju-Wook et. al., 2019). Several researchers have confirmed that lead can be bioacumulated in different tissues of the fish and can also be biomagnified with the food chain (Shaukat et al., 2018; Ju-Wook et al., 2019; Khan et al., 2020). Apart from the defecation very small amount of lead have been found to be eliminated out through bones and scales during depuration (Łuszczek-Trojnar et al., 2013; Ju-Wook et al., 2019).

Mercury: Mercury, a highly toxic and non-essential metal, also termed as quicksilver, is prevalent in the environment as a result of natural and anthropogenic activities. Exposure of mercury is considered to be the second highest cause of toxic metal poisoning. Best known accident related to mercury pollution is Minamata disaster of Japan (Vasanthi et al., 2019).

Mercury is present in three states with different metabolic fate: mercury vapour or metallic mercury or elemental mercury (Hg0), mercury salts or inorganic mercury (including mercurous chloride or Hg_2cl_2, mercuric chloride or $HgCl_2$ and mercuric sulfide or HgS) and organic mercury (methyl mercury, ethyl mercury, phenyl mercury and alkyl mercury). There are some natural sources of mercury pollution like elemental mercury vapour from volcanoes and forest-fire, inorganic mercury by rock weathering etc. (Martinez-Finley and Aschner, 2014; Raihan et al., 2020). But after industrial revolution, source of mercury in the environment is mainly anthropogenic (Rice et. al., 2014). viz., gold mining, fossil fuel combustion, paper and pulp industries, electronic wastes, medical wastes, electroplating, metal industries, pharmaceutical industries etc.

In fishes, mercury can be taken up through gills, skin or digestive tract (Sweet and Zelikoff, 2001; Morcillo et al., 2017). Metallic mercury or mercury vapour (Hg0) can be oxidized into water soluble inorganic mercury (Hg^{2+}) which is basically taken up by the fishes or can be reduced back to metallic mercury (Hg0) (Tokar et al., 2015). Metallic mercury often can be converted into organic mercury (Methyl mercury or phenyl mercury) by microorganisms (Rodriguez et al., 2015). Toxicity of mercury depends upon the state of mercury, environmental media, conditions, age and life history of the specimen and sensitivity of the organism. Organic form of mercury is most toxic to the aquatic organisms as it can be biomagnified through food chain (Fig 2) (Vasanthi et al., 2019).

Figure 2: Forms of mercury in environment



Mercury uptake can be energy-dependent or passive depending on state of mercury (Aschner et al., 2010). Water soluble inorganic mercury (Hg2+) or mercury in mercuric or mercurus salt can be absorbed (15%) through digestive tract whereas, methyl mercury absorption is 90-95% in food. Most of the methyl mercury is found in the muscles (80-100%). Mercury can be absorbed through gills, gastrointestinal tract and very little amount through skin. The inorganic Hg can cross the epithelia and bound with plasma proteins and transported to different organs via systemic circulation (Aschner et al., 2010; Rodriguez et al., 2015).

In Rainbow trout (*Oreochromis mykiss*) major part of whole blood methyl mercury (90%) efficiently binds with beta chain of haemoglobin of RBC (Jasim et al., 2016). Due to the lipophilic property of methylmercury, it can easily pass the gut cell membrane and enters into the cell. Then methyl mercury can bind reversibly to sulphur containing amino acid (Cysteine). Therefore, cellular molecules like glutathione (GSH) can easily bind with methyl mercury (Morcillo et al., 2017). The cysteine bound form facilitates its transport to sensitive tissues like brain by an L-neutral amino acid transport system.

Table 2. Histopathological alteration of gill, liver and kidney due to chronic exposure to sub-lethal concentrations of selected heavy metals

Selected Heavy Metals	Major histopathological alterations of different organs due to chronic metal exposure to sub-lethal concentrations			References
	Gill	Liver	Kidney	
Arsenic	Epithelial hyperplasia, lifting and oedema, lamellar fusion, desquamation aneumerism, and necrosis	Focal lymphatic and macrophage infiltration, congestion, sinusoid dilation a & swelling, vacuolization and shrinkage of hepatocytes, necrosis	Pycnotic nuclei, vacuolization of tubular cells, glomerular shrinkage, lumen enlargement, necrosis.	Ahmed et al. (2013b) ; Morcillo et al. (2017)
Cadmium	Hyperplasia, increase in chloride cells, reduced and shortened length of secondary gill lamellae	Dissociation of hepatocytes, Necrosis, blood congestion in liver sinusoids, vacuolization	Disorganization and degeneration of renal epithelial cells, reduction in glomerulus, hypertrophy, dialation of bowman's capsule, focal necrosis	Ahmed et al. (2014); Kaur et al. (2018)
Chromium	Lamellar disorganization, Necrosis in epithelial cells, atrophied central axis.	Hyperplasia, Necrosis of hepatocytes, Reduced N-C ratio	Highly fenestrated Bowman's capsule, Constricted lumen of renal tubes, glomerular disorganization,	Velma et al. (2009); Velma and Tchounwou, (2009); Bakshi, (2016); Bakshi and Panigrahi, (2018)
Lead	Hyperplasia, hypertrophy and destruction or disintegration of lamellar architecture, lamellar clubbing and fusion of lamellae.	Disarrangements of hepatic cords, shrinkage of hepatocytes, dialation of sinusoids, exudation of blood, loss of cell adherence of hepatocytes.	Degeneration of renal epithelium, vacuolization, nuclear pycnosis. Renal tube atrophy, oedema, necrosis	Mobarak and Sharaf, (2011); Ahmed et al. (2014); Mustafa (2020)
Mercury	Mild congestion and oedema in primary lamellae, hyperplasia, desquamation in epithelial lining secondary lamellae, hyperactivity of chloride and mucous cells, Increase in RBC, macrophages	Vacuolization, Hypertrophy of hepatocytes, intravascular hemolysis, nuclear pycnosis, congestion in central vein, necrosis	Hydropic swelling of tubules, pycnotic nuclei, swelling of proximal convoluted tubule with necrotic nuclei.	Kaviraj, (1983); Kaoud and El-Dahshan, (2010); Selvanathan et al. (2013)
Copper	Lifting of Lamellar epithelium, RBC exudes, Necrosis, fusion of adjacent lamella , hyperplasia, oedema	Necrosis, vascular hemorrhage, dilated sinusoids and vacuolar degeneration	Damage and degeneration of renal tubules, glomerular oedema, Necrosis,	Nandan and Kumar, (2014); Atabati et al. (2015); Al-Tamimi et al. (2015)

In the digestive tract, methyl mercury is absorbed and transported to blood plasma and started to distributed in different tissue. Jasim et al., (2016) reported that liver accumulate more amount of mercury than gill and muscles (liver> gills> muscles) in *Oreochromis niloticus*. Methyl mercury can readily bind to metalloproteins and metallothioneines. About 10% of total ingested methyl mercury entrapped into central Nervous system (CNS) as it can pass blood brain barrier and the rest is transported to liver and kidney, from where it is excreted through urine or bile (Rodriguez et al., 2015). It is also proved that not only methyl mercury but also total mercury amount also put some deleterious impact on freshwater fishes (Subhavana et al., 2020).

Copper: Copper is an essential trace element for the living organisms. This is very much necessary for completing the metabolic reaction for growth of any organism. Copper is particularly important in activating cuproenzymes that catalyses many important metabolic reactions of living organisms. However, this element may be converted to hazardous substance if exposed beyond its permissible limit (50 ppm.) for long time. Extensive use of copper in agriculture, and industries like textile, tanneries, paints, battery, laundry, photographic studio, copper ware manufacturer, pipe making industries introduce the copper in high amount in the environment, becoming the principal source of contamination (Table 1).

Copper is essential element for living organisms for its involvement in many biological processes like oxidative phosphorylation, gene regulation and also acts as cofactor for enzymes but the metal becomes toxic when it exceeds its tolerance level in the surrounding aquatic medium. The main route of entry is through gill and dietary uptake whereas a very little amount of the metal can be taken up through skin (Padrilah et al., 2018). After entry copper bind to the plasma proteins and carried to different organs of the fish. Particularly, copper becomes toxic when an excessive amount of copper entered into the cell and binds to the cellular proteins and nucleic acid altering the natural metabolic reactions and gene expression. During chronic exposure at high concentration, Copper first accumulates in gill at higher concentration at which it may be toxic (Padrilah et al., 2018).

Then the metal gets absorbed into the plasma. Similarly, plasma takes the metal from the gut cells as well (Annabi et al., 2013). Then the metal is distributed in other organs like liver, spleen and kidney through the blood and bioaccumulated. Several researchers showed that liver is the main depot for the copper accumulation (Rajkowska and Protasowicki, 2013). Das and Gupta (2013) reported the accumulation of copper in the selected fish (*Esomus danricus*) organs as follows: liver>gill>kidney>fles h>bones>brain. Uptake and accumulation of copper in fish body is highly regulated by physico-chemical parameters of water such as pH, hardness, alkalinity, presence of inorganic and organic matter etc. (Malhotra et al., 2020).

Major histopathological alterations in gill, liver and kidney due to heavy metal accumulation in fresh water fishes: Accumulation of heavy metals leads to cellular level, tissue level or organ level toxicity. Chronic exposure to different heavy metals causes various deleterious impact on fish organs. Histopathological study proves the degree of metal infestation though impacts are dose/concentration and time of exposure dependent, organ sensitive and organism specific. In ecotoxicological studies, histopathology of the sensitive organs has been highly recommended as a biomarker of evaluation of stress due to metal contamination. Gill, liver and kidney are most sensitive to metal pollution thus histopathological studies of these organs become an unavoidable tool for evaluation of metal stress in the environment (Table 2). Several researchers have reported different types of heavy metal induced tissue degradation in different piscian models (Bakshi, 2016; Morcillo et al., 2017; Bakshi and Panigrahi, 2018). Long-term exposure to heavy metals even in very low amounts generally leads to leakage of cellular pathology marker enzymes in diferent tissues of fsh (Islam, 2019; Mustafa, 2020).

CONCLUSION

In this review we have compiled the uptake and accumulation process of some heavy metals (viz., Arsenic, Cadmium, Copper, Chromium, Lead and Mercury) of fishes. The bioaccumulation process of these metals poses serious impact on the aquatic food chain also. The magnified concentration of different heavy metals leads to higher mortality rate in fish eating organisms especially aquatic birds. Fish is consumed as a primary source of protein thus contamination of heavy metals can be very dreadful to human being also. To cope up with the serious environmental threat effective legislation guidelines and regular monitoring are highly required. Failure to control the contamination will lead to severe complication in near future because of the imposed adverse impact of the heavy metals. Monitoring the exposure, release of the heavy metals and probable intervention for reducing additional exposure in environment can become a momentous step towards control measures. State, National and international co-operation is very important for framing ideal tactics to avoid the consequences of heavy metal toxicity.

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Identification of Phytochemicals from Seed Extract of Custard Apple, Annona squamosa

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ABSTRACT

Custard apple has many alkaloids, such as aporphine, roemerine, norcorydine, corydine, norisocorydine, glaucine and anonaine in different parts of the plant. The roots are used to treat acute dysentery, depression and spinal marrow diseases, while leaves have been used in cases of prolapse of the anus, sores and swelling. Ripe fruit is sweet and good tonic for human health and it is enriching the blood, increases muscular strength and lessens vomiting. Its seeds are used as abortifacients. Seed oil is used in paint and soap industry. This investigation involves preliminary screening, detection, and separation of secondary metabolites from the seed extract of *Annona squamosa* Linn. Oil was extracted from seeds of *Annona squamosa* Linn. by Soxhlet extraction method; methanol was used as a solvent for extraction. Absorbance and functional group detection were studied using FTIR, seed oil supernatant was used to detect the functional groups of secondary metabolites those were presented in sample. A potent Cyanidin-3-O-(2-O-beta-xylopyranosyl-6-O-acetyl)-beta-galactopyranoside, 4-[4-(2aminoethyl)-2,6Diiodophenoxy]2-iodophenol, Amikacin. The study was done using atmospheric pressure chemical ionization (APCI) Liquid Chromatography mass Spectroscopy technique for identification of Phytochemical. Various phytochemicals were detected using this technique. The analysis by APCI-LC-MS reveals presence of several compounds like cyclopeptides and acetogenins. Cyclosqamosin A, Cyclosqamosin B, Cyclosqamosin H, Acetogenins (polyketides); Annonacin, Squamocin, Annonin VI, which were detected by peaks of m/z ratio between 605 to 640 positive ions shift and Tenacissoside F (steroid) at 667 m/z negative ion shift. Identified compounds were compared with reference of earlier investigations, it was clear that this compound played a major role as anti-diabetic, anticancer, anti-inflammatory and have insecticidal property. However, further Research is required to study phytochemicals.

KEY WORDS: FTIR, APCI-LC-MS, TENACISSOSIDE F, CYANIDIN-3-O-BETA-GALACTOPYRANOSIDE, SQUAMOCIN.

INTRODUCTION

Custard apple is tropical fruit which belongs to genus Annona and family Annonaceae and are collectively known as annonaceous fruits. There are over 120 species of genus Annona and are commonly found in India as a fruit consuming plant. This plant is commonly known as custard apple in English and Sitafal in Gujarati. Custard apple contains alkaloids, such as aporphine, roemerine, norcorydine, corydine, norisocorydine, glaucine and anonaine in various parts of the plant (Kowalska and Puett, 1990; Pinto et al., 2005; Hiwale, 2015). The Custard apple roots are used in the treatment of acute dysentery, spinal marrow diseases and some cases of depression. The leaves of Custard apple are used in cases of prolapse of the anus, sores and swelling. Custard apple contains alkaloids, such as aporphine, roemerine, norcorydine, corydine, norisocorydine, glaucine and anonaine in various

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parts of the plant (Kowalska and Puett, 1990; Chao-Ming et al., 1997; Pinto et al., 2005). Seeds of Custard apple contains acetogenins namely; squamocins B to N, coumarinoligans, annotemoyin-1, annotemoyin-2, squamocin and cholesteryl, glucopyranoside (Zahid et al., 2018).

Seeds of Custard apple are toxic, but they are used to treat head lice as they have insecticidal properties (its preparation causes eye irritation and can cause blindness). Seeds of *Annona* also have insecticidal properties. Farmers use pesticides to protect their crops from pest infestation, using chemical pesticides is no longer preferable. Seeds of custard apple can be used as biopesticide which can be used as suitable alternatives for pest control. Oil content is high in seeds which can be used to make soap or, if treated to remove the toxic alkaloids, it can be used as a cooking oil (Das et al., 2016; Vetal and Pardeshi, 2019). Earlier studies have been done to study phytochemicals obtained from seeds, leaves.

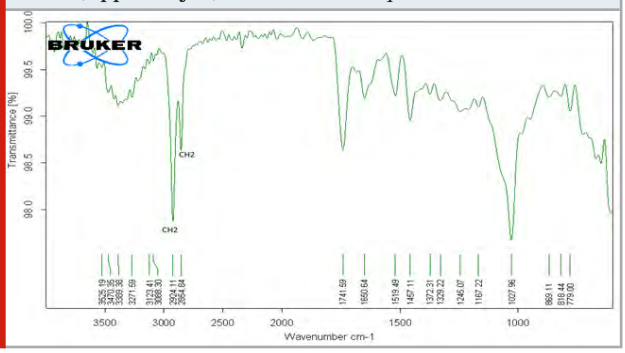
Table 1. Analysis of functional group present in first separated layer (Upper leyer) from crude oil of *Annona squamosa* L. by FTIR peaks values.

Sr. No	Peak Value	Bond	Functional group
1	3625.19	O-H free hydroxyl, Aromatic O-H free	Alcohols, Phenols
2	3470.35	O-H stretch, Aromatic O-H H-bonded	Alcohols, Phenols
3	3389.38	Dimer OH, Aromatic O-H H-bonded	Carboxylic acids, Phenols
4	3271.59	#C-H stretch, Dimer OH, Aromatic O-H H-bonded	Alkenes, Carboxylic acids, Phenols
5	3123.41	=C-H stretch, Dimer OH	Alkenes, Carboxylic acids,
6	3088.30	=C-H stretch, Dimer OH, Aromatic-H stretch	Alkenes, Carboxylic acids, Aromatics
7	2924.11	C-H stretch, Dimer OH	Alkanes, Carboxylic acids
8	2854.84	C-H stretch, -CH2, Dimer OH,	Alkanes, Carboxylic acids
9	1741.59	C=O stretch doublet, C=O stretch	Ketones, Amides
10	1650.64	C=C stretch, C=O stretch (H-Bond), C=N, =NOH	Alkenes, Amides, Quinone or conjugated ketone, Imine, Oxime
11	1519.49	N-O asymmetric stretch, N=O	Nitro compounds, Nitroso compounds
12	1457.11	Aromatic C-C stretch, $-CH_3$, -CH2	Aromatics, Alkanes
13	1372.31	$-CH_2$ and $-CH_3$, S=O	Alkanes, Sulphate ester
14	1329.22	R-F (C-F stretch), S=O (sulfone), N-O Symmetric stretch	Alkyl halides, Sulfone, Nitro Compound
15	1245.07	R-F (C-F stretch), C-O stretch, P-H bending (phosphine), P=O (phosphonate), P=O (phosphoramide), Si-CH3, N-O (aromatic)	Alkyl halides, Ethers, Phosphine, Phosphonate, Phosphoramide, Trimethylsilyl, Amine Oxide, Carboxylic acids, Esters
16	1167.22	R-F (C-F stretch), C=S (thiocarbonyl), P-H (phosphine), P=O (phosphine oxide), P=O (phosphate), C-O stretch	Alkyl halides, Thiocarbonyl, Phosphine, Phosphine oxide, Phosphate, carboxylic acid, esters
17	1027.96	R-F, P-H phosphine, P-OR esters, Si-OR, C-O Stretch	Alkyl halides, Phosphine, Phosphite Esters, Organosilicon, Carboxylic acids, Esters
18	869.11	C-H out of Plan, S-OR, RNH2, RNH	Aromatics, esters, Amines
19	818.44	R2C=CHR (=CH out of plan), R-Cl, C-H out of Plan, S-OR, RNH2, RNH	Alkenes, Alkyl halides, Aromatics, esters, Amines
20	779.00	R-Cl, C-H out of Plan, S-OR, RNH2, RNH	Alkyl halides, Aromatics, esters, Amines

Investigations done in past suggests that the alkaloids from Annona species have rarely been explored for their medicinal applications (Nugraha et al., 2019). Many volatile components have been isolated from *A. squamosa* such as bullatacin, 12,15-cis-squamostatin-A, α-Pinene, β-caryophyllene, camphene, β-pinene, myrcene, anonaine, spathulenol, germacrene Duvariamicin-III, annonacin, squamocin, liriodenine,

and molvizarin (Alkazman, Harnett and Hanrahan, 2020). This investigation involves preliminary screening, detection, and separation of secondary metabolites from the seed extract of *Annona squamosa* Linn. By comparing with reference of earlier studies, it was clear that seed extract contains compound that played a major role as anti-diabetic, anticancer, anti-inflammatory and have insecticidal property (Mangal et al., 2015; Ribeiro et al., 2018). The present investigation involves identification of such compounds that can be used for medicinal purposes.

Figure 1: FTIR Spectral pattern of Methanolic extracts of seeds (Upper Layer) from *Annona squamosa* L.



MATERIAL AND METHODS

Seeds of *Annona squamosa* were collected from fruit of *Annona squamosa* L. seeds were tested and determined viability, viable seeds were selected for identification of phytochemicals as previous study (Patel et al., 2019). Seeds were crushed using mixer grinder and powdered seeds were filled in a paper cup for further oil extraction. Finely crushed seed powder packed in paper cup was taken for oil extraction using Soxhlet extraction method, methanol was used as a solvent for extraction. After few cycles the crude oil was obtained and stored in dark bottles for further phytochemical analysis.

FTIR spectral analysis was conceded from the *Annona squamosa* seed oil supernatant to detect the functional groups of secondary metabolites those were presented in sample. It was performed on BRUKER-FITR instrument in Department of chemistry, P. S. Science & H. D. Patel Arts College, Kadi. We used atmospheric pressure chemical ionization (APCI) Liquid Chromatography mass Spectroscopy technique for identification of Phytochemicals. According to APCI-LC-MS technique we have obtained major peaks, APCI (Positive) m/z at 623, 605, 587, 639 and APCI (Negative), we have obtained major peaks of m/z at 667, 683. The oil was used to check antibacterial activity against *Bacillus subtills* & *Staphylococcus aureas*, (Chavan, 2006).

Table 2. Analysis of functional group present in Second separated (lower level) layer from crude oil of *Annona squamosa* L. by FTIR peaks values.

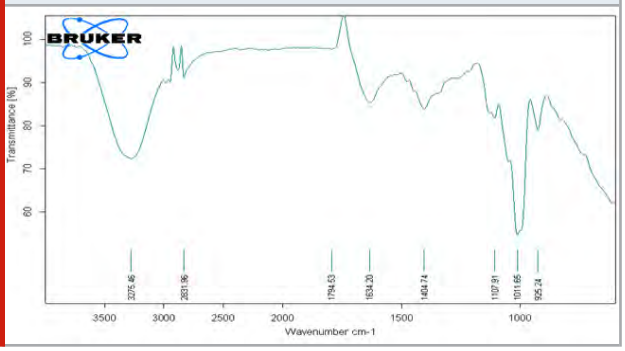
Sr. NO	Peak Value	Bonds	Functional Group
1	3275.46	#C-H stretch, Dimer OH, Aromatic O-H H-bonded	Alkynes, Carboxylic acids, Phenols
2	2831.96	Dimer OH	Carboxylic acids
3	1794.53	Unknown	Acid halide, Aryl carbonate, Five-membered ring Anhydride
4	1634.20	NH out of plan, C=N	Amides, Imine
5	1404.74	C-O stretch, S=O (Sulfate ester), Aromatic C-C stretch	Carboxylic acids, Sulfate esters, Aromatics
6	1107.91	R-F (C-F stretch), C-O stretch, C=S (thiocarbonyl), P-H bending (phosphine), P=O (phosphine oxide), P=O (phosphate), Si-OR,	Alkyl halides, carboxylic acid, esters, ethers, Thiocarbonyl, Phosphine, Phosphine oxide, Phosphate, Organosilicon
7	1011.65	R-F (C-F stretch), P-H bending (phosphine), P-OR (esters), Si-OR, C-O stretch	Alkyl halides, Phosphine, Phosphite Esters, Organosilicon, Carboxylic acids, Esters
8	925.24	P-OR (esters), RCOOH O-H bend	Phosphite Esters, Carboxylic acids

RESULTS AND DISCUSSION

The Upper layer of Methanolic extract FTIR spectra had 20 peaks. The Peaks at 3625.19 cm-1, 3470.35 cm-1, 3389.38 cm-1, 3271.59 cm-1, 3123.41 cm-1, 3088.30 cm-1, 2924.11 cm-1, 2854.84 cm-1, 1741.59 cm-1 indicates the presence of O-H stretch, O-H free hydroxyl, Aromatic O-H free, Aromatic O-H (H-bonded), Dimer OH, #CH stretch, =CH stretch, CH stretch, C=O stretch doublet, Aromatic H stretch and Functional group free hydroxy Alcohol, Phenols, Carboxylic acids, Alkanes, Ketones and Aromatics. The Peaks formed at 1650.64

cm-1, 1519.49 cm-1, 1457.11 cm-1, 1372.31 cm-1, 1329.22 cm-1, 1245.07 cm-1, 1167.22 cm-1, 1027.96 cm-1, 869.11 cm-1, 818.44 cm-1, 779.00 cm-1 specify the presence of C=C stretch, C=O stretch (H-Bond), C=N, =NOH, N-O asymmetric stretch, N=O, Aromatic C-C stretch, -CH3, -CH2, S=O, R-F (C-F stretch), S=O (sulfone), N-O Symmetric stretch, P-H bending (phosphine), P=O (phosphonate), P=O (phosphoramide), Si-CH3, N-O (aromatic), C-O stretch, R-F (C-F stretch), C=S (thiocarbonyl), P-H (phosphine), P=O (phosphine oxide), P=O (phosphate), C-O stretch, P-OR esters, Si-OR, C-H out of Plan, S-OR, RNH2, RNH, R2C=CHR (=CH out of plan), R-Cldemonstrated for the presence of Alkenes, Amides, Quinone or conjugated ketone, Imine, Oxime, Nitro compounds, Nitroso compounds, Aromatics, Sulphate ester, Alkyl halides, Sulfone, Ethers, Phosphine, Phosphonate, Phosphoramide, Trimethylsilyl, Amine Oxide, Carboxylic acids, Esters, Thiocarbonyl, Phosphine oxide, Phosphate, Phosphite Esters, Organosilicon, Amines respectively (Table-I) (Harnett and Hanrahan, 2020).

Figure 2: FTIR Spectral pattern of Methanolic extracts of seeds (Lower Layer) from *Annona squamosa* L.



The Second separated (Lower layer) layer of Methanolic extract FTIR spectra had 8 peaks. The Peaks at 3275.46 cm-1, 2831.96 cm-1, 1794.53 cm-1, 1634.20 cm-1, 1404.74 cm-1, 1107.91 cm-1, 1011.65 cm-1, 925.24 cm-1 indicated the presence of Alkynes, Carboxylic acids, Phenols, Acid halide, Aryl carbonate, Five-membered ring Anhydride, Amides, Imine, Sulfate esters, Aromatics, Alkyl halides, esters, ethers, Thiocarbonyl, Phosphine, Phosphine oxide, Phosphate, Organosilicon, Alkyl halides, Phosphine, Phosphite Esters respectively (Table-II) (Harnett and Hanrahan, 2020). The third separated (Crystal) layer of Methanolic extract FTIR spectra had 11 peaks.

Table 3. Analysis of functional group present in Crystal (lower level) from crude oil of *Annona squamosa* L. by FTIR peaks values.

Sr. NO	Peak Value	Bonds	Functional Group
1	3040.94	=C-H stretch, Dimer OH, Aromatic H stretch	Alkenes, Carboxylic acids, Aromatics
2	2878.65	C-H stretch, Dimer OH	Alkanes, Carboxylic acids
3	2378.22	P-H (Phosphine)	Phosphine
4	1700.72	C=O stretch, C=O stretch, C=N	Aldehydes, Amides, Imine
5	1571.66	C=C stretch, C-O stretch, N=O	Alkenes, Carboxylic acids, Nitroso Compounds
6	1424.56	S=O (Sulfate ester), Aromatic C-C stretch	Sulfate esters, Aromatics
7	1341.64	R-F (C-F stretch), S=O (sulfone 1), S=O, N-O symmetric stretch,	Alkyl halides, Sulfone, Sulfonic acid, Nitro Compounds
8	1303.88	R-F (C-F stretch), S=O (sulfone 1), N-O symmetric stretch, C-O stretch	Alkyl halides, Sulfone, Nitro Compounds, Carboxylic acids, Esters
9	1158.56	R-F (C-F stretch), C=S (thiocarbonyl), S=O (sulfone 2), P-H bending (phosphine), P=O (phosphine oxide), P=O (phosphate), C-O stretch,	Alkyl halides, thiocarbonyl, Sulfone, Phosphine, Phosphine oxide, Phosphate, Carboxylic acids, Eesters
10	1050.54	R-F (C-F stretch), C-O stretch, C=S (thiocarbonyl), P-H bending (phosphine), P-OR (esters), Si-OR,	Alkyl halides, Alcohols, carboxylic acids, esters, ethers, Thiocarbonyl, Phosphine, PhosphiteEsters, Organosilicon,
11	908.56	=CH out of plan, P-OR (esters)	Alkenes, PhosphiteEsters

Figure 3: FTIR Spectral pattern of Methanolic extracts of seeds (Crystal) from *Annona squamosa* L.

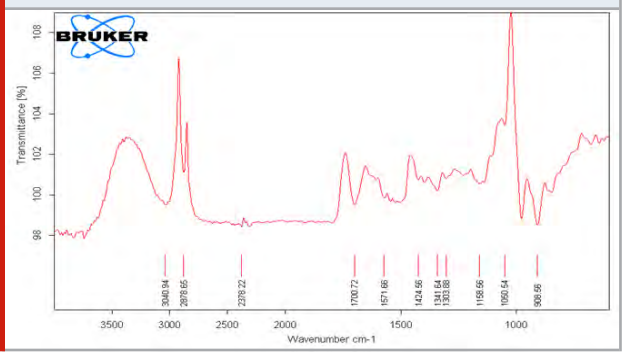


Figure 4: MS analysis of *Annona squamosa* L. seed oil. (positive ion shift)

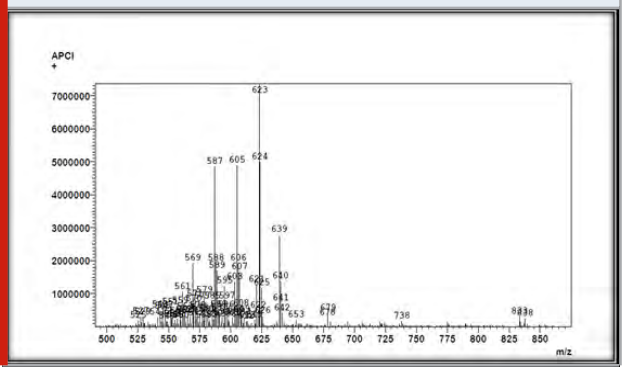
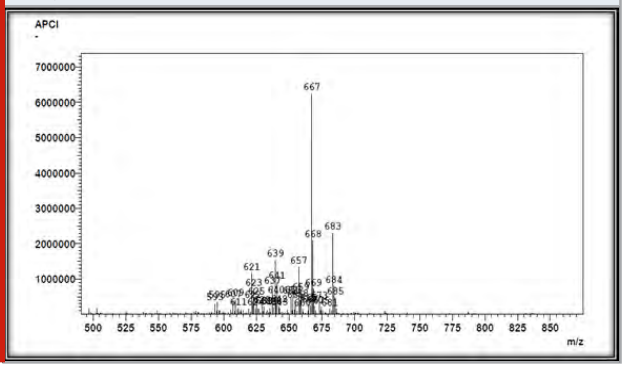


Figure 5: MS analysis of *Annona squamosa* L. seed oil. (Negative ion shift)



The Peaks at 3040.94 cm-1, 2878.65 cm-1, 2378.22 cm-1, 1700 cm-1, 1571.66 cm-1, 1424.56 cm-1, 1341.64 cm-1, 1303.88 cm-1, 1158.56 cm-1, 1050.54 cm-1, 908.56 cm-1 shows the presence of Alkenes, Carboxylic acids, Aromatics, Phosphine, Aldehydes, Amides, Imine, Nitroso Compounds, Sulfate esters, Alkyl halides, Sulfone, Sulfonic acid, Nitro Compounds, Esters, thiocarbonyl, Phosphine oxide, Phosphate, Phosphite Esters, Organosilicon (Harnett and Hanrahan, 2020).

According to LC-APCI-MS technique we have obtained major peaks, APCI (Positive) m/z at 623, 605, 587, 639 (Fig 4) and APCI (Negative), we have obtained major peaks of m/z at 667, 683 (Fig 5). The analysis by APCI-LC-MS revealed presence of several compounds namely cyclopeptides and acetogenins (Zahid et al., 2013). Cyclopeptides like Cyclosqamosin A, Cyclosqamosin B, Cyclosqamosin H, Acetogenins (polyketides) like Annonacin, Squamocin, Annonin VI, which were detected by peaks of m/z ratio between 605 to 640 positive ions shift and Tenacissoside F (steroid) at 667 m/z negative ion shift. Investigations done in past suggests that the alkaloids from *Annona* species have rarely been explored for their medicinal applications. Many volatile components have been isolated earlier from *A. squamosa* such as Annonacin, Squamocin which suggest that these chemicals have medicinal properties (Nugraha et al., 2019; Alkazman, Harnett and Hanrahan, 2020). By comparing with reference of earlier studies, it was clear that this compound played a major role as anti-diabetic, anticancer, anti-inflammatory and have insecticidal property (Mangal et al., 2015; Ribeiro et al., 2018).

CONCLUSION

The present study explicates the therapeutic application of the seeds which is a rich source of antioxidants such as phenols and flavonoids. The analysis by APCI-LC-MS and FTIR reveals presence of several compounds; cyclopeptides and acetogenins. Cyclopeptides like Cyclosqamosin A, Cyclosqamosin B, Cyclosqamosin H and other groups of cyclopeptides. Acetogenins (polyketides), Annonacin, Squamocin, Annonin VI and Tenacissoside F. By comparing with reference of earlier studies, it was clear that this compound played a major role as anti-bacterial, anti-diabetic, anticancer, anti-inflammatory and have insecticidal property. Research and development would be an important area to focus on medicinal importance of plant. The isolated compounds can be used in future to make antidiabetic, anticancerous, anti-inflammatory medicines, further in future this compound can be used in anti-leaching cream, anti-dandruff shampoo, hair oil and seed oil can also be used as biopesticide.

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Conflict of interest: On behalf of all authors, the corresponding author states that there is no conflict of interest.

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On the Hypoglycemic and Antioxidant Activities of Root Extract of Asparagus racemosus in Alloxan-Induced Diabetic Rats

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ABSTRACT

Oxidative stress induced by the rise in free radicals is the pivotal cause of many dreadful diseases in which Diabetes mellitus is one of them. Diabetes mellitus results in hyperglycemia leading to an increase in oxidative stress in the body due to the generation of free radicals that cause complications such as nephropathy due to oxidative damage. Many plant-derived phytomedicines are known to reduce diabetes-related complications. Asparagus is one such medicinal plant, which is widely used as phytomedicine for many diseases by traditional healers. In the present study, *Asparagus racemosus* crude methanolic root extract (ACMRE) was assessed for its hypoglycemic and antioxidant properties. The crude methanolic root extract of Asparagus was prepared using soxhlet apparatus. Wistar albino rats were divided into three groups viz. Normal control, Diabetic and Asparagus treated diabetic rats. Diabetes was induced by administering Alloxan (100 mg/kg body weight) in the tail vein. Diabetic rats were orally treated with 500 mg per kg body weight dose of crude methanolic root extract of *Asparagus racemosus* for 30 days using gavage. Blood glucose, creatinine and tissue antioxidants levels were analyzed at an interval of 10, 20 and 30 days respectively. Enzymic antioxidants such as Superoxide dismutase (SOD), Glutathione-S-Transferase (GST), Glutathione Peroxidase (GPx), Glutathione Reductase (GR), Catalase (CAT) and nonenzymic antioxidant molecule like Reduced Glutathione (GSH) were analysed along with serum creatinine and blood sugar using UV-Vis Spectrophotometer. Treatment with crude root extract significantly reduced the blood glucose and increased the enzymic and non enzymic antioxidant significantly ($p< 0.05$) of kidney tissue as compared to the diabetic rat group which was further confirmed by the decrease in the level of serum creatinine. Thus, the results indicate that the plant has hypoglycemic as well as antioxidant potential.

KEY WORDS: ANTIOXIDANT, ASPARAGUS RACEMOSUS, DIABETES MELLITUS, HYPOGLYCEMIC, OXIDATIVE STRESS.

INTRODUCTION

Oxidative stress occurs in the living body when reactive species outnumbers the antioxidant buffering capacity, Reactive species include Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). It causes several degenerative diseases such as Parkinson's, Rheumatoid arthritis, cardiovascular, Diabetes mellitus etc. Diabetes mellitus, commonly known as Diabetes, is a metabolic disorder that is marked by symptoms such as hyperglycemia, glycosuria, etc. resulting from lack of insulin or action (Dandekar, 2002; Galli et al., 2005; Amira, 2010). Hyperglycemia-induced oxidative stress is reported to be associated with the initiation and progression of Diabetes and its complications (Maritim, 2003; Matough et al., 2012 and Asmat et al, 2016).

In addition to this, the generation of free radicals associated with diabetes is reported to cause oxidative damage in organs such as the kidney, liver, eyes,

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gastrointestinal, cardiovascular system.. Insufficient glycemic control a major public health concern and therefore needs research on new complementary medicine derived from plants. Phytomedicine has been reported to ameliorate secondary complications of diabetes such as kidney damage, oxidative stress etc. The World Health Organization has reported that 80% of the developing countries population is beyond the reach of pharmaceutical drugs relies on plant-based traditional medicines for their health care needs (Juarez-Rojop et al., 2012; Khatune et al., 2016, Ramar et al., 2012; Buko et al., 2018; Yin et al., 2018).

In India, several plant products have been reported to be used by the tribal community and practitioners of the Ayurvedic system of medicine to treat diabetes and other diseases; one such plant is Asparagus, *Asparagus racemosus* Willd. (family Asparagaceae; Liliaceae), is commonly called Satavari, Satawar or Satmuli in Hindi; Satavari in Sanskrit; Shatamuli in Bengali; Shatavari or Shatmuli in Marathi; Satawari in Gujarati; Toala-gaddalu or Pilli-gaddalu in Telegu; Shimaishadavari or Inli-chedi in Tamil; Chatavali in Malayalam; Majjigegadde or Aheruballi in Kannada; Kairuwa in Kumaon; Narbodh or Satmooli in Madhya Pradesh; and Norkanto or Satawar in Rajasthan (Bopana et al., 2007; Alok et al., 2013; Tanwar et al., 2017; Tanwar et al., 2017).

The plant grows throughout the tropical and subtropical parts of India up to an altitude of 1500 m. The plant is a spinous under-shrub, with tuberous, short rootstock bearing numerous succulent tuberous roots (30–100 cm long and 1–2 cm thick) that are silvery-white or ash coloured externally and white internally. It has been reported that these roots are used in various medicinal preparations and possess various pharmacological activities such as antioxidant and free radical scavenging activity, anti-inflammatory property etc. (Bopana et al., 2007; Vadivelan et al., 2018). However, the effects of methanolic root extract on the various antioxidant enzyme in animal models have been meagrely reported. The present study is designed to evaluate the in-vivo hypoglycemic and antioxidant activity of crude methanolic root extract of *Asparagus racemosus* to understand how the extract acts against diabetes-induced oxidative stress (Vadivelan et al., 2018).

MATERIAL AND METHODS

The Plant part was authenticated by Prof. S. R. Padmadeo, Former Head of the Department of Botany, Patna University. The roots of *Asparagus racemosus* were purchased from the local market. Roots of *A. racemosus* were carefully washed with distilled water 3-6 times to remove dirt and other contaminating material. The plant materials were shade dried at ambient temperature and pressure until no moisture was left in it. The plant material was converted to fine powder using a kitchen grinder followed by sieving with the help of muslin cloth to remove coarse particles. The powdered form of roots of *Asparagus racemosus* was stored in a well-labeled airtight container for further use. The methanolic crude extract of roots of *Asparagus racemosus* was prepared using a Soxhlet apparatus (Riviera, India). 100 grams of fine powder of plant material was weighed using a digital weighing machine (Wensar, India) and placed in the cellulose thimble using gloves. The thimble was carefully placed in the extraction chamber of the Soxhlet apparatus while 500 ml of Methanol (100%) was placed in the boiling flask attached to the heating mantle (Nafisa et al., 2007).

The Soxhlet apparatus was run for 48 hours at 60oC to ensure that all phytochemicals in the plant material have dissolved in methanol. After 48 hrs cycle, the methanolic extract was collected from the Soxhlet apparatus and was further filtered using Whatman filter paper to get rid of any solid particle. The methanolic extract was concentrated by Rotavapour (Popular, India) at 60 °C and reduced pressure to one-twentieth volume (5 ml). it was further lyophilized to get thick yellowish-brown coloured residue which was stored in a well-labeled vial at 4 °C. Alloxan monohydrate used in this study was a product of Sigma Chemical Company, St Louis, U.S.A. Gluco-one glucometer was a product of Dr.Morepen, Delhi, India. UV-Vis Spectrophotometer (Systronics, India) was used to analyse enzymes and molecules. All other chemicals and assay kits used were products of Sigma-Aldrich Inc. and Merck, Germany, respectively. Healthy Wistar male albino rats (100–150 g) were kept under well-ventilated standard environmental conditions (temperature 25±2 °C, relative humidity 50±5 %) with a 12 h light / dark cycle. Animals were allowed to acclimatize for 7 days before the commencement of the experiment (Nafisa et al., 2007).

The experiments were designed and conducted as per the current ethical norms and guidelines approved by the Ministry of Social justices and Environment, Government of India. The rats were fed on Laboratory prepared pellet having the composition suggested by Subcommittee on Laboratory animal nutrition, National Research Council, USA and water ad libitum to ensure proper growth and nourishment. The extra supplement that was given was carrot, sprouted Bengal gram and Green gram. Alloxan monohydrate 100 mg/kg body weight dissolved in 0.9% sterilized NaCl solution of pH 7.0 was administered in the tail vein of rats to induce diabetes mellitus. After 48 hours, their fasting blood glucose levels were monitored using a glucometer by collecting blood from the tail artery of animals. Those rats having fasting glucose levels in the range of 250 and 400 mg/dl were considered diabetic and used for the experiment (Nafisa et al., 2007). The pure breed rats were kept in new polypropylene cages and were categorized into the following groups:Group I – Normal Control, Group II – Alloxan treated Diabetic rats, Group III – *Asparagus racemosus* Crude Methanolic root extract (ACMRE) treated diabetic rats.

ACMRE of 500mg/kg.body weight was prepared from the stock solution according to the weight of the rats by dissolving in olive oil. Oral administration of the desired herbal extract was made through oral gavages for 10,20 and 30 days. For the present research work blood sample were collected by tail clipping for fasting glucose estimation and after an interval of 10, 20, and 30 days rats were sacrificed for organ collection and preservation. For the entire research work, tissue samples of the kidney for the antioxidant assay of different parameters were kept in Tris-buffer at -20 °C. The kidney tissue was isolated, washed in 0.2 M Tris buffer solution, blotted dry and weighed. A 10% tissue homogenate was prepared in 0.2 M Tris buffer solution by a Potter-Elvehjem Homogenizer. The tissue homogenate was centrifuged at 10,000 g for 20 min, to remove cell debris and then the supernatant was centrifuged at 35,000 g for 30 min. The supernatant obtained was used for various antioxidant assays. The tissues collected at each interval were immediately processed and each tissue sample was analyzed separately. Superoxide Dismutase (SOD) activity was measured by the method of Marklund and Marklund (Marklund and Marklund, 1974) based on the inhibition of the autoxidation of pyrogallol.

Catalase (CAT) activity was determined by measuring the rate of decomposition of H_2O_2 by the method of Claiborne, 1985. The Glutathione Peroxidase (GPx) activity was determined using H_2O_2 as a substrate according to the method of Rotruck et al., 1973. Glutathione Peroxidase enzyme catalyzes the decomposition of H_2O_2 or other peroxides (-OH) with the simultaneous oxidation of GSH into GSSH (Rotruck et al., 1973). The tissue GSH content was estimated by the method of Beutler (Beutler et al., 1967) based on the development of a stable yellow coloured complex, with 5,5'-dithio, bis-2, nitrobenzoic acid (DTNB) or Ellman's reagent. The activity of GSH-R was measured by the oxidation of NADPH as described by Horn,1963. The activity of GST was determined using 1-chloro 2,4-dinitrobenzene (CDNB) as substrate (Habig et al., 1974). Data were expressed as the Mean ± SEM. For statistical analysis of the data, group means were compared by analysis of variance (ANOVA) followed by Tukey and Duncan post hoc test for multiple comparisons using Graph Pad Prism 8 software. $P < 0.05$ was considered to be statistically significant (Habig et al., 1974).

RESULTS AND DISCUSSION

Hyperglycemia is the major cause of structural changes (Somania et al., 2012). In the present study, Alloxan treated rats at a dose of 100 mg/kg body weight caused elevation in blood glucose up to 489% as compared to control leading to loss of weight and lethargic activity. Nevertheless, when the crude Asparagus methanolic root extract at a dose of 500 mg/kg body weight was administered to diabetic rats caused a significant decline in blood glucose level up to -70% (Fig.1) which is in agreement with the findings of Taepongsorat et al., (2018) and Vadivelan et al., (2011).

Figure 1: Effect of Crude *Asparagus* methanolic root extract on blood glucose

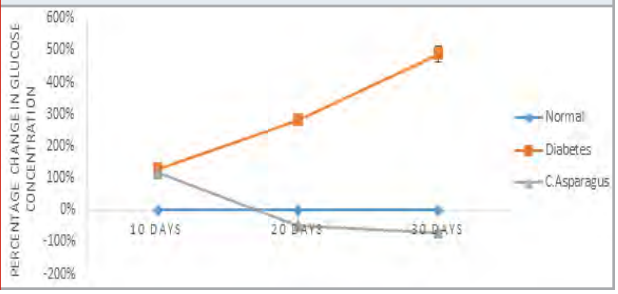

Table 1. Effect of ACMRE on SOD (U/ mg of protein) in Kidney Tissue

Days	Normal	Diabetes	Crude Asparagus
10 days	172.156± 0.494	130.396± 0.471*	25.686± 1.055*#
20 days	172.156± 0.494	97.0633± 0.087*	42.94± 0.390*#
30 days	172.156± 0.494	27.5966± 0.8*	61.11± 0.061*#

Values indicate mean ± SEM (n=3)
*p<0.05, compared with normal control values, # p<0.05, compared with Diabetic values

The antioxidant effects of crude Asparagus were studied in terms of antioxidant enzymes like SOD, GST, GPx, Catalase and Glutathione Reductase along with antioxidant molecules like Reduced Glutathione (GSH). Superoxide dismutase plays a key role during oxidative stress. It catalyses the dismutation of superoxide radicals. In the diabetic rat group, SOD level considerably decreased (-84%), Glycosylation of proteins may be responsible for degradation in SOD activity in the diabetic group (Satheesh et al., 2004; Jabeen et al., 2006). Nonetheless, ACMRE treatment leads to a significant increase in enzyme activity (+121%) on the 30th day ($p<0.005$) as compared to the diabetic group. (Table.1). Glutathione S-transferases (GSTs) is another significant antioxidant enzyme that helps to overcome oxidative stress. GST catalyse addition or substitution reactions of the substrate through the nucleophilic attack of the tripeptide glutathione to electrophilic substrates (Armstrong, 1997; Jabeen et al., 2006).

GST activity in the alloxan-induced diabetes group illustrated -49% decrease as compared to normal on

day 30. However, on treatment with methanolic crude Asparagus root extract, there was a noticeable elevation ($p<0.05$) in enzyme activity by 1.5 fold from day 10 to day 30 showing the beneficial effect of the extract (Table 2). GPxs have been reported to catalyze the reduction of H_2O_2 or organic hydroperoxides to water or the corresponding alcohols, respectively, typically using Glutathione (GSH) as a reducing agent (Brigelius-Flohé et al., 2013). Its activity decreased substantially by 90% in the diabetic group, nonetheless, on treatment with crude extract caused recovery of enzyme activity by 3.47 fold on day 30 ($p<0.05$) with respect to the diabetic group (Table 3).

Table 2. Effect of ACMRE on GST (U/ mg of protein) in Kidney Tissue

Days	Normal	Diabetes	Crude Asparagus
10 days	0.612± 0.004	0.481± 0.001*	0.694± 0.001*#
20 days	0.612± 0.004	0.462± 0.003*	0.949± 0.001*#
30 days	0.612± 0.004	0.311± 0.002*	1.046± 0.008*#

Values indicate mean ± SEM (n=3)
*p<0.05, compared with normal control values, # p<0.05, compared with Diabetic values

Table 3. Effect of ACMRE on GPx (U/ mg of protein) in Kidney Tissue

Days	Normal	Diabetes	Crude Asparagus
10 days	12.286± 0.014	4.26± 0.05*	1.386± 0.026*#
20 days	12.286± 0.014	2.093± 0.027*	2.13± 0.011*
30 days	12.286± 0.014	1.23± 0.0057*	4.813± 0.001*#

Values indicate mean ± SEM (n=3)
*p<0.05, compared with normal control values, # p<0.05, compared with Diabetic values

Catalase which is a significant antioxidant enzyme against H_2O_2 was analyzed and there was a marked decline in catalase activity up to 98% in the diabetic group as compared to normal although when treated with ACMRE, enzyme activity increased 3.46 times as compared to the diabetic group showing recovering trend ($P<0.05$) (Table 4) (Tehrani et al., 2018). Glutathione reductase which helps to maintain a consistent supply of reduced glutathione in the cell was observed to follow the declining trend in the case of the diabetic rat group (-58%) (Couto et al., 2016). Nevertheless, on treatment with ACMRE, there was 59% increase in enzyme activity as compared to the diabetic group on day 30 ($P<0.05$) (Table 5) (Tehrani et al., 2018).

Table 4. Effect of ACMRE on Catalase (U/ mg of protein) in Kidney Tissue

Days	Normal	Diabetes	Crude Asparagus
10	413.756± 57.748	271.85± 1.668	95.596± 1.189#
20	413.756± 57.748	51.48± 0.931	168.04± 0.843#
30	413.756± 57.748	8.703± 0.275	330.886± 1.206#

Values indicate mean ± SEM (n=3)
*p<0.05, compared with normal control values, # p<0.05, compared with Diabetic values

Table 5. Effect of ACMRE on GR (U/ mg of protein) in Kidney Tissue

Days	Normal	Diabetes	Crude Asparagus
10 days	0.774± 0.001	0.693± 0.003*	0.192± 0.0005*#
20 days	0.774± 0.001	0.462± 0.0005*	0.281± 0.001*#
30 days	0.774± 0.001	0.323± 0.001*	0.515± 0.001*#

Values indicate mean ± SEM (n=3)
*p<0.05, compared with normal control values, # p<0.05, compared with Diabetic values

Reduced Glutathione (L-γ-glutamyl-L-cysteinyl glycine, GSH) is at the core of one of the most significant antioxidant enzyme systems of the cell which in its reduced form is efficient of neutralizing reactive oxygen and nitrogen species, thus assisting in the control of redox homeostasis. Treatment with ACMRE leads to 3.07 fold increase in GSH level in the diabetic rat ($p<0.05$) in contrary to the diabetic group without treatment, where GSH level fell drastically to -24% as compared to Normal (Table 6).

High production of reactive oxygen species is a significant reason behind the advancement of diabetic complications like diabetic nephropathy and some reports illustrated the regulation of oxidative stress using

antioxidants to reduce diabetic complexities (Kataya and Hamza, 2008; Couto et al., 2016). In the present study, diabetic rats showed an overall decreasing level of enzymic antioxidants such as SOD, GPx, GR, GST, CAT and non-enzymic antioxidant like GSH however on treatment with crude methanolic Asparagus root extract elevated the enzymic as well as non-enzymic antioxidant which is in concordance with the findings of Vadivelan et al., (2011) and Purena et al., (2018). The result is also in congruence with the findings of Kamat et al., (2000) and Acharya et al., (2012) which were reported in liver tissue suggesting the protective role of crude root extract of Asparagus on SOD enzyme against free radicals (Acharya et al., 2012; Purena et al., 2018).

Table 6. Effect of ACMRE on GSH (µg/ml of sample homogenate) in Kidney Tissue

Days	Normal	Diabetes	Crude Asparagus
10 days	11.569±0.024	10.216±0.006*	6.666± 0.041*#
20 days	11.569± 0.024	9.613± 0.024*	9.057± 0.001*#
30 days	11.569± 0.024	8.767± 0.041*	20.482± 0.024*#

Values indicate mean ± SEM (n=3)
*p<0.05, compared with normal control values, # p<0.05, compared with Diabetic values

Table 7. Effect of ACMRE on Creatinine (mg/dl) in blood serum

Days	Normal	Diabetes	Crude Asparagus
10 days	0.011± 0.001	0.03± 0.001*	0.91± 0.004*#
20 days	0.011± 0.001	0.12± 0.002*	0.62± 0.005*#
30 days	0.011± 0.001	1.109± 0.005*	0.58± 0.002*#

Values indicate mean ± SEM (n=3)
*p<0.05, compared with normal control values, # p<0.05, compared with Diabetic values

Serum creatinine is the most widely used endogenous renal biomarker which increased 36.96 fold in the diabetic group. Elevated Creatinine levels may be due to the damage caused to the podocyte foot process leading to degradation of filtration (Ahmed et al., 2015; Hokamp et al., 2016). In contrast to this, when treated with crude asparagus extract there was -37 % decrease in creatinine level on the 30th day as compared to the diabetic group ($p<0.05$) (Table 7). This finding suggests that Asparagus root extract provides nephroprotection against renal deterioration and is in corroboration with the findings of Somania et al., (2012) (Sachdeva et al., 2014; Hokamp et al., 2016). Possibly, the phytoconstituents of Asparagus root extract reduced the blood glucose through its insulin secretory activity and complemented the activity of enzymic and non-enzymic antioxidant. Thus establishing its hypoglycemic and antioxidant properties (Hannan et al., 2007).

CONCLUSION

Based on our result it may be concluded that roots of *Asparagus racemosus* possess the hypoglycemic and antioxidant potential hence can be utilized as a significant source of antioxidant that has the ability to deal with diabetic complications such as nephropathy. Therefore may be promoted as a food supplement in the treatment of diabetes. However further researches are needed to analyse its actual therapeutic capability and compounds involved in its medicinal value.

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Screening and Isolation of Plant Growth-Promoting Bacteria from Forest and Coastal Regions of Saurashtra, Gujarat

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ABSTRACT

Chemical fertilisers have been used intensively in recent years leading to the degradation in the quality of the soil. Diversity of microorganisms is important, as their unique features can be utilized for crop production and environment. Microorganisms are usually inhabited in all parts of the plant from the roots to the shoot and internal regions of the plants. Rhizosphere microbial variety conveys an assortment of microorganisms which offer advantageous properties to the plant environments. In the present study, an attempt has been made for the screening of bacteria for plant growth-promoting activities such as nitrogen fixation, phosphate solubilization and indole acetic acid production. Soil samples were collected from thirty-four different places of districts Junagadh, Gir Somnath, Amreli, Diu, Dwarka and Jamnagar. Twenty soil samples were from forest region and fourteen soil samples were from the coastal region of Saurashtra. The nitrogen-fixing capability of the isolates was evaluated using Ashby's media containing bromothymol blue. Total 57 nitrogen-fixing bacteria based on their colony morphology were isolated, of which 49 bacterial isolates were able to solubilize phosphate and 27 were able to produce indole acetic acid. Of 57 bacterial isolates, 23 isolates showed positive results for nitrogen fixation, phosphate solubilization and indole acetic acid production. Nitrogen and phosphorus are one of the major essential macronutrients for plant growth and development. Indole acetic acid serves as one on the plant hormone for growth of plants. The present study indicates 23 bacterial isolates can have the potential for plant growth-promoting bacteria and as a greater number of isolates were from forest region which also indicates the fertility of the soil.

KEY WORDS: BACTERIA, PLANT-GROWTH PROMOTING, NITROGEN FIXATION, PHOSPHATE SOLUBILIZATION, INDOLE ACETIC ACID.

INTRODUCTION

Farmers are currently using chemical fertilisers to intensively supplement the basic nutrients of the soil-based plant system. The advantage of accessibility and intensive use create environmental issues of chemical fertilizers today's agriculture. The use of chemical fertilizers does however have their advantages and drawbacks in agriculture. Hence, there's an increasing demand for various ways to support the crop production and to maintain the nutrient within the soil environment for ecological equilibrium in an agroecosystem. The engagement of microorganism as inoculants or for plant growth-promotion is promising and are widely accepted practices which are been employed in agriculture for the agricultural produce. Symbiotic / non-symbiotic soil bacterium that colonizes root rhizosphere of plant and promotes the expansion in terms of crop yields, (Gouda et al., 2018; Santos et al., 2019, Lebrazi et al., 2020).

Diversity of microorganisms is important, as their unique features can be utilized for crop production and environment (Costa et al., 2018). Variety of microorganism

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assists with developing the biological system comprises of an organism, soil, and plant. The working of this environment is significantly represented by microbial elements. Microorganisms are usually inhabited in all parts of the plant from the roots to the shoot and internal regions of the plants (Harman and Uphoff, 2019). In all structures, most of these microorganisms help and raise the plant to live emphatically and offer significant great conditions to the plants. In all structures, the greater part of these organisms helps and elevate the plant to live soundly and offer useful focal points to the plants. Among all these plant growth-promoting bacteria undertake a significant job and are a focal situation in quality and the quantity of yield. Rhizosphere microbial variety conveys an assortment of microorganisms which offer advantageous properties to the plant environments (Thakur et al., 2020).

Plant growth-promoting effect of the PGPB is usually explained by the discharge of metabolites which directly promote the plant growth (Rilling et al., 2019). There are several ways to elucidate the activities of PGPB benefit to the host plant. PGPB have potential to supply plant growth regulators like cytokinins, indole acetic acid (IAA) and gibberellins, enhancing organic process, promote solubilization of inorganic and organic phosphate. The inoculation with PGPB strain like *Azotobacter* could help to scale back the utilization of nitrogen-based chemical fertilizer (Sharma et al., 2016; Roriz et al., 2020).

Plant growth-promoting bacteria (PGPB) have been studied as a sustainable alternative to the use of chemical fertilizers to increase crop yields, and effective PGPB have been isolated from diverse plants and soil compartments. Naturally occurring bacteria, commonly found in the soil associated with the roots of plants, positively affect the growth of plants in a number of different ways (Rilling et al., 2019; Glick, 2020). This includes increases in plant yield, nutritional content, tolerance to various abiotic and biotic stresses, and the production of useful secondary metabolites.

Due to its topographic state, the Saurashtra region, Gujarat India has a wide variation. The region has a range that ranges from both forest and coastal areas to wetlands. Saurashtra region has shallow, medium black, calcareous soils with a rainfall range of 400mm to 700mm and dry sub-humid climate. Groundnut, cotton, sesamum, sugarcane, rice, pulses, jowar and bajra are major crops produced in the Saurashtra region (Gondaliya et al., 2017; Ravi and Fulekar, 2018). In the present study, an effort has been made to screen for free-living plant growth-promoting bacteria from the forest and coastal region of Saurashtra.

MATERIAL AND METHODS

Soil samples from Gir forest and Coastal areas of Saurashtra region, Gujarat were collected. Soil samples were collected from thirty-four different places of districts Junagadh, Gir Somnath, Amreli, Diu, Dwarka and Jamnagar from Saurashtra region, Gujarat India. Of the thirty-four soil samples, 20 were from forest region and 14 were from the coastal region of Saurashtra. The sampling area for the soil was dug to a depth of about 25-30 cm and then collected and transferred to sterile polyethene bags. The soil samples for further use were stored in a refrigerator at low temperatures. For the isolation and screening of nitrogen-fixing bacteria from soil, serial dilution technique and spread plate method using Jenson agar medium was used (Sahoo et al., 2014).

In case of soil samples from coastal regions Jenson agar medium with varying salt concentration of 0.5%, 2%, 4%, 6%, 8% and 10% respectively. Different components of Jenson agar medium were weighed, dissolved in an appropriate amount of water, pH was adjusted and autoclaved at 1210C (15 psi) for 15 minutes. Ten gram of soil sample was suspended in 90 ml of sterilized distilled water blank and kept on a rotary shaker for 30 minutes so that microorganism adhered to the soil particles get dispersed uniformly into the water. Using serial dilution technique serial dilution were made up to 10-7. From dilution of 10-5 to 10-7, 100 µl was spread on Jenson agar medium plates in triplicates. The spreaded plates were incubated at 30±1°C till the visible colonies appeared. Individual colonies of different bacterial isolates showing different morphological features were picked up, purified by streaking on solidified Jenson agar medium plates.

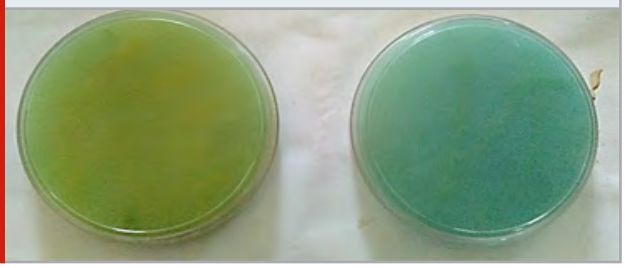
The isolated colony of each isolate, colony characters were described according to Microbiology: A Laboratory Manual (Cappuccino and Welsh, 2017). Individual isolated pure colonies were picked up and maintained on Jenson agar slants for further use. They were streaked on freshly prepared nutrient agar plates and incubated for 3 days at 30±1 °C. Gram's staining of isolates was done according to the procedure given by (Brown and Heidi, 2015). Cell shape was also recorded. Nitrogen-fixing capability of the isolates was evaluated using Ashby's media containing bromothymol blue (Hingole and Pathak, 2016).

Plates containing medium were prepared and streaked with different isolates in triplicates. Plates were incubated at 30±1°C. Isolates fixing nitrogen showed growth on the medium with a change in colour from green to blue. Uninoculated plates in triplicates served as control. Different bacterial isolates were screened for their phosphate solubilizing ability by growing them on Pikovskaya agar medium (Gupta and Pandey, 2019). Fifty microlitres of two days old culture suspension of selected isolates were spotted on the solidified agar medium plates incubated at 30±2°C for 5-6 days. The plates were examined for the production of a clear zone around the bacterial growth. As a result of acid production, isolates which used tricalcium phosphate developed a clear zone around the colony (Gupta and Pandey, 2019).

The bacterial isolates were screened for their ability to produce IAA, in the absence and presence of tryptophan. The bacterial isolates were inoculated in 5 ml Jensen's

liquid medium incubated at 30±2°C. Cultures were centrifuged at 3000 rpm for 30 minutes. Two ml of Salkowski's reagent and two drops of ortho-phosphoric acid was mixed with 2 ml of supernatant. The presence of the pink colour indicated the production of IAA. Further study was performed for ammonia production and other biochemical properties, such as capsule staining, indole analysis, oxidase test and catalase test, in isolates that have shown positive results for nitrogen fixation, phosphate solubilization and indole acetic acid production (James Cappuccino and Welsh, 2017; Gupta and Pandey, 2019).

Figure 1: Green coloured plate (negative control) and Blue coloured plate which indicated the production of ammonia and nitrogen fixation.



RESULTS AND DISCUSSION

Screening of nitrogen-fixing bacteria from soil: The accession number given to the isolates were GFS for the isolates obtained from the soil of forest region of Saurashtra and SCS for the coastal region of Saurashtra. Different isolates were isolated based on colony characteristics like morphology, size, and shape. From all the soil samples, about 57 bacterial isolates have been isolated. Twenty-seven isolates were from the forest region and thirty were from the coastal region of Saurashtra. It was observed that out of 57 isolates 34 were Gram-negative coccobacilli,6 were Gram-negative bacilli, 10 were Gram-positive bacilli, 6 were Gram-positive coccobacilli and one was Gram-positive cocci. They were grouped based on Gram reaction and shape of the bacterial cell (Table1). Upon capsule stain of 57 isolates, 30 were capsulated and 27 were non-capsulated.

Table 1. Gram's Stain, Morphology, and presence of capsule of all bacterial Isolates.

Sr.	Accession no.	Gram's stain	Morphology	Capsule	Sr.	Accession no.	Gram's stain	Morphology	Cap-sule
1	GFS01C1	Negative	Cocco Bacilli	Yes	30	SCS01C3	Negative	Bacilli	Yes
2	GFS01C2	Negative	Cocco Bacilli	No	31	SCS02C1	Negative	Bacilli	Yes
3	GFS02C1	Negative	Cocco Bacilli	No	32	SCS03C1	Positive	Cocco Bacilli	Yes
4	GFS03C1	Negative	Cocco Bacilli	Yes	33	SCS03C2	Negative	Cocco Bacilli	No
5	GFS04C1	Negative	Bacilli	Yes	34	SCS04C1	Positive	Bacilli	No
6	GFS05C2	Negative	Cocco Bacilli	Yes	35	SCS05C1	Positive	Cocco Bacilli	No
7	GFS05C1	Negative	Cocco Bacilli	No	36	SCS05C2	Positive	Cocco Bacilli	No
8	GFS06C1	Negative	Cocco Bacilli	Yes	37	SCS06C1	Negative	Cocco Bacilli	No
9	GFS07C1	Positive	Bacilli	Yes	38	SCS07C1	Positive	Bacilli	No
10	GFS07C2	Negative	Cocco Bacilli	No	39	SCS07C2	Negative	Cocco Bacilli	Yes
11	GFS08C1	Negative	Cocco Bacilli	Yes	40	SCS07C3	Negative	Cocco Bacilli	No
12	GFS10C1	Negative	Cocco Bacilli	Yes	41	SCS08C1	Negative	Cocco Bacilli	No
13	GFS11C1	Positive	Bacilli	Yes	42	SCS09C1	Negative	Cocco Bacilli	Yes
14	GFS12C1	Negative	Cocco Bacilli	No	43	SCS10C1	Positive	Bacilli	Yes
15	GFS13C1	Positive	Cocci	No	44	SCS11C1	Negative	Cocco Bacilli	Yes
16	GFS13C2	Negative	Cocco Bacilli	No	45	SCS11C2	Negative	Bacilli	No
17	GFS14C1	Negative	Cocco Bacilli	No	46	SCS12C1	Positive	Bacilli	No
18	GFS15C1	Positive	Bacilli	Yes	47	SCS12C2	Negative	Cocco Bacilli	No
19	GFS15C2	Negative	Cocco Bacilli	Yes	48	SCS12C3	Positive	Bacilli	No
20	GFS16C1	Positive	Bacilli	Yes	49	SCS12C4	Negative	Cocco Bacilli	No
21	GFS16C2	Negative	Cocco Bacilli	Yes	50	SCS12C5	Positive	Cocco Bacilli	Yes
22	GFS17C1	Negative	Cocco Bacilli	No	51	SCS13C1	Negative	Bacilli	No
23	GFS18C1	Negative	Cocco Bacilli	No	52	SCS13C2	Negative	Bacilli	Yes
24	GFS18C2	Negative	Cocco Bacilli	Yes	53	SCS13C3	Positive	Bacilli	Yes
25	GFS19C1	Negative	Cocco Bacilli	Yes	54	SCS13C4`	Negative	Cocco Bacilli	Yes
26	GFS19C2	Negative	Cocco Bacilli	No	55	SCS13C1	Negative	Cocco Bacilli	No
27	GFS20C1	Negative	Cocco Bacilli	Yes	56	SCS13C2	Negative	Cocco Bacilli	No
28	SCS01C1	Negative	Cocco Bacilli	Yes	57	SCS13C3	Positive	Cocco Bacilli	Yes
29	SCS01C2	Positive	Cocco Bacilli	Yes					

Nitrogen fixation: Nitrogen is required for the synthesis of amino acids, chlorophyll, nucleic acids, and ATP which are required for the growth and survival of plants (Chakraborty and Tribedi, 2019). All the 57 isolates indicated by growth on Ashby's medium and turned the greenish colour of the medium to blue (Table 2). The development of blue colour was due to the production of ammonia in the medium making it alkaline (Figure 1). It has been previously observed, that Azospirillum possess high nitrogenase activity allowing for the possibility of using this bacterium as a biofertilizer to improve soil fertility for improved and efficient farming (Richard et al., 2018). However, the confirmatory test of nitrogenase activity using acetylene reduction assay (ARA) needs to be performed to establish their nitrogen-fixing capability (El-Khaled et al., 2020).

Table 2. Plant Growth Promoting activities of the bacterial isolates.

Sr.	Accession no.	Nitrogen fixation	Phosphate Solubilization	Indole Acetic Acid	Sr.	Accession no.	Nitrogen fixation	Phosphate Solubilization	Indole Acetic Acid
1	GFS01C1	Positive	Positive	Positive	30	SCS01C3	Positive	Positive	Negative
2	GFS01C2	Positive	Positive	Negative	31	SCS02C1	Positive	Positive	Negative
3	GFS02C1	Positive	Negative	Negative	32	SCS03C1	Positive	Positive	Positive
4	GFS03C1	Positive	Positive	Positive	33	SCS03C2	Positive	Positive	Positive
5	GFS04C1	Positive	Positive	Positive	34	SCS04C1	Positive	Positive	Positive
6	GFS05C2	Positive	Positive	Positive	35	SCS05C1	Positive	Positive	Negative
7	GFS05C1	Positive	Positive	Negative	36	SCS05C2	Positive	Positive	Negative
8	GFS06C1	Positive	Negative	Negative	37	SCS06C1	Positive	Positive	Negative
9	GFS07C1	Positive	Positive	Positive	38	SCS07C1	Positive	Negative	Negative
10	GFS07C2	Positive	Negative	Positive	39	SCS07C2	Positive	Positive	Positive
11	GFS08C1	Positive	Positive	Positive	40	SCS07C3	Positive	Positive	Positive
12	GFS10C1	Positive	Positive	Positive	41	SCS08C1	Positive	Negative	Negative
13	GFS11C1	Positive	Positive	Positive	42	SCS09C1	Positive	Positive	Negative
14	GFS12C1	Positive	Positive	Positive	43	SCS10C1	Positive	Positive	Negative
15	GFS13C1	Positive	Positive	Positive	44	SCS11C1	Positive	Positive	Negative
16	GFS13C2	Positive	Negative	Negative	45	SCS11C2	Positive	Positive	Negative
17	GFS14C1	Positive	Negative	Negative	46	SCS12C1	Positive	Positive	Positive
18	GFS15C1	Positive	Negative	Positive	47	SCS12C2	Positive	Positive	Positive
19	GFS15C2	Positive	Positive	Positive	48	SCS12C3	Positive	Negative	Positive
20	GFS16C1	Positive	Positive	Positive	49	SCS12C4	Positive	Negative	Positive
21	GFS16C2	Positive	Positive	Positive	50	SCS12C5	Positive	Positive	Positive
22	GFS17C1	Positive	Positive	Negative	51	SCS13C1	Positive	Negative	Negative
23	GFS18C1	Positive	Positive	Negative	52	SCS13C2	Positive	Positive	Negative
24	GFS18C2	Positive	Negative	Negative	53	SCS13C3	Positive	Positive	Negative
25	GFS19C1	Positive	Negative	Negative	54	SCS13C4	Positive	Positive	Negative
26	GFS19C2	Positive	Positive	Positive	55	SCS13C1	Positive	Positive	Negative
27	GFS20C1	Positive	Positive	Negative	56	SCS13C2	Positive	Positive	Negative
28	SCS01C1	Positive	Positive	Positive	57	SCS13C3	Positive	Negative	Negative
29	SCS01C2	Positive	Positive	Negative					

Figure 2: Solubilization of phosphate as seen around the bacterial isolate.

Phosphate solubilization: Phosphorus is one of the major essential macronutrients for plant growth and development. However, the concentration of soluble P in the soil is very low (Zhu et al., 2011). The use of phosphate solubilizer bacteria as inoculants will increase P intake by plant and cultivation at the same time (Olanrewaju et al., 2017). Of the 57 bacterial isolates, 49 isolates solubilized tri-calcium phosphate as indicated by the production of clearance zone around the bacterial colony on Pikovskaya's agar medium plates (Table 2). Solubilization of tri-calcium phosphate requires either acid production or chelate formation by the bacterium in the medium. Probably other isolates did not produce acid insufficient amount or chelate to solubilize tri-

calcium phosphate in the medium (Figure 2). Several studies showed that PGP bacteria were responsible for solubilizing the insoluble P. It was also reported that excretion of organic acids was one of the most important factors in phosphate solubilization (Hemambika et al., 2013; Alori et al., 2017; Pérez-Rodriguez et al., 2020).

Production of Indole Acetic Acid: Out of 57 bacterial isolates 27 produced IAA from tryptophan (Table 2). These broth cultures containing tryptophan showed red colouration on the addition of Salkowski reagent. Indole acetic acid production is characteristic of the production of plant growth promoters.Bacterial IAA contributes to the growth of the lateral and adventitious root lead and triggers the bacterial proliferation of roots by exuding the root in order to increase their absorption of minerals and nutrients (Glick, 2010). In previous studies it has been indicated that IAA-producing rhizobacteria could be harnessed to improve plant growth (Das et al., 2019; Lebrazi et al., 2020).

Table 3. List of bacterial isolates which showed plant promoting properties nitrogen fixation, phosphate solubilization & indole acetic acid production.

Sr.	Accession no.	Nitrogen fixation	Phosphate Solubilization	Indole Acetic Acid	Sr.	Accession no.	Nitrogen fixation	Phosphate Solubilization	Indole Acetic Acid
1	GFS01C1	Positive	Positive	Positive	13	GFS16C2	Positive	Positive	Positive
2	GFS03C1	Positive	Positive	Positive	14	GFS19C2	Positive	Positive	Positive
3	GFS04C1	Positive	Positive	Positive	15	SCS01C1	Positive	Positive	Positive
4	GFS05C2	Positive	Positive	Positive	16	SCS03C1	Positive	Positive	Positive
5	GFS07C1	Positive	Positive	Positive	17	SCS03C2	Positive	Positive	Positive
6	GFS08C1	Positive	Positive	Positive	18	SCS04C1	Positive	Positive	Positive
7	GFS10C1	Positive	Positive	Positive	19	SCS07C2	Positive	Positive	Positive
8	GFS11C1	Positive	Positive	Positive	20	SCS07C3	Positive	Positive	Positive
9	GFS12C1	Positive	Positive	Positive	21	SCS12C1	Positive	Positive	Positive
10	GFS13C1	Positive	Positive	Positive	22	SCS12C2	Positive	Positive	Positive
11	GFS15C2	Positive	Positive	Positive	23	SCS12C5	Positive	Positive	Positive
12	GFS16C1	Positive	Positive	Positive					

Biochemical tests: All the 57 isolates were able to produce ammonia, oxidase and catalase. Isolates tabulated in the Table 3 can act as potential plant growth promoting bacteria. Of the 23 isolates tabulated in Table 3, 14 are from forest region and 9 were from coastal region of the Saurashtra region. These isolates have potential for biofertilizers which can be useful in agricultural practices.

CONCLUSION

In conclusion the present study attempt was made to isolate plant growth promoting bacteria which could be harnessed to improve plant growth. Nitrogen fixing bacteria, and phosphate solubilizing bacteria and Indole acetic acid producing bacteria were isolated. So it can be stated that presence of growth promoting bacteria are responsible for the beneficial effects on plant growth and they can be used as potential biofertilizers. However quantitative analysis of the above parameters can help us to better understand the efficiency of the bacterial isolates.

Conflict of Interest: The authors declare no conflict of interest among themselves.

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Assessment of Knowledge and Practice of Menstrual Hygiene Among Adolescent Girls of Tamil Nadu State India

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ABSTRACT

The menstrual hygiene problem is inadequately understood and has not received adequate attention. The use of sanitary pads and genital washing are important practices for preserving menstrual hygiene. The goal of the study is to assess the knowledge and practice of menstrual hygiene management among the adolescent gilrs in Dharmapuri district of Tamilnadu. This is a cross-sectional study was administered with adolescent girls in Sri Vijay Vidyalaya Arts and Science College, Dharmapuri district, Tamil Nadu. This study was done among 200 adolescent girls under the age group of 19-24 years. A self-administered structured questionnaire was developed to obtain information from school students. Descriptive analysis was done to analyses the knowledge and practice among the adolescents on menstrual hygiene management. The results of that study revealed that, 53% and 41% of them were under the age group of 23-24 years in the control group and experimental group. The control group subjects secured 44% of them having poor knowledge (30-39) and 36% were having regular (40-49) nutritional knowledge and remaining 16% and 4% were very poor (20-29) and bad nutritional knowledge category. In experimental group, 24% of the adolescent was shifted to a good knowledge, 11% of them were under satisfactory (50-59), and 63% of them were in good knowledge and had a positive attitude towards menstrual hygiene management related issues. From this study it was concluded that, knowledge of menstrual hygiene management among adolescents is very fair, still attitude and practice has to be improve and need a healthy awareness campaigns to improve behavior alongside regular enhancement of school health education schemes.

KEY WORDS: KNOWLEDGE, ATTITUDE, PRACTICE, MENSTRUAL HYGIENE, MANAGEMENT

INTRODUCTION

Adolescence has been described as a period between 10-19 years by the WHO (WHO, 1997). The word adolescence comes from the term "to grow to maturity" in Latin. By 2025, the teenage population in developed and developing countries are going to be about 19% and 27%, respectively (Bansal and Mehra, 1998; Kulkamai and Baride, 2002). Adolescent girls constitute not only a vulnerable group in terms of their social standing, but also in terms of their health. In this respect, in society, menstruation is considered impure or filthy (Dasgupta and Sarkar, 2008). Consistent with the UNICEF (The Status of World's Children 2011) survey, there are an approximate 1.2 billion teenagers within the world aged 10-19 years, making up 18% of the world's population, and 88% of them sleep in developing countries. In Haryana, teenagers structure 21% of the entire population (Census 2011).

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Adolescence accounts for 20% of the nation's population in India (UNICEF, 2011). Hygiene-related practices of girls during menstruation are of considerable importance, because it features a health impact in terms of increased vulnerability to reproductive tract infections (RTI). The interplay of socio-economic status, menstrual hygiene practices, and RTI are noticeable. Today many women are sufferers of RTI and its complications and sometimes the infection is transmitted to the offspring of the pregnant mother (Dasgupta and Sarkar, 2008 Sharma 2017 ICDS 2019).

There are over 355 million menstruating women and girls in India, but many girls across the globe still face major problems with a relaxed and dignified menstrual hygiene management experience. In India, during their menstrual cycle, about 88 percent of girls use homemade items (e.g., old cloth or rags). The key reasons for using the cloth-based product are personal preference and familiarity, lack of approach to or affordability for good-quality commercial sanitary pads, and lack of sufficient knowledge about pads. A locally produced cotton fabric is additionally employed by some girls. The incidence of reproductive tract infection (RTI) was 70% more prevalent among women and girls if hygienic sanitary practices weren't practiced during menstruation (Garg et al., 2012). Menstrual hygiene is an issue that every girl has to deal with in her life, but there is lack of awareness on the process of menstruation, the physical and psychological changes associated with puberty and proper requirement for managing menstruation. The taboos surrounding this issue in the Indian society prevent girls and women from articulating their menstrual needs. The problems of poor menstrual hygiene management have been ignored or misunderstood by the society as well the policy makers till now (Juyal et al., 2012, ICDS 2019).

Adolescence is that the most vulnerable period within the human life cycle after puberty, marked by rapid development and growth with a transition from infancy to maturity. The teenage term is taken from the Latin word 'adolescence,' aiming to mature into adulthood (ICDS, 2012). For girls, menstruation may be a physiological process which begins in puberty and is unprecedented for ladies . it's periodic discharge of blood and mucosal tissue from the uterus for 4-5 days (average) occurs regularly every 28-30 days of the cycle (Roy et al., 2014). Women with improved menstrual hygiene skills and good practices are less prone to reproductive tract infections and their effects. Increased awareness of menstruation right from childhood will also escalate healthy practices and can also alleviate the misery of millions of women. The social stigma attached to menstruation causes dangerous grooming activities to be carried out by many girls and women.

Girls and women frequently suffer from discomfort and infection, avoiding urination during menstruation, and using any kind of cloth available old (or) unwashed as an, but still girls do not visit medical practitioners, lacking a forum to share menstrual hygiene problems. Hence, the present study was planned to assess the menstrual hygiene knowledge and self-care practice among the adolescent gilrs and to seek out the impact of knowledge and practice among the adolescents (Roy et al., 2014 Sharma 2017).

MATERIAL AND METHODS

In phase I clinical trial a quantitative research approach was adopted for the study. The research design chosen for this study was a descriptive cross-sectional research design. The population chosen for this study was, adolescent girls studying in Sri Vijay Vidyalaya Arts and Science College, Dharmapuri district, Tamil Nadu. Adolescent girls who fulfill the sampling criteria were included within the study. The sample size for the study was 200 (control group n=100; experimental group n=100). The technique adopted for this study was the straightforward sampling technique. An in depth interview schedule was developed by the investigator to elicit information about the socio-economic status, History of menstruation deals with menarche, menorrhea and menstrual duration, health status and private habits of the chosen subjects. Before starting the study, a pilot was adopted among 10 percent (n=10) of the entire subjects to seek out validation within the formulated interview schedule. In phase II clinical trial , nutrition education was given to the chosen adolescent groups through PowerPoint presentation and therefore the knowledge and practice were evaluated using the questionnaire. A nutritional knowledge questionnaire was formulated which consisted of 10 questions of multiple-choice and 10 nutritional practice questions that were developed to seek out the menstrual hygiene knowledge and practice between both the control group and therefore the experimental group.

Within the control group, 100 adolescent subjects were chosen and there was no intervention was adopted during this group. During this experimental group, 100 adolescent subjects were grouped during a big hall. Before starting the assessment of data pretest was assessed using the structured questionnaire. the themes were projected with the Power Point presentation about menstrual hygiene and self-care materials. Posttest about menstrual hygiene and self-care was assessed after one week through an equivalent questionnaire. The collected questionnaire was validated and every correct answer is scored and therefore the total score obtained by each subject is noted right down to test their pre and post nutritional knowledge. The difference between the initial and final scores was assessed to seek out the impact of nutrition education. Questions were scored as followed 1 mark for the right answer and 0 marks for wrong or no answer. the entire score of every aspect equal 60% or Quite → Adequate or satisfactory knowledge and practice). the entire of every aspect but 60% → inadequate e or unsatisfactory knowledge and practice.

Table 1. Socioeconomic status of the selected subjects

Socioeconomic details	Control group (n=100)		Experimental group (n=100)	
	Number	%	Number	%
Age (years)				
19-20	22	22	28	28
21-22	25	25	31	31
23-24	53	53	41	41
Ethnic				
OC \FC	10	10	21	21
BC	45	45	40	40
MBC	35	35	35	35
SC\ST	10	10	14	14
Religion				
Hindu	68	68	59	59
Christian	24	24	24	24
Muslim	8	8	17	17
Others	-	-	-	-
Educational qualification of parents				
Elementary school	10	10	-	-
High school	55	55	74	74
College	35	35	26	26
Illiterate	-	-	-	-
Employment				
Sedentary	49	49	34	34
Moderate	35	35	47	47
Heavy	16	16	19	19

RESULTS AND DISCUSSION

The results and discussion of this study was discussed below with the relevant tables, which describes the datas in details.

Socioeconomic status of the selected subjects:

Data about the study revealed that 53 percent and 41percent of them were under the age group of 23-24 years in the control group and experimental group. Nearly 22percent and 28percent of them were belonged to 19-20 years of age. In our society menstruation is considered as very personal and private matter of discussion. In the present study most of the girls (80.82%) had attained menarche between 13-15 years of age. Other studies by Sharma (2017) showed comparative findings where 57.35 percent of girls had menarche between the ages of 13-15 years. Majority of 40% of the selected subjects were under backward ethnic group. The religion said that most (above 50%) of the subjects were Hindu. About 35 percent of them under MBC and 45 percent of the girls belonged to BC in the control group. In the experimental group, 40percent of them belonged to the BC category, and 14percent were Sc/ST group (Drakshayani et.al., 1994).

The majority of 68percent of them were Hindu and only 8percent of them were Muslim in the control group and the experimental group 59percent them and 17percent of them were Hindu and Muslim respectively. 10 percent did their elementary level, 55 percent had studied up to high school and 35 percent of them were graduates in the control group. Whereas in the experimental group, 74 percent of them did their high school level education and 26 percent of them were finished their college studies. 49percent were as sedentary workers, 35percent were as moderate workers and 16 were as heavy workers in the control group and 47percent of them were moderate worker and 19percent of them were a heavy worker in the experimental group. Menstruation-related information and activities are often based on socio-economic circumstances (Drakshayani et.al., 1994).

Percentage of distribution of menstruation and menstrual hygiene knowledge among study participants:

About 81 percent (control) and 50 percent (experimental) of participants were able to answer that Menstruation was a physiological process and 44 percent and 52 percent of girls were knowing about dysmenorrheal. Dysmenorrhea is one of the most common complaints and gynecological problems among worldwide women (George and Bhaduri, 2002; Harel, 2006 and Agarwal and Agarwal, 2010). Among the study participants, 48 percent and 755 answered the definition of menstruation. About 80 percent of girls able to open up their time of ovulation. In the control group, 55 percent of the girls do not have signs and symptoms of menstruation. In the experimental group, 77 percent of them know the causes of menstruation. Almost 70 percent of the girls knew about the menstrual flow organ. About 67 percent and 82 percent of the group girls knew that poor menstrual hygiene practices lead to infections. Almost70 percent of girls were aware that menstruation indicates fertility (Sapkota et al., 2014).

The knowledge score was demarcated into Poor, Average, and Good and is given in the table. The pre and post-test Knowledge Mean initial scores were 15, 20, 8, and 18 for poor scores, 75, 70, 32, and 67 for average scores and 10, 10, 60, and 15 were good scores of both groups. In the present study 39 (23.64%) of the adolescent girls were found to be absent from school during their menses in comparison to a study done by Kumar et al where 42.8% adolescent girls were absent from school during menses. Regarding the percentage of distribution of menstruation and menstrual hygiene knowledge, 48 percent and 75% were answered the definition of menstruation. Nearly about 67% and 82% of girls knew that poor menstrual hygiene practices lead to infections. There is limited knowledge and many misconceptions about menstruation among young women in India before and even after the menarche. This usually leads to undue fear, anxiety, and undesirable practices (Mahon and Fernandes, 2010). Juyal et al., (2012) and Sapkota et al.,

(2014) stated in their study that 83% of the respondent had the idea that menstruation is a physiological process, which is significantly higher than findings (Juyal et al., 2012; Sapkota et al., 2014).

Table 2. Percentage of distribution of menstruation and menstrual hygiene knowledge among study participants

S. No	Questions	Control group (n=100)		Experimental group (n=100)	
		Pre test (%)	Post test (%)	Pre test (%)	Post test (%)
1.	Menstruation is a regular process				
	Physiological process	81	81	50	60
	Disease	2	2	31	10
	Sin	10	10	2	18
	Do not know	7	7	17	12
2.	Definition of dysmenorrheal				
	Painful	34	34	47	50
	No periods	44	44	52	42
	Do not know	22	22	1	8
3.	Definition of menstruation disorder				
	No periods	30	30	14	15
	Normal menstrual cycle	48	48	75	85
	Do not know	22	22	11	0
3.	Time of ovulation				
	Yes	85	85	89	92
	No	15	15	11	8
4.	Signs and symptoms before or during menses				
	Yes	45	45	62	84
	No	55	55	38	16
5.	What is the cause of menstruation?				
	Hormones	50	50	77	67
	Diseases	1	1	24	33
	Do not know	39	39	0	0
6.	From where does the menstrual blood flow?				
	Uterus	85	85	73	85
	Vagina	15	15	27	10
	Abdomen	0	0	0	5
	Do not know	0	0	0	0
7.	Do food habits affect menstrual cycle?				
	Yes	67	67	82	88
	No	23	23	18	12
8.	Have you heard about menstrual hygiene?				
	Yes	72	72	69	72
	No	28	28	31	28
9.	Do poor menstrual hygiene practices lead to infections?				
	Yes	67	67	82	88
	No	23	23	18	12
10.	Menstruation indicates fertility				
	Yes	79	79	88	94
	No	21	21	12	6

From the above table -3 it was found that about 76 percent and 86 percent of the participants in all two groups use a sanitary napkin during menstruation. Regarding several pads per day, 80 percent of participants answered that they change it more than 3 times in a day. They were asked whether they change pad before sleep for which 60 percent of participants responded yes. Burying, burning, disposing of in waste bin after proper wrapping was considered to be fair practice and 83.8 percent girls were practicing it. About 80 percent of participants of both groups were cleaning their genitalia regularly.

Table 3. Percentage distribution of menstruation and menstrual hygiene practice among subjects

S,no	Questions	Control group (n=100)		Experimental group (n=100)	
		Pretest (%)	Posttest (%)	Pretest (%)	Posttest (%)
1.	What absorbent do you use during menstruation?				
	Sanitary pad	76	76	85	92
	New cloths	24	24	15	8
	Old cloths	0	0	0	0
2.	How many times do you change pad/cloths per day?				
	One time	67	67	82	52
	Two time	23	23	18	20
	Three time	0	0	0	28
	Four time	0	0	0	0
3.	Do you change pad/cloth before sleep?				
	Yes	72	72	69	80
	No	28	28	31	20
4.	If you are using cloth or absorbent (re-usable), How do you dry it?				
	Outside room in sunlight	-	-	-	9
	Inside room with sunlight	67	67	82	74
	Without sun light	23	23	18	17
	Not using reusable absorbent	0	0	0	0
5.	Type of pads used				
	Piece of clothes	0	0	0	0
	Piece of new cloths	8	8	2	0
	Piece of cotton	0	0	0	2
	Sanitary pad	92	92	98	98
6.	Number of pads per day				
	Single per day	85	85	89	50
	Twice per day	15	15	11	50
	Thrice per day	0	0	0	0
	Four or more per day	0	0	0	0
7.	How do you dispose your sanitary pads?				
	Buried	30	30	14	30
	Burned	48	48	75	65
	Dustbin	22	22	11	5
	Latrine	0	0	0	0
	Throw on road	0	0	0	0
8.	When do you clean your genitalia?				
	Every time use toilet	85	85	89	85
	During bathing	15	15	11	15
	Do not clean regularly	0	0	0	0
9.	Material used for cleaning of external genitalia				
	Water and antiseptic	22	22	15	10
	Soap and water	48	48	75	80
	Only water	30	30	10	10
	Not cleaning regularly	0	0	0	0
10.	Do you practice any restriction during menstruation				
	Yes	85	85	89	92
	No	15	15	11	8

A study conducted by Dasgupta and Sankar (2008) in which just 48.75% knew the use of a sanitary pad. Mudey et.al., (2010) stated that poor genital hygiene negatively afects adolescents' health. Most girls are unaware and unprepared for menarche as they are not informed or ill-informed about menstruation. This increment in knowledge indicates the exposure and readiness of school adolescents to adopt hygiene behavior. Though the majority of students know about menstruation which might be attributed to the inclusion of reproductive health education in school curricula and exposure to a wide range of information media like television, radio, internet; still misperceptions persist in this matter (Dasgupta and Sankar 2008; Mudey et.al., 2010).

The results of the 't' value of the control group were 0.44 which is more than the 5% level of significance. The two-tailed p-value was 0.76 which means that the mean nutritional knowledge was increased (2.28) which was not significant at a 5% level (Lawan et al., 2010).

The results of the 't' value of the experimental group were 47.2 which is less than a 1% level of significance. The two-tailed p-value was 0.00 which means the mean final nutritional knowledge was increased (2.87) which was significant at a 1% level. Dasgupta and Sankar (2008) pointed out that the increased awareness and knowledge about menstruation from the early days is most likely to inculcate healthy practices and help in lowering the sufferings of millions of women. Results of the 't' value of the control group were 12.4 which is more than the 5% or 1% level of significance. The two-tailed p-values were 0.81 which means the mean final attitude was increased (0.44) which is not significant at 5% or 1% level. The results of the 't' value of the experimental group were 1.04 which is less than a 1% level of significance (Lawan et al., 2010).

Table 4. Mean comparison of the control group and experimental group regarding menstrual hygiene knowledge and practice

Aspects studied	Total scores	Groups	Pretest Mean ± SD	Post-test Mean ± SD	't' value	Significance
Nutritional knowledge	10	Control Group	4.48 ± 1.26	6.76±4.26	0.44	0.76NS
		Experimental Group	5.36± 2.32	8.23±1.92	47.2	0.00**
Practice	10	Control Group	3.51±0.95	3.95±0.69	12.4	0.81NS
		Experimental Group	4.35±0.91	7.52±0.71	1.04	0.02**

*- significant at 5% level, ** -significant at 1% level; NS -not significant

Table 5. Nutrition knowledge of adolescents based on Z scores

Standard Mean scores*	Control group (N=100)		Experimental group I (N=100)	
	Pre test	Post Test	Pre test	Post test
Excellent >80	-	-	-	-
Very good 70-79	-	-	-	2
Good 60-69	-	-	-	63
Satisfactory 50-59	-	-	-	11
Regular 40-49	-	45	-	24
Poor 30-39	-	15	-	-
Very poor 20-29	56	20	62	-
Bad	44	20	38	-

The two-tailed p-value was 0.02 which means the mean final attitude was increased (3.71) which was significant at a 1% level. The promotion of adolescent sexual and reproductive health and the prevention of diseases are among the key reasons for menstrual hygiene. Our study found that the majority of adolescent girls used sanitary pads (commercial or reusable) during their menstruation. This is similar to reports from Lawan and colleagues from Nigeria but in contrast to the study conducted in India and Adinma's study where the majority was found to be using toilet rolls to manage menstrual blood (Lawan et al., 2010). Ciccone et al. (2010) study has clearly demonstrated that educating the subject on health and management will have greater impact in reducing the burden of risk. The outcome of the work warrants a strong partnership between the care manager and the subject and collaboration between the physician and the care manager in the health management. Our

results are in agreement with the above study (Ciccone et al., 2010).

Nutrition knowledge of adolescents based on Z scores:

Results of the Z score of adolescents reported that in the control group initially 44 percent of them were in bad nutritional knowledge category and 56 percent came under very poor (20-29) nutrition knowledge and after one week. 44 percent of the subjects were shifted to poor category (30-39) and 36 percent were regular (40-49) nutritional knowledge and remaining 16 percent and 4 percent were very poor (20-29) and bad nutritional knowledge category respectively. Results of the experimental group indicated that initially 44% of the subjects were in the bad nutritional knowledge category and 56 percent were in very poor (20-29) nutritional knowledge after nutrition education 24 percent of the adolescent were shifted to a regular category, 11 percent to satisfactory (50-59) and 63% of them were in a good category. Following the findings from our study, 55.4% believed menstruating females should not consume poultry and sour food items. Sapkota et al., (2014) done his finding in rural Nepal regarding food taboos and this was agreed. Despite the expansion of the knowledge horizon, cultural taboos in society prevent a shift of attitude; thus, practice among adolescents on menstrual hygiene management. In the name of history, this case shows the desperate need to counter harmful practices (Sapkota et al., 2014).

CONCLUSION

Nearly three forth of the participants had good knowledge of menstruation and menstrual hygiene but they were not following due to the taboos. The practice of menstrual hygiene was satisfactory (80%). In conclusion, although adolescents' attitude toward menstruation was relatively positive; they mostly had poor knowledge about menstrual hygiene; consequently, a poor practice was expected. Also, the results indicated that students' mothers were the main source of their information on mensuration. It is very important to become aware of the need for knowledge about good menstrual practices. Health data on menstrual hygiene should also be stressed by the mass media. Policymakers and stakeholders should also set up a health education campaign to raise knowledge of good menstrual hygiene and practice. Hence, awareness through the change in curriculum and more friendly relationship between the students and the teachers will contribute significantly to the improvement in the status of menstrual hygiene and overall health of the adolescent.

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Effect of Punicalagin on Antioxidant Enzymes on Colon Cancer Cells Stimulated with T-BOOH

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ABSTRACT

Stress can initiate by many factors incorporated in the daily life such as long-term health issues, immune system suppression and obesity. Free radicals as well as endogenous antioxidants are in balance as part of body's natural functioning. Oxidative stress occurs when endogenous defence system not capable to maintain the balance, therefore natural antioxidant was in-need to help the endogenous antioxidant in scavenging the excess of free radicals. Punicalagin is a natural antioxidant compound that extracted from pomegranate husk. Punicalagin affect the activities of endogenous antioxidant enzymes which was investigated by measuring the level of catalase, superoxide dismutase (SOD), glutathione peroxidase and reductase of Caco-2 cells under stress conditions. Caco-2 cells were exposed to 3 mMtert-butyl hydroperoxide (T-BOOH) for 2 hours to initiate oxidative stress when pretreated with 5 and 10 µM punicalagin for 24 hours. Both doses (5 µM and 10 µM) of punicalagin significantly elevated the level of catalase (15.05 U/ml; $p < 0.05$ and 20.95 U/ml; $p < 0.001$, respectively) compared to cells treated with only T-BOOH (11.79). On the other hand, punicalagin had no effect SOD, glutathione peroxidase and glutathione reductase. In conclusion, under stress conditions punicalagin significantly enhanced endogenous antioxidant enzymes of Caco-2 cells.

KEY WORDS: PUNICALAGIN, OXIDATIVE STRESS, GLUTATHIONE, SUPEROXIDE DISMUTASE, REACTIVE OXYGEN SPECIES.

INTRODUCTION

Free radicals in living cells are naturally produced as a result of cell metabolism and detoxification process (Halliwel and Gutteridge., 2015). Oxidative stress occurs as consequences of imbalance between antioxidant capacity in biological system and the excess of free radicals initiated in the system. Excessive radical species production causes cell injury by reacting with lipid membrane, cell proteins and DNA (Ahmad et al., 2019). Attacking of biological molecules with excessive radicals leading to pathogenesis and many diseases that include cancers and aging disease ((Valko et al., 2004, Liguori et al., 2018).

Cellular antioxidant system, including antioxidant enzymes are capable to diminish the harmful effect of free radicals and hence protecting cells from being damage (Ray et al., 2012). Some of the important endogenous cellular antioxidant enzymes are catalase, superoxide dismutase (SOD), glutathione peroxidase and glutathione reductase. Superoxide dismutase is one of the endogenous antioxidant enzymes that protecting cells from oxidative stress by catalysing the decomposition of superoxide radicals (O_2^-) to oxygen (O_2) and (H_2O_2). Both of catalase and glutathione peroxidase are protecting cells from oxidative damage by catalysing the decomposition of harmful hydrogen peroxide (H_2O_2) to safe products as water and oxygen. Moreover, Glutathione reductase is one of the important antioxidant defence enzymes at cellular

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levelas it is responsible for maintaining the reducing environment of the cell by optimizing the concentration level of reduced glutathione (GSH) in cellular system (Aguilar et al., 2016).Under certain conditions, excess reactive species formation overwhelms the oxidant/ antioxidant balance despite the presence of cellular antioxidant defense system (Georgieva et al., 2017).

Therefore, scientific researches have been focused heavily on finding natural antioxidants that can protect biological system from free radical-mediated oxidative stress and enhance the action of endogenous antioxidant system to overcome the side effect of excessive free radicals (Jamshidi-Kia et al., 2020). Some plant polyphenol has been suggested to have antioxidant effect by upregulating intracellular defence systems or/and intercepting free radical formation (Losada-Barreiro et al., 2017). Polyphenols have also shown ability to diminish the decrease of GSH levels that induced by T-BOOH (Lima et al., 2006, Cianfruglia et al., 2020). One of the plant polyphenol naturaly rich with antioxidant compounds is pomegranatefruit.The high antioxidant properties of pomegranate are due to the presence of punicalagin isomers which form when both gallic and ellagic acid link to glucose molecules, and hydrolysable tannins (Gil et al., 2000). Punicalagin is a yellowwater-soluble compound that is mainly present in the pomegranate husk. Omar et al., (2016) stated that punicalagin from pomegranate has regulating effect on total glutathione in Caco-2 cell stressed by T-BOOH.

The current study has been carried out on human intestinal Caco-2 cell line which displays morphological and physiological characteristics that are similar to intestinal epithelial cells when differentiated (Meunier et al., 1995). T-BOOH was also used to generate oxidative stress that results in cell injury (Lashpina et al., 2005). Once T-BOOH gets penetrated in the cell membrane and is easily decomposed to produce alkoxyl and peroxyl radicals which subsequently continue to generate reactive species (Kim et al., 2013). Prevention or delaying the adverse effect of excess ROS on cells is important to protect cellular membrane and its content (Cianfruglia et al., 2020). Therefore, the aim of this study was to investigate the effect of punicalaginas a natural plant derived antioxidant on the activity of catalase, SOD, glutathione peroxidase and glutathione reductase using stressed Caco-2 cells as an epithelial model.

MATERIAL AND METHODS

Cell culture and treatment: Human colon epithelial cells (Caco-2) was obtained from European Collection of Cell Cultures (ECACC, UK). Cells were stressed for 2 hours with 3 mM tert-butyl hydroperoxide (T-BOOH) and treated with 5 and 10 µM Punicalagin according to (Omar et al., 2016).

Preparation of cell lysate: Cells were collected in 15 ml centrifuge tubes and centrifuged for 3 minutes at 150 x g. The supernatant was decanted and cell pellets were lysed using 300 µl lysis buffer (50 mM Tris HCl, 1% Nonidet P40 (NP-40), 150 mM NaCl, 0.2% SDS solution, 1 µg/ml Aprotinin, 20 µM PMSF, 1 µg/ml Leupeptin, and 1mM Na3VO4). Cells were then kept in ice for 20 minutes and stored at – 80°C for further use in the following experiments.

Catalase: Catalase level was measured in Caco-2 cell lysate using the OxiSelectTM Catalase activity Assay Colorimetric Kit (catalog number STA-341, Cell Biolabs, INC., Cambridge, UK) according to the manufacturer's instructions.

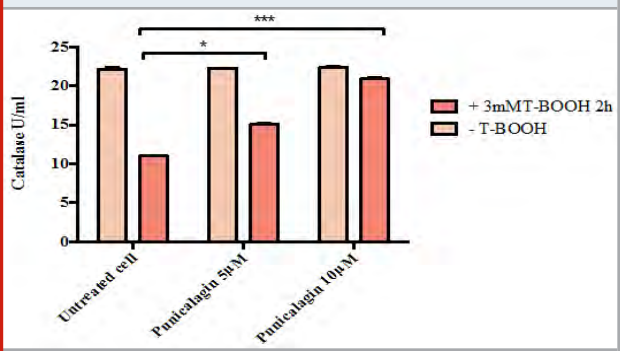
Superoxide Dismutase assay: Superoxide dismutase (SOD) activity was measured using the SOD Activity Assay Colorimetric Kit (catalog number ab65354, Abcam®, Cambridge, UK) according to the manufacturer's instructions.

Glutathione Peroxidase: Glutathione peroxidase was measured quantitatively using Glutathione PeroxidaseAssay Kit (catalog number 703102, Cayman chemical, Michigan, USA) according to the manufacturer's instructions.

Glutathione Reductase: Glutathione reductase was measured quantitatively using OxiSelectTM Glutathione Reductase Assay Kit (catalog number STA-812, Cell Biolabs, INC., Cambridge, UK) according to the manufacturer's instructions.

Statistical Analysis: Results were analyzed using GraphPad Prism software version 6.0. Data are presented as the mean (±SD). ANOVA was performed in Graphpad Prism version 6.0, followed by Bonferroni's multiple comparisons test versus treated cells with T-BOOH alone * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Figure 1: Effect of Punicalagin onlevel of catalase in Caco-2 cells. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0. followed by Bonferroni's multiple comparisons test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ versus treated cells with T-BOOH alone



RESULTS AND DISCUSSION

Figure 1 shows that there was a significant depletion of catalase level in cells treated with T-BOOH (11.29 U/ml) as compared with untreated cells (22.42 U/ml;

p <0.001). Catalase level was significantly elevated in stressed cells pretreated with 5 µM punicalagin (15.05 U/ml) in comparison to cells treated with T-BOOH alone (p <0.05). Cells treated with 10 µM punicalagin prior to oxidative induction exhibited significantly higher catalase level (20.95 U/ml) when compared with cells treated with t-BHP (p < 0.001).

Figure 2 illustrates that there was no significant difference in the percentage of SOD activity between untreated cells (53.19 %),cells treated with T-BOOH alone (49.92 %), cells treated with 5 uMpunicalagin prior to oxidative induction (47.22 %) and cells pretreated with 10 µM punicalagin prior to oxidative conduction (45.86 %).

Figure 2: Effect of Punicalagin on the percentage of superoxide dismutase (SOD) activity in Caco-2 cells. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0. followed by Bonferroni's multiple comparisons test.

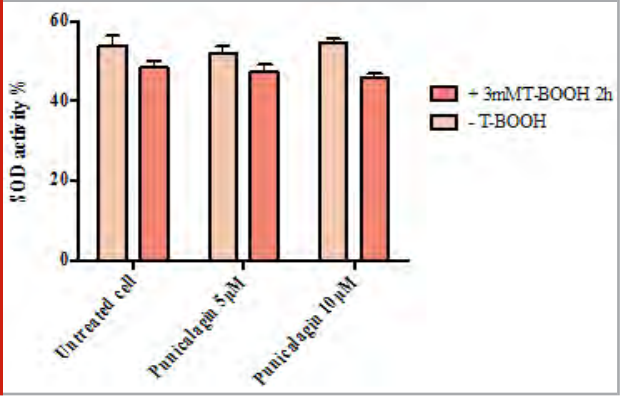


Figure 3 shows that there was no significant difference in the concentration of glutathione peroxidase between untreated cells (0.68), cells treated with T-BOOH alone (0.74), cells treated with 5 µM punicalagin prior to their exposure to T-BOOH (0.71) and cells pretreated with 10 µM punicalagin prior to oxidative conduction (0.70).

Figure 3: Effect of Punicalagin on the amount of glutathione peroxidase in Caco-2 cells. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0. followed by Bonferroni's multiple comparisons test.

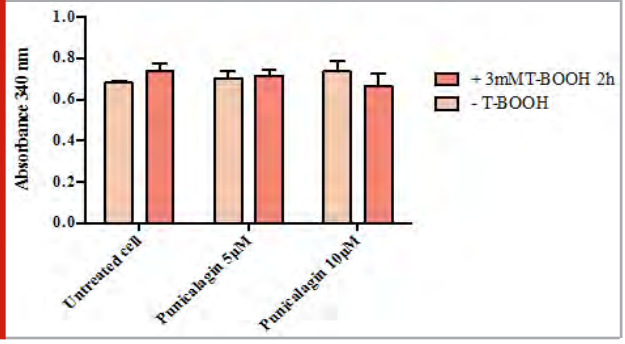
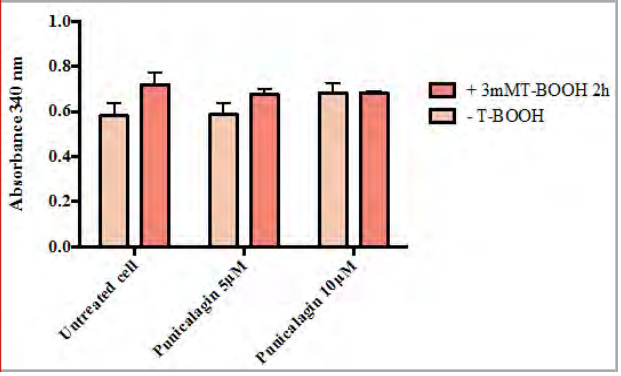


Figure 4 shows that there was no significant difference in the concentration of glutathione reductase between untreated cells (0.62), cells treated with T-BOOH alone (0.72), cells reated with 5 µM punicalagin prior to their exposure to T-BOOH (0.68) andcells pretreated with 10 µM punicalagin prior to oxidative conduction (0.68).

Figure 4: Effect of Punicalagin on the amount of glutathione reductase in Caco-2 cells. Data correspond to the means ± SD of three independent experiments. ANOVA was performed in Graphpad Prism version 6.0. followed by Bonferroni's multiple comparisons test.



Natural antioxidants play a crucial role in helping the endogenous antioxidants in scavenging the excess of free radicals (Breinholt, 1999; Duthie et al., 2000; Ro 'hrdanz et al., 2002; Alia et al., 2006, Yusof et al., 2005). Scientific research considering the effect of punicalagin on the activity of antioxidant enzymes in stressed culture cells is scarce. Therefore, this study is considered first to none in measuring the effect of punicalagin in very small doses (5 and 10 µM) on the activity of four main endogenous antioxidant enzymes. The activity of Punicalagin as an antioxidant was previously highlighted by Omar et al., (2016). Punicalagin at low concentrations (5 and 10 µM) succeeded to protect Caco-2 cells against cell death which exposure to stress using 3 µM T-BOOH. The protection offered by this phenolic compound highlighted by scavenging cellular ROS, preventing lipid oxidation and prohibiting GSH depletion (Omar et al., 2016).

These findings led to further examination of other cellular antioxidant enzymes as catalase, SOD, glutathione peroxidase and glutathione reductase in Caco-2 cells using the same conditions. In a healthy system, cellular antioxidative enzymes can protect cells either their membranes or their cellular content by scavenging free radicals. However, under certain conditions excess radical formation overwhelms the antioxidant/ prooxidant balance and then weakens the antioxidant defence system (Cimen, 2008).Catalase is one of the main endogenous antioxidant enzymes that responsible for the dismutation of hydrogen peroxide to safe products such as water and oxygen (Aguilar et al., 2016).

In this study, cells treated with either 5 or 10 µM punicalagin prior to oxidative induction, significantly elevated catalase level to (15.05 U/ml, p<0.05 and

20.98 U/ml, p< 0.001; respectively) as compared with cells treated with T-BOOH alone. This result illustrated the effect of punicalagin on enhancing the activity of Catalase under oxidative stress. In contrast, there was no significant difference in SOD activity between pre-treated stressed cells and untreated stressed cells.Catalase and SOD results might be explained by the effect of stressed condition on the activity of each enzyme. Researcher suggested that 5×10^{-2} mM T-BOOH organic peroxides can activate catalase enzyme while inactivate SOD, although both enzymes undergo the same stressed condition (Pigeolet et al., 1990). Many studies confirmed that some natural antioxidants either derived from plant or animal source able to enhance the endogenous defence system by increase the enzyme activity level or its gene expression.

Recently Talaei et al., (2020) have suggested that quercetin either alone or with exercise significantly elevated the level of catalase in the heart tissue of rats stressed with 15 mg/kg Azoxymethane in comparison with saline group (p< 0.0001). Another study found significant increase in the expression of catalase and SOD genes was found in HepG2 cells treated with peptide isolated camel milk (Homayouni-Tabrizi et al., 2017). Maalej et al. (2017) suggested that ethanolic olive fruit extract and its phenolic compound exhibited hepatic and renal protection against the toxicity induced by a synthetic pyrethroid (deltamethrin) on Wistar rats. This protection was highlighted by improving the activities of catalase and SOD. Another study revealed that ginger extract (Zingiber officinale) has a possible chemoprotective effect by enhancing the activity of catalase, SOD and glutathione peroxidase in HepG2 cell line (Seyidoglu and Aydin 2020).

Glutathione peroxidase is another enzyme regulating the presence of free radicals in human body. This enzyme may exist in tow forms: selenium-dependent and selenium-independent Glutathione peroxidase reduce hydrogen peroxides or any organic peroxides to water (Maiorino et al., 1995), in the presence of reduced glutathione (GSH) which is converted into oxidised glutathione (GSSG). It is well known that glutathione peroxidase that there is a competition between this enzyme and catalase for scavenging the hydrogen peroxide (Aguilar et al., 2016). In the current study, although the significant effect of punicalagin on catalase activity, there is no significant difference between the glutathione peroxidase level between pre-treated stressed cells and untreated one. This outcome might be affected by the dose of T-BOOH that used in the current study and/ or incubation time that used for stress. Pigeolet et al., (990) stated that 5×10^{-2} mM T-BOOH inactivated glutathione peroxidase by 50% when incubated for 11 minutes at 37 °C.

Glutathione reductase enzyme is responsible for converting GSSG to GSH which is an important molecule in resisting oxidation stress (Duthie et al., 2000). Under oxidative stress, elevation of peroxideslevel leads to a shift in thiol redox status which defined by a severe reduction in the reduced form of glutathione (GSH) and rise in the level of the oxidised form (GSSG). Although Omar et al., 2016 stated that punicalagin as a radical scavenger could maintain the GSH level against oxidative stress occur due to T-BOOH in caco-2 cells, in the current study punicalagin did not exhibit any effect on glutathione reductase level and there was no significant difference between untreated stressed cells and treated one.

This result can also be linked to the dose of T-BOOH and its incubation time that used while stressing caco-2 cell. Organic peroxide might inactivate the enzyme activity as stated previously by Pigeolet et al. (1990). Endogenous antioxidants operate together under one system that complements its main constituents to maintain the redox balance in the body. Regulation the level of reactive oxygen species is the target of researcher but not diminished them completely as they may lead to degenerative diseases (Lobo et al., 2010). According to the current research, it is highly likely that punicalagin can enhance the endogenous antioxidant enzyme system by activating catalase enzyme which able to scavenge the excess hydrogen peroxide and hence protect cellular system from damage.

CONCLUSION

Punicalagin enhanced the activity of endogenous antioxidative enzymes causing protective effect to the oxidatively stressed Caco-2 cells. The protective effect of punicalagin was demonstrated by the significant elevation of Catalase level when the stressed cells either treated with 5 µM punicalagin (15.05 U/ml; p <0.05) or with 10 µM punicalagin (20.95 U/ml; p < 0.001) compared to cells treated with T-BOOH alone. Punicalagin has no effect on SOD, glutathione peroxidase and glutathione reductase levels. Highlighting the effect of Punicalagin on the activity of endogenous antioxidant enzymes in addition to previous findings on its antioxidant activity that conducted by the same researcher (Omar et al., 2016) might elucidate the actual mechanism of its protective action. These novel findings may offer punicalagin potential applications in the nutraceutical market.

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Modified Particle Swarm Optimization Method for Handling Renal Disease

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ABSTRACT

Renal disease is a common issue in everyone's life. The toxin produced in our body is deposited in the kidneys. Kidneys will maintain the fluid level of the body. Abnormal in the fluid level causes the renal disease. Pathologic proteinuria is the most common cause of membranous nephropathy (MN). The disease is very progressive. Blood pressure encompasses a dramatic effect on the speed at which the disease progresses. Even alight rise in pressure level can quickly make nephropathy worsen. Low salt intake will reduce the blood pressure. Healthcare industry along with the rigorous growth in computer field analyzes the disease and give a boon support to the medical field. Machine Learning Methods is one of the smart manifestation practical significance for medicine. It is often accustomed classify various objects supported a series of coaching data whose result value is known. Classifiers methods have been used to identify the important attributes and uses different techniques to identify the disease. The classifier performance and also the length of selected feature subset was used as heuristic information for the proposed PSO-based method. The classifier performance and also the length of selected feature subset was adopted as heuristic information. The present work selected the best feature subset without any prior knowledge of features. Particle swarm optimization is a technique used for multidimensional space. In order to achieve good performance modified particle swarm optimization was introduced to achieve better algorithm overall performance. The proposed modified particle swarm optimization approach makes use of the class techniques Adaboost and KNN techniques for better handling the management of renal diseases.

KEY WORDS: MACHINE LEARNING, PARTICLE SWARM OPTIMIZATION, MODIFIED PARTICLE SWARM OPTIMIZATION.

INTRODUCTION

Our body has a wide range of cell types. Among the cell types most heterogeneous type of tissues are identified in the kidneys. Each area of the kidney contains a defined segment called the nephrons and portion of the collecting duct system. The filtering portion of the kidney is named as glomeruli and have a more complex structure comprising capillaries epithelium and intraglomerular mesangial cells. Endocrine functions play a vital role in kidney function. Abnormalities as a result of poisonous chemical compounds or other interventions may have profound outcomes on those functions and consequently, on overall capabilities (Padmavathi and Senthilkumar, 2020).

Membranous nephropathy (MN) is a continual sickness and its development embraces impulsive diminutions and recurrent deteriorations (Bomback et al., 2018). Nephrotic patients who do not know how to warmth into attenuation are susceptible to course to cease stage renal morbidity. People who are susceptible to high blood pressure, diabetes are more perspective to chronic kidney disease. As a symptom of membranous nephropathy, it

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triggers problem in breast for women and it is addressed that if any one of the diseases is recognized it leads to primary membranous nephropathy in lungs (Silva et al., 2018; Padmavathi and Senthilkumar 2020).

Diagnosing the disease plays an important role. Proteins with a molecular weight of much less than 20,000 skips easily throughout the glomerular capillary wall. Conversely, albumin, with a molecular weight of 65,000 Daltons and a negative charge, is confined under everyday conditions. The smaller proteins are in large part reabsorbed at the proximal tubule, and the best small amounts are excreted. Lack of protein, urine excretion greater than grams per day in 24 hours is an end result of glomerular disease. Shi and Eberhart (2001) states that the performance of the classification techniques is improved by using particle swarm optimization methods Particle Swarm Optimization (PSO) is a heuristic optimization approach displaying a relationship with evolutionary algorithms and strongly primarily based on the concept of the swarm. Normally the particle swarm optimization is used for the non-linear functions. The researchers use this particle swarm optimization method to get better performance (Shi and Eberhart, 2001; Silva et al., 2018).

PSO is based totally on the principle that every solution may be represented as a particle inside the swarm. every particle has a role inside the search space, which is represented by way of a vector xi = (xi1, x i2,..., xi D), where D is the dimensionality of the search space, debris pass within the seek space to look for the most suitable solutions therefore, each particle has a velocity, which is represented as vi = (vi1, v i2,...,vi D). At some point of the motion, each particle updates its function and speed in keeping with its very own enjoy and that of its associates. The quality preceding position of the particle is recorded as the personal satisfactory p high-quality, and the high-quality position obtained via the populace so far is known as g nice primarily based on p pleasant and g fine,

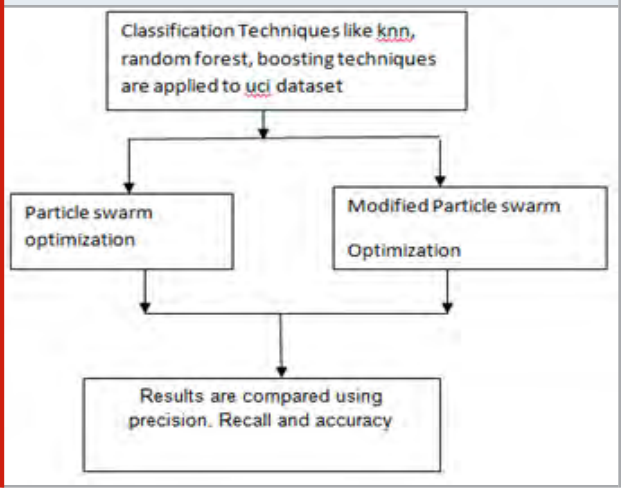
PSO searches for the most effective solutions through updating the velocity and the placement of every particle consistent with the subsequent equations. K-Nearest Neighbor, Random forest and boosting methods have been taken for the optimization techniques. The accuracy degree of every classifier has been calculated earlier (Silva et al., 2018; Padmavathi and Senthilkumar 2020). Then the modified particle swarm optimization has been used to get higher performance. Assessment of PLA2R autoimmunity is crucial for an affected man or woman management. The classifiers are applied to the AntiPLA2R dataset to enhance the overall performance. Normally, feature choice is a multi-goal problem. It has two foremost objectives, which can be to maximize the type overall performance (reduce the classification errors rate) and to reduce the variety of features (Padmavathi and Senthilkumar 2020).

MATERIAL AND METHODS

The research work has been implemented on MATLAB software tool and utilized it as a user-friendly interface. For the experimental tests, UCI Repository dataset was used in this framework. Monitoring the PLA2R and diagnosing the disease plays a key role in this research work. Kaplanmeier statistical analysis was done and hypothesis method was introduced and proved that p value was significant. Machine learning methods was introduced and the performance of the classifiers was done along with classifiers bagging and voting, which was introduced and the performance of the bagging gave better result for the disease prediction. Weka toolkit 3.8.2 was used to obtainbetter results modified using methods of earlier workers (Rakhlin, 2006; Padmavathi and Senthilkumar, 2019).

Optimization based feature selection method has also been used, where teaching learning-based optimization method has been used. Hybrid based learning method was used for one of the supervised learning method support vector machines, (SVM) and its performance was calculated, based on the time period following the methods of workers like, (Dhayanand and Vijayarani1, 2015; Padmavathi and Senthilkumar, 2019). Class problems often have a big wide form of functions in the records units, but now not they all are beneficial for class irrelevant and redundant functions may additionally even reduce the overall performance. Characteristic selection targets to choose a small number of relevant skills to gain similar or perhaps higher magnificence normal overall performance than the use of all capabilities (Padmavathi and Senthilkumar, 2019).

Figure 1: Optimization Based Selection System



It has fundamental conflicting objectives of maximizing the category performance and minimizing the quantity of capabilities. In the proposed method, the modified PSO technique we alternate the fitness feature (distance calculation) for every statistic. Here we alternate the match cost each new release, each new release the threshold cost is increased. Ultimately, we discover the

solution of the iteration value based totally on the p best and b best values. Every iteration discovers the solution of facts based totally on generation and locates the first-class answer value in a few particular iterations and then we set the edge cost.

The particle swarm optimization was found to be fee effective and its technique is being used in many fields. Modified-PSO algorithm becomes a parameters optimization method, progressing to improve the sitting of the parameter values of system learning algorithms KNN, RF, Boosting, this model was designed to help the physicians reliably in identifying the abnormalities in pancreatic cancer. KNN, Random Forest and Boosting methods have been taken for the optimization methods. The source of data was taken from the UCI Repository dataset. The particle swarm optimization was applied to this dataset. A total of twenty-five attribute was taken for the calculation of the accuracy (Padmavathi and Senthilkumar, 2019).

This model is designed to handle the renal disease membranous nephropathy. The classification techniques like knn, Random forest and boosting techniques are considered for optimization-based selection system. Particle swarm optimization and modified particle optimization methods are applied. Then the accuracy of the classification techniques is calculated. PSO algorithm work details: PSO is an evolutionary algorithm inspired from the flocks of birds or schools of fish in coordinated motion.

In PSO, individuals are called particles and the population is called a swarm. Each and every particle search for the best point and this is based on the particle movement and intelligence. Thus, each particle motion is to find the particle current location (lbest), particle best location (pbest), sum of best location (gbest). The current location of the particle is estimated by the fitness function which is obtained from the fitness value (Padmavathi and Senthilkumar, 2019).

Steps: 1) Find the Objective (target to be achieved), 2) Let as Assume the Fitness value as 1 by Objective 3) Initialize Velocity and number of Iteration a) For each iteration calculate the local best from the population. b) Compare the local best with the previous local best to update the current lbest and velocity 4) Recalculate the Global best.5) Compare with the fitness if reached stops the iteration 6) Else continue to the next step. The accuracy level of each dataset is considered and it is tabulated.

Table 1 Describes the performance of the classification techniques by PSO. Based on the time factor accuracy is calculated for the Anti PLA2R DATASET.

Table 2 describes the performance of the classification techniques by PSO algorithm. Based on the time factor accuracy is calculated.

RESULTS AND DISCUSSION

PSO provides a valuable high level data points for the initial selection for further classification. Particles or potential solutions are represented having a position and rate of the change in d-dimensional space. In PSO, a number of solutions are encoded as a swarm of particles in search space. The initial values of a particle are randomly chosen. Each particle maintains a record of its best achieved since the beginning of the iteration. Also, each particle has a defined neighborhood. Particles make decision based on the performance of its neighbor and itself (Padmavathi and Senthilkumar, 2019).

Table 1. Accuracy level of the particle swarm optimization-based classification techniques

Algorithm	Accuracy	Precision	Recall	F measure	Time period
PSO_KNN	92	89	90	90	4.2
PSO_RF	95	92	94	93	3.7
PSO_BOOSTING	97	95	94	95	3.3

Table 2. Accuracy level of the optimization-based classification techniques

Algorithm	Accuracy	Precision	Recall	F measure	Time period
PSO_KNN	90	87	89	89	4.6
PSO_RF	93	90	91	92	4.2
PSO_BOOSTING	95	91	93	94	3.8

This method without feature selection total 25 attributes features based data given UCI data set (age, Level of specific gravity, sugar, rbc, pus cell clumps, bacteria, hypertension, diabetes mellitus, coronary artery disease, Appetite, pedal edema, pus cell clumps, age, blood pressure, blood urea, serum creatinine, sodium,

potassium, hemoglobin, packed cell, wbc).Using modified pso algorithm improve the algorithm and optimized the features there given 25 it reduced the feature attributes our proposed method selected most relevant feature 19 from 25.

Table 3 describes the performance of the classification techniques by modified particle swarm optimization. Based on the time factor accuracy is calculated. In this modified pso method the distance is measured and its threshold values is calculated.

Figure 1: Showing the accuracy of the particle swarm optimization being determined.

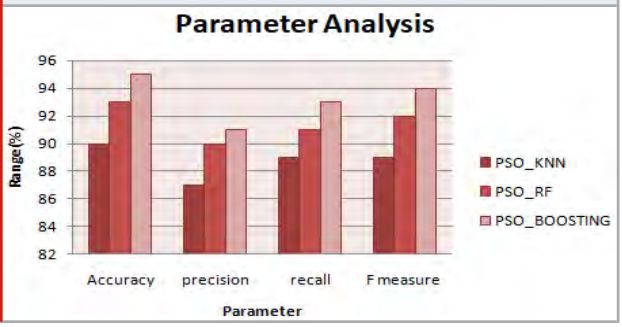


Figure 2: The accuracy of the particle swarm optimization determined on the basis of time.

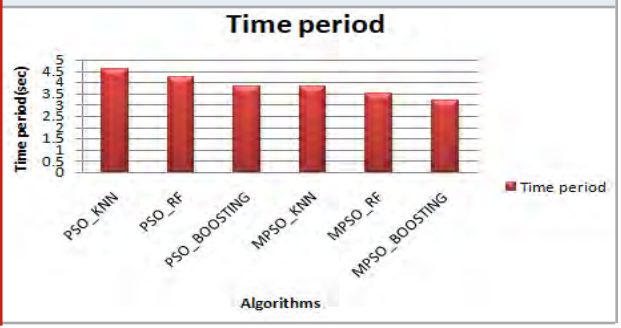


Table 3. Accuracy level of the Modified Particle swarm optimization algorithm for the classification techniques

Algorithm	Accuracy	Precision	Recall	F measure	Time period
MPSO_KNN	94	93	92	93	3.8
MPSO_RF	96	93	94	93	3.2
MPSO_BOOSTING	98	96	94	95	2.9

Table 4. Accuracy level of the optimization based classification techniques

Algorithm	Accuracy	Precision	Recall	F measure	Time period
MPSO_KNN	93	90	92	92	3.8
MPSO_RF	95	92	94	94	3.5
MPSO_BOOSTING	97	95	95	96	3.2

Figure 3: Applying parameter analysis for the machine learning techniques using PSO

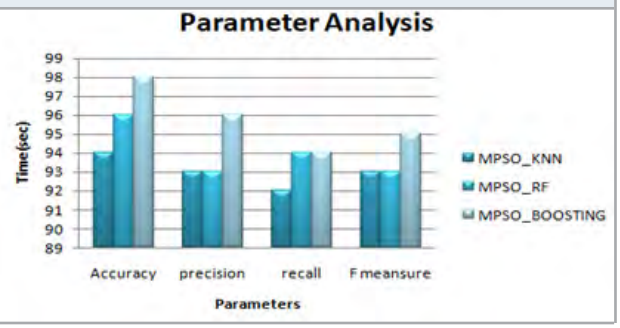


Figure 4: Applying parameter analysis for the machine learning techniques using pso for Anti –Pla2R dataset.

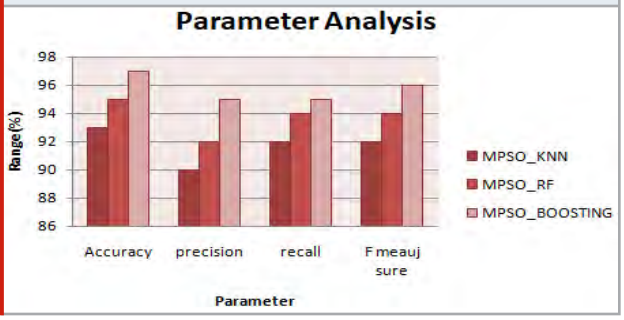


Table 4 describes the performance of the classification techniques by modified pso algorithm. Based on the time factor accuracy has been calculated

Figure 4: Applying parameter analysis for the machine learning techniques using pso for Anti –Pla2R dataset.

This present model has been designed to help the physicians reliably in identifying the abnormalities in pancreatic cancer. KNN, Random Forest and Boosting methods were taken for the optimization methods. The source of data is taken from the UCI Repository dataset.

The particle swarm optimization was applied to this dataset. A total of twenty-five attribute was taken for the calculation of the accuracy as the method of Padmavathi and Senthilkumar, (2019). The above process discusses about the comparison of previous work with the proposed work Modified particle swarm optimization. The accuracy of the ensemble methods bagging and boosting proves to be higher with the time factor 3.8.

Recently Trujillo et al (2020) have provided a new way of understanding membranous nephropathy, similar to our work where two datasets are used to improve the classification performance. The input parameters for boosting methods were optimized using modified version of PSO algorithm. In both the datasets the boosting algorithm performance is very high compared with the other classification techniques knn and Ada boost techniques

Table 5. Comparison of previous work.

Description	Algorithm used	ACCURACY	PRESCISION	RECALL
Feature selection process	SVM	90	89	90
	DT	93	91	92
	ES	96	93	95
Modified Optimization techniques	KNN	93	90	92
	RF	95	92	94
	Boosting	97	95	95

CONCLUSION

Membranous nephropathy may be a relatively common autoimmune disorder with a heterogeneous prognosis and its detection persists within the timely treatment of the patients. Different classification techniques have been used to improve the performance. KNN method was applied to boost up the accuracy. In this proposed method classifiers along with the particle swarm optimization methods were used. Two datasets were used to improve the classification performance. The input parameters for boosting methods were optimized using modified version of PSO algorithm. In both the datasets the boosting algorithm performance was very high as compared with the other classification techniques, the knn and Ada boosted techniques. We anticipate that future research will specialize for a far better understanding of autoimmune antibodies and to enhance with the applications of Artificial Neural Network.

Author Contribution: All authors contributed to the content of this manuscript:

Conflicts of Interest: The authors declare that they have no conflict of interest.

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Scientometrics of the Emerging Trends in Nursing Research Related to Perceived Needs of Breast Cancer Survivors

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ABSTRACT

As a critical point to promote breast cancer survivors' health care, the perceived needs are inevitable to this scenario. The purpose of this analysis is to provide a dynamic and longitudinal Scientometric study of the perceived needs of breast cancer survivors on nursing research. The Web of Science (WoS) Core Collection was searched to retrieve all existing and highly cited perceived needs research papers published in English between 1991 and November 2020. Based on the bibliometric indicators, the growth rate of publications, countries, and institutions was engaged on research, characteristics of research activities, keyword analysis, document co-citation analysis (DCA) for research hotspot tendencies were computed using the CiteSpace Software. The search identified 898 articles, which were included in the analysis. The United States (501; 55.791%) and the National Cancer Institute (NCI; 38) were the most productive and active research conducting in this knowledge domain. The United States also produced more than half of the publication in the last 30 years on the part of perceived needs. Cluster-based analysis of document co-citation analysis (DCA) was conducted to know the emerged trends in this domain. This bibliometric analysis concludes that (1) internet-based health-related information; (2) quality care of cancer patients; (3) breast cancer patient-physician relationships; (4) experience of psychosocial and physical needs after oncological treatment; and (5) eHealth system, which are found the major nursing research trends on this domain. This finding helps researchers, policymakers, and practitioners better understand the current trends in breast cancer survivors' perceived needs.

KEY WORDS: BREAST CANCER SURVIVORS, INFORMATION NEEDS, NURSING RESEARCH, PERCEIVED NEEDS, SCIENTOMETRIC ANALYSIS.

INTRODUCTION

The self-perception of health among patients and caregivers with chronic diseases had changed drastically in recent times. Notably, in breast cancer, both the woman and her partner affects in different ways. The high prevalence of this chronic disease combined with psychiatric morbidity leads to various psychosocial impacts on the patient's life. Specifically, isolation, depression and anxiety, and lack of social support, which are all not only worsen the quality of life but also reduces the possibilities of the treatment outcome.

Even though various psychosocial interventions help overcome the psychiatric symptoms of breast cancer survivors, very few studies have focused on perceived needs from patients' and caregivers' perspectives. Without understanding the perceived needs, helping breast cancer

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patients and caregivers have become more critical. To increase the rate of survival and quality of life, perceived needs play a significant role in breast cancer survivors. It helps to understand the gap between survivors' experiences and expectations (Spiegel, 1996; Macleduff et al., 2004; Burgess et al., 2005; Boyes, et al., 2009; Melvin et al., 2016, Mishra and Saranath, 2019; Kern et al., 2019 and Shen et al., 2020).

Self-perception of survivors' needs and issues remains unachieved but expected to experience, which are considered essential factors underlying to measure cancer survivors' perceived needs. Based on this, five significant factors had identified and included to measure perceived needs such as psychological needs, health system and information needs, physical and daily living needs, patient care, and support needs and issues with sexuality (Boyes, et al., 2009). The domains specifically focused on emotions and coping of survivors (Lebel et al., 2009). Followed by, needs related to treatment center including information in regards to disease severity, diagnosis, treatment and follow-up (Melvin et al., 2016).

Furthermore, coping on physical symptoms, side effects of treatment and performing routine daily activities, health care providers and importance given to sensitivity to physical and emotional needs, and privacy, finally, the sexuality domain focused on needs connected with sexual relationships (Rietman et al., 2003; Boyes et al., 2009; Mao et al., 2013; Yoo et al., 2014; Fang et al., 2015; Crowley et al., 2016; Fong et al., 2017). Although these domains were playing a vital role in perceived needs, the emerging trends in this field, and the key contributors of this area are not known from any nursing research studies. Hence, to get a solution to these research questions, conducting a Scientometric analysis is inevitable to the present scenario with CiteSpace software's help (Chen, 2017).

MATERIAL AND METHODS

For the data sources, scientometric articles were collected from advanced search in the WoS Core collection (Web of Science) incorporating with Science Citation Index Expanded (SCI-E), Social Science Citation Index (SSCI), and Arts and Humanities Citation Index (AandHCI). All the articles were written in English, only taken for analysis. The dataset was collected through the strategies; TS= ("breast cancer survivors" AND "perceived needs") or ("breast cancer" AND "health information") or ("breast cancer survivors" AND "daily living") or ("breast cancer" AND "patient care support") or ("breast cancer" AND "interpersonal communication") or ("breast cancer" AND "nursing care")) that articles with those words in the title or abstract, or keywords were retrieved. The period was taken between 1991 and 2020* (*-November 2020). 898 articles in total were retrieved from more than 50 Web of Science categories and the top five categories as 'Oncology' (281), followed by 'Public environmental occupational health' (205), 'Nursing' (153), 'Health care sciences services' (141), and 'Communication' (77).

For the software, CreateSpace was used, as developed by Chen (Chen, 2004; Chen, 2017), to analyze and visualize the Scientometric properties from the retrieved documents. Co-citation analysis network containing authors, countries, document references, and institutions as well as it helps to find the research patterns and detects the research core spots in the knowledge domain of perceived needs among breast cancer survivors.

RESULTS AND DISCUSSION

Publication years: Until 2000, a minimal number of articles were only published in this field. Significantly, in 1991, no paper was published related to perceived needs among cancer survivors or breast cancer survivors. In 1992, there were only two articles published related to this area. And the subsequent year, only one article was published. After 2001, the number of publications was increased gradually. In 2019, the highest publication was recorded (72). Annual research publications are illustrated in Figure 1. The findings show that the continued growth of publications on perceived needs was noticed.

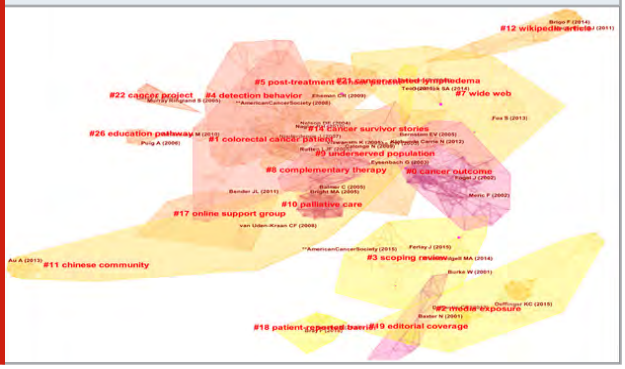
Figure 1: The number of annual publication on perceived needs of breast cancer survivors

Countries distribution: Among the published articles, the top 10 most productive countries were ordered based on publication production and their percentage such as USA (501; 55.791%), followed by England (71; 7.906%), Canada (63; 7.016%), Australia (54; 6.013%), Peoples R China (42; 4.667%), South Korea (24; 2.673%), Netherlands (19; 2.116%), Turkey (19; 2.116%), Sweden (18; 2.004%) and Brazil (17; 1.893%). Within these records, India (1; 0.111%) was published only an article in the Web of Science during the period of 1991 to 2020. Besides, more than half of the records were published only in the United States shows that this country plays a crucial role in publishing more records regarding the perceived needs of breast cancer survivors.

Institutions / Organizations: The top 10 institutions based on the number of publications related to perceived needs among breast cancer survivors were listed. It is worth noting that the National Cancer Institute (NCI) ranked first in total publications (38). Harvard University ranked as the second on in this list (28), followed by University of Michigan (26), University of Pennsylvania (24), University of Wisconsin (21), Memorial Sloan Kettering

Cancer Center (19), University of North Carolina (18), University of Toronto (17), University of Illinois (16) and Dana-Farber Cancer Institute (15). All of the top 10 institutions were from the United States only. Hence, the United States playing prepotency in researching with perceived needs among cancer survivors.

Figure 2: Scientometric mapping of document co-citation analysis (DCA) based on clusters with data derived from WoS between 1991 to 2020



Authors distribution: Based on the publication records on perceived needs published uniquely by the authors, the top five authors were listed and author Gustafson, DH (11) as the first, followed by Moser, RP (9), Pingree, S (9), Klein, WMP (7) and Li, XM (7).

Keyword distribution: By using CiteSpace analyzing software, based on citation burst, the keywords viz., 'world wide web' (10.07), 'health literacy' (5.80), 'quality' (5.77), 'chemotherapy' (5.39), and 'symptom' (4.82) were the top five keywords used popularly and recently emerged. It shows that around these keywords, particularly, 'world wide web' (10.07) as the most robust citation burst on cancer survivors' perceived needs.

Document co-citation analysis (DCA): The 898 records from 1991 to 2020 were visualized, and a one-year time slice was selected for analysis. Cluster-based analysis of document co-citation analysis (DCA) was illustrated in Figure 2. The top fifty most cited articles in each year were chosen for the Scientometric study and summarized in table 1 with the top five clusters within that top five cited references of cancer survivors' perceived needs.

Table 1. The five largest DCA clusters on perceived needs with top 5 most cited references DOI

Cluster #	Cluster Size	Silhouette Value	Mean (Year)	cc	Burst	σ	ΣTop 5 Most Cited references DOI
Cancer outcome	83	0.910	2002	14	6.66	0.00	1.0110.1037/0278-6133.21.4.398
				12	5.70	0.00	1.0110.1001/jama.285.20.2612
				12	4.92	0.00	1.0010.1136/bmj.324.7337.577
				11	4.46	0.01	1.0310.1136/bmj.324.7337.573
				10	4.27	0.06	1.2610.1136/bmj.328.7439.564
Colorectal cancer patient	65	0.892	2006	11	4.36	0.06	1.3110.1016/j.pec.2004.06.006
				7	4.00	0.02	1.0810.1080/10410230701454189
				6	-	0.24	1.0010.2196/jmir.1035
				6	-	0.04	1.00 -
				6	3.46	0.03	1.1010.1002/cncr.23445
Media exposure	54	0.998	2015	5	-	0.08	1.0010.3322/caac.21412
				5	-	0.07	1.0010.1001/jama.2015.12783
				4	-	0.00	1.0010.3322/caac.21349
				4	-	0.04	1.0010.2196/jmir.5729
				4	-	0.00	1.0010.3322/caac.21387
Scoping Review	43	0.944	2015	8	4.05	0.01	1.0610.1002/ijc.29210
				8	4.05	0.15	1.7510.3747/co.21.1932
				4	-	0.14	1.0010.1002/cncr.28951
				4	-	0.00	1.0010.1016/j.breast.2017.10.004
				4	-	0.03	1.0010.1002/cncr.28737
Detection behavior	37	0.914	2006	18	8.55	0.04	1.4010.1080/10810730490504233
				4	-	0.01	1.0010.7326/0003-4819-144-8-200604180-00004
				4	-	0.02	1.0010.1111/j.1460-2466.2008.00383.x
				4	-	0.00	1.0010.1146/annurev.clinpsy.3.022806.091428
				4	-	0.02	1.00 -

Source: The data were derived from analysis of document co-citation network using dataset retrieved from WoS. CC-Citation-count, σ-Centrality, Σ-Sigma.

The top five clusters based on cluster size are Cancer Outcome (83), Colorectal cancer patient (65), Media exposure (54), Scoping review (43), and Detection behavior (37). Each cluster was labeled by the method of log-likelihood ratio. The range of silhouette value of the first five largest clusters is between 0.910 and 0.998. It represents each cluster as different from one another and has a high homogeneity level for each cluster. The mean year of each cluster is 2002, 2006, 2015, 2015 and 2006 respectively. The clusters, both Media exposure, and Scoping review are very recently emerged.

The most-cited references of Cancer Outcome based on citation count and burst strength as Fogel, J_2002 (14; 6.66), followed by, Berland, GK_2001 (12; 5.70), Meric, F_2002 (12; 4.92), Eysenbach, G_2002 (11; 4.46), and Ziebland, S_2004 (10; 4.27). This cluster article mainly focused on how the internet plays a crucial role in health related information. In this digital world, most patients try to search for health-related information with the internet's help. Therefore, wide people depend more on the internet.

Eysenbach and Kohler first conducted observational study to investigate the retrieval strategies of people searching for health information on the website (Eysenbach and Köhler, 2002). Further, another study revealed that internet use for breast health issues was highly associated with greater social support and the survivors felt less loneliness (Fogel et al., 2002). "The impact of the internet on cancer outcomes" is the most active citer article on this cluster (Eysenbach, 2003).

The second-largest cluster as Colorectal cancer patient (65) labeled by the method of log-likelihood ratio. The top five most cited references as Rutten, LJF_2005 (11; 4.36), Niederdeppe, J_2007 (7; 4.00), Atkinson, NL_2009 (6), American cancer society_2007 (6), and Hesse, BK_2008 (6; 3.46). This cluster mainly focused on ensuring quality care of cancer patients. The most active citer article as "Differences in information seeking among breast, prostate, and colorectal cancer patients: results from a population-based survey" (Nagler et al., 2010; Tan and Goonawardene, 2017).

Further, the third cluster is Media exposure (54), and the top five most-cited authors as DeSantis, CE_2017 (5), Oeffinger, KC_2015 (5), Miller, KD_2016 (4), Tan, SSL_2017 (4) and Siegel, RL_2017 (4). This cluster mainly focused on reviewing breast cancer screening guidelines, patient-physician relationship (Tan and Goonawardene, 2017), and reviewing most recent data on cancer incidence, mortality, and survival (Siegel, Miller and Jemal, 2017). The most active citer article in this cluster is "Effects of media exposure to conflicting information about mammography: results from a population-based survey experiment" (Nagler, et al., 2019).

The fourth cluster is labeled as Scoping review (43), and the mean year of this cluster is 2015. The top five cited authors as Ferlay, J_2015 (8; 4.05), followed by, Shea-Budgell, MA_2014 (8; 4.05), Burg, MA_2015 (4), Arif, N_2018 (4), and Champion, VL_2014 (4). This cluster reference articles concentrate on the experience of psychosocial and physical needs related to cancer experience after their treatment (Burg et al., 2015), also focused on better prevention approaches and clinical responses. The article "A scoping review of consumer needs for cancer information" as the most citer active article on this cluster (Jo, et al., 2019).

And finally, the fifth cluster is labeled as Detection behavior (37). The top five most-cited references are Nelson, DE_2004 (18; 8.55), followed by, Smith-Bindman, R_2006 (4), Gustafson, DH_2008 (4), Strecher, V_2007 (4) and American Cancer Society_2008 (4). This cluster concentrates on cancer-related information, eHealth system, that provides integrated information, support, and bioinformatics and deliver behavioral and health-related interventions through the internet (Strecher, 2007; Gustafson et al., 2008). The most active citer to the cluster is "Topics and sources of memorable breast cancer messages and their impact on prevention and detection behaviors and a cluster-randomized trial of a primary care informatics-based system for breast cancer screening" (Smith et al., 2009; Atlas et al., 2011).

CONCLUSION

The present study aimed to provide a bird's-eye view of the entirety of cancer survivors' perceived needs. This analysis depicted trends on perceived needs among breast cancer survivors; (1) internet's role on health-related information; (2) ensuring quality care of cancer patients; (3) patient-physician relationships; (4) experience of psychosocial and physical needs related to cancer experience after treatment; and (5) eHealth system. However, there are minimal studies found in the developing countries in sexuality among cancer survivors, relationship between cancer survivors and partners, and caregiver's perspective on the partner. Future research should be dedicated to filling the gap in this domain.

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Analysis on the Advantages and Disadvantages of Coronavirus Disease 19 on-Going Treatments

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ABSTRACT

Corona viruses COVID-19 is a novel and highly infectious virus emerged in December 2019, (Wuhan) China and affected a wide range of global population with deaths worldwide till date. Lack of knowledge regarding the clinical management and treatment of infected patient has augmented this epidemic of China to get transformed into a global pandemic. This review outlines briefly about the novel coronavirus, its taxonomic classification, associated symptoms, origin, life cycle and the treatment strategy which includes diagnosis as well as on-going trials related to antiviral drugs and vaccine with its pros and cons to combat SARS-CoV 2 infection. Presently, to deal with the infection therapeutic strategies are only supportive and prevention is the best weapon to abate transmission in the community. Efforts have been made to develop vaccines against human coronavirus (CoV) infections such as MERS and SARS in the past decades. However, to date, no licensed antiviral treatment or vaccine exists for MERS and SARS. Researchers are searching for effective and suitable vaccine candidates and therapeutics for controlling the deadly COVID-19. There are no effective vaccines or specific antiviral drugs for COVID-19. Hence, we have to rely exclusively on enforcing strict preventive and control measures that minimize the risk of possible disease transmission. There is an urgent requirement of vaccine to stop the spreading of SARS-CoV2 infection. Since there is no antiviral drug or vaccine so it is important to enhance the host immune response against the infection. We except our analysis to become a milestone for future studies helping with acting as a credible groundwork for their results.

KEY WORDS: INFECTITIOUS, ANTI-VIRAL, THERAPEUTICS, EXCLUSIVELY, VACCINE, IMMUNE.

INTRODUCTION

Corona viruses are characterised by their largest genome with single positive stranded RNA (27-32Kbs) that is packaged in nucleo-capsid and the membrane protein as well as has been named as 'Corona' or crown like morphological appearance with the spike proteins. These enveloped viruses recently became associated with humans and animals respiratory and gastrointestinal infections. In the year 2002 to 2003, viral epidemic severe acute respiratory syndrome (SARS-CoV), HINI in 2009 and in 2012 Middle East respiratory syndrome coronavirus in Saudi Arabia have been recorded by World Health Organization. Among coronaviruses severe acute respiratory syndrome (SARS- CoV) and MERS-CoV are found zoonotic and highly pathogenic causing global outbreaks (Luk et al. 2020).

Based on nucleotide sequence SARS-CoV 2 are new human infecting betacoronavirus 2019-nCoV originated probably from bats to humans after passing in one or

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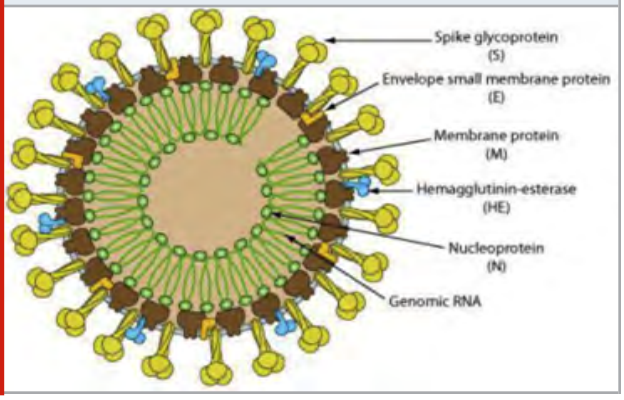
more intermediate hosts as evidenced by the molecular data and experiments (Chen et al. 2015; Luk et al. 2020). However, the original source and association with the animal host still remains indefinable. Additionally, Xiong et al. (2020) on the basis of molecular evolution data suggested that two different viral strains of SARS-CoV 2 might be involved in the outbreak (Xiong et al. 2020). This mini review describes briefly the novel corona viruses COVID 19 and summarises its historical background, probable source of origin, life cycle and the treatment strategies describing the ongoing trials to combat SARSCoV2 infection. Therefore, this review focussed to assemble updated information and evidences to understand the evolving virus.

A brief history and origin: In December (2019) pneumonia cases were recorded in Wuhan, China and the cause identified was due to novel Beta-coronavirus. WHO named this virus initially as the 2019-novel coronavirus on 12 January, 2020 and Corona Study Group (CSG) of the International committee proposed the name SARS-CoV-2 on 11 February, 2020. On 7thJanuary 2020, Chinese Scientists isolated SARS CoV-2 and sequenced the virus genome. After virus genome and evolutionary history analysis, bat has been considered as the possible origin of corona virus infection initiation. 96.2% genome sequence identity showed that Bat CoV Ratg13 and human COVID 19 genomes probably shared the same ancestors and is also in a way identical to 79.5% with SARS-CoV. However, it is clear now to infect humans, SARS-CoV-2 could use angiotensin-converting enzyme 2 (ACE2), the same receptor as SARS-CoV (Zhou et al. 2020). Interestingly, on the basis of similar residue of receptors apart from protein homology and phylogenetic analysis, few studies also reported alternative intermediate hosts such as pangolin, turtles and snakes as the possible reservoir hosts of corona virus. However, the origination of Corona virus still remains uncertain.

Model of life cycle and transmission: The regions that are encoded for viral replication, nucleocapsid and spike formation is present in ORF 1a/b downstream region in all coronaviruses. The spike of viruses (Figure 1) that is made of glycoprotein is meant for entry and attachment of the virus with the host cell (Cui et al. 2019; Perlman, 2020). Among viruses loosely attached receptor binding domain is present due to which it can attack multiple hosts. A typical corona virus structure consists of spike proteins as well as other polyproteins, nucleoproteins and membrane proteins that include RNA Polymerase, papain like protease, chymotrypsin like protease, helicase, glycoproteins and accessory proteins (Raj et al. 2013; Elfiky et al. 2017).

The three-dimensional structure in the receptor binding domain in SARS-CoV 2 is recognised by human ACE2 receptor that facilitates viral envelop fusion with the host cell. The RNA of SARS-CoV2 is released in the host cell. Viral gRNA by viral replicase is translated into polyproteins and cleaved by viral proteases. Thus, viral genome and proteins are assembled in virions in ER and Golgi and by vesicle trafficking release out of the cell (Wong et al. 2015). The virus spread from one person to another via droplets or aerosols or by fomite transmission. Doremanlan et al. (2020) in his study have shown that virus survive in different surfaces for days and in aerosols for hours. Incubation period of virus has been reported to be of 5.1days and the initiation of symptoms up to 14 days after incubation (period of self-isolation/ quarantine) (Doremanlan et al. 2020; Lauer et al. 2020).

Figure 1: Novel Coronavirus structure HCoVs: structural proteins, such as Spike (S) marking all coronaviruses, Nucleocapsid (N), Matrix (M), and Envelope (E) (From Biowiki: http://ruleof6ix.fieldofscience.com/2012/09/a-new-coronavirus-should-you-care.html).



SARS CoV and COVID 19: Outbreak of Severe Acute Respiratory Syndrome (SARS-CoV) was in the year 2003, MERS –CoV in 2012 and COVID 19 in 2019. Bat is thought to be the source of origin of SARS-CoV and spread by an intermediate host before jumping to humans. Compared to SARS-CoV, COVID 19 transmits more easily probably because viral load appears initially to be higher in nose and throat before the symptom develop. Some researchers from Centre of Disease control suggested that COVID 19 might spread without the carrier showing any symptom of the virus.

Additionally, the transmission of SARS-CoV and MERS-CoV has been testified to occur mostly through nosocomial transmission (Gao et al. 2020). Infections of healthcare workers in 33–42% of SARS cases and transmission between patients (62–79%) were the most common route of infection in MERS-CoV cases (Chowell et al. 2015). Direct contact with intermediate host animals or consumption of wild animals was also supposed to be the main route of SARS-CoV-2 transmission. Caution should be, however, exercised to promptly identify asymptomatic viral carriers. Additionally, fomite transmission of SARS-CoV-2 might have predisposed to the rapid spread globally (Cornman et al. 2020).

Treatment strategies: Diagnostic techniques: Fever, cough and shortness of breath are the specific noticeable symptoms to start the diagnosis. Patient with epidemiological link includes basically the travel history

of an individual in an outbreak area, or contact with an infected individual being asymptomatic within 14 days as estimated incubation time of viruses and close contact with an individual having infection. SARS-CoV2 has been detected in urine, gastric mucosa, saliva, stool (Xie et al. 2019; To et al. 2019; Guan et al. 2019) and accordingly different types of corona virus test involved: swab test, nasal and tracheal aspirate test, sputum, blood test as well as rapid test that ensure speedy and rapid diagnosis.

Rapid Diagnostic test detects viral protein from the samples taken from respiratory tract of the COVID 19 infected person. If the target antigen is present in the sample, it will bind to the antibodies fixed in a paper strip generating a visual detectable signal. However, this test is not 100% accurate as this test may show false positive results (antibodies on the strip recognise other viral antigens) also concentration of the virus in the specimen, reagents in the test kit might give a false result. Apart from this rapid diagnostic test based on host antibody detection has been widely exploited, (Cornman et al. 2020).

During recovery stage of the corona virus infected individual antibodies are produced in the blood in response to viral infection and therefore, target the COVID 19 virus. PCR testing has been recommended by WHO for identification and laboratory confirmation of COVID 19 virus. Along with, RT-PCR (Real Time Reverse Transcription Polymerase Chain reaction) has been a reliable diagnostic tool in terms of sensitivity and specificity for COVID 19 virus detection (Cornman et al. 2020).

On-going trials: SARS and MERS outbreak in 2003 and 2012 has been guidance for doctors and researchers to treat the present COVID 19 infected patients. For treating corona virus infection initially HIV drugs such as lopinavir/ritonavir that is a protease has been administered and inhibit corona virus replication but this drug combination caused diarrhoea and jaundice like situation and in some COVID 19 infected patients no beneficial effect was observed (Cao et al. 2020). Nucleotide analog like ribavirin (Table 1) that has been used to treat SARS might also have antiviral effect by inhibiting nucleotide biosynthesis. Additionally, combination of ribavirin and interferons (IFN-α) have been used to treat SARS. Ramdesvir is a novel nucleotide analog is presently under clinical trials. It has been recognised as a promising anti -viral drug for SARS/ MERS-CoV and its activity inhibit RNA dependent RNA polymerase from MERS-CoV (Gordon et al. 2020; de Wit et al. 2020).

Table 1. Therapeutic targets in clinical trials against SARS-CoV 2 infection

Drugs	Mode of action against SARS-CoV2	Probable pros and cons
Ribavirin Remdesvir Sofosbuvir Galidesvir Tenofovir	Act against RNA dependent RNA polymerase (Elfiky, 2020)	Yet to establish Safety and efficiency.
Lianhuaqingwen	Inhibit virus replication (Runfeng et al. 2020)	Antiviral and anti -inflammatory. Might have potential to inhibit cytokine storm
Oseltamivir	Neuraminidase inhibitor	No exact evidence regarding its effectiveness against COVID 19
Lopinavir Atazanavir Darcenavir Nelfinavir Sequinavir Tipranavir	HIV protease inhibitor	Invitro activity against SARS-CoV2 in Vero E6 cells but no data to support COVID 19
Favilavir (Avigan)	Inhibit RNA dependent RNA polymerase (Wang et al. 2020)	Used in Japan and China for treating influenza. Showed invitro activity against SARS-CoV 2 in Vero E6 cells but its use contradicted in pregnant women.

For inhibiting viral infection from SARS-CoV, it is essential to block the binding of S-protein to ACE2 (acetyl-converting enzyme 2) and an anti-malarial drug Chloroquine /Hydroxychloroquine have been highly commercialized as potent inhibitor of SARS-CoV and a study reported by Wang et al. chloroquine has been found to be highly effective against COVID 19 (Wang et al. 2020; Gao et al. 2020). Chloroquine and hydroxychloroquine block channels on heart muscle cells that control the flow of ions, which governs the heart's electrical recharging between beats. Immune system modulating agents have also been proposed to combat virus COVID 19 infection. The entry of virus in the host cell activates an immune response to increase in cytokines which is responsible for disease severe condition. Individuals with low number of lymphocytes CD^{4+} T cells, those having some chronic illness in the history are badly affected by SARS-CoV 2 (Beigel et al. 2019; Chen et al. 2020).

Convalescent Plasma Therapy: In the year 1890, Emil Von Behring discovered Convalescent plasma therapy for which he got Nobel Prize in medicine. This therapy presently has been widely used to treat COVID 19 patients by taking out the plasma of an individual who has recovered from COVID 19 infection and thus has sufficient antibodies and thus transferring plasma to freshly infected individual. This therapy has been used to treat MERS and SARS and EBOLA virus diseases but this therapy renders passive immunization and is impermanent as it cannot provide lifelong immunity (Burnouf et al. 2016; Chen et al. 2020).

BCG vaccine and COVID 19: BCG vaccine has been given to children to prevent tuberculosis and being heterologous also provides protection against other non-related infections. BCG vaccination primes histone modifications and epigenetic reprogramming of human monocytes resulting in a more active innate immune response in terms of trained immunity. Professor Netea pointed out those studies that were done among adults, also showed lower incidence of respiratory tract infections in those who were given the BCG vaccine (Kleinnijenhuis et al. 2012; Netea et al. 2016). BCG (Bacillus Calmette Guerin) an attenuated strain of Mycobacterium bovis studies are in progress in order to investigate this vaccine against COVID 19 infections. Redelman (2020) explained why BCG could be a candidate vaccine for treating life threatening SARS-CoV2 infections are as follows:

1. Molecular similarity in BCG antigens and viral antigens
2. Long term activation and reprogramming of innate cells
3. Developing B and T cell memory for recognizing BCG antigen and another respiratory antigen
4. BCG vaccination increases the expression of surface markers producing high

cytokines such as IL-1B, IFNY, TNF.

Need of the hour: Viruses, an obligate intracellular parasite evolved to hijack the host cell machinery. In the same manner the emergence of SARS-CoV 2 resulted in high mortality all over the world by overtaking the host cell function thus threatening the global public health. So, it is very important to gain more insights in understanding pathogenesis of human infecting corona viruses for developing targeted therapeutics and vaccines. At present everyone should strictly follow the preventive measures recommended by WHO and other health organizations to protect oneself from SARS-CoV2 infection, (Chen et al. 2020). Frequent hand washing, use of portable sanitizers, avoid public gathering, maintaining hygiene, and use of N95 mask, gloves, etc by the health- workers to prevent pathogen transmission. Convincingly, 'each one treats one for one' is the only strategy to overcome the corona virus global pandemic, (Chen et al. 2020).

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Conflict of Interest: There was no conflict in the interests of the associated people.

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Diversity of Spiders in Microhabitats of a Tropical Reserve Forest of Amravati, Maharashtra, India

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ABSTRACT

Spiders are among the most abundant insectivorous predators of terrestrial ecosystem and consume large number of preys without damaging the plants. Spiders, are the most common ubiquitous animals on land, constitute an essential portion of the predatory arthropods in several ecosystems. They play an important role in insect pest control without any harm to ecosystem. Spider species abundance in ecosystem can be high as undisturbed natural ecosystem, as they act as pest control creatures, which feed on destructive insects. The information on spider's diversity is becoming increasingly important in the context of a global decline in the spider population. A survey of spiders was carried out in a tropical reserve forest of Pohra Malkhed, Amravati District during the years 2017-19. We have selected five microhabitats for observations in the study area viz; grassland, bush land, woodland, agricultural land and wetlands. Spiders were collected by adapting standard sampling techniques and collected spiders were photographed and later preserved in 75% ethyl alcohol. Spiders were observed using stereo zoom microscope for study and identification of spiders was confirmed with the help of available keys. During the present study, we have reported 120 species of Spiders belonging to 14 Families and 37 genera, families such as Araneidae, Clubionidae, Eresidae, Gnaphosidae, Lycosidae, Oecobiidae, Oxyopidae, Pholcidae, Salticidae, Sparassidae, Tetragnathidae, Theridiidae, Thomisidae and Uloboridae were abundant. This study provides updated checklist and base-line data of spider fauna from Pohra-Malkhed Tropical Reserve forest of Maharashtra State India. Moreover, we expect this research to become a suitable milestone by providing credible information to the future analysis on the similar topics.

KEY WORDS: SPIDER DIVERSITY, POHRA-MALKHED, TROPICAL RESERVE FOREST, SPIDER.

INTRODUCTION

Spiders belong to order Araneae, class Arachinidae and are members of phylum Arthropoda, the largest assemblage of animal with jointed legs and hard exoskeleton. They are the largest group of arachnids comprising more than 44,000 species distributed over 110 families, worldwide as of the World Spider Catalog .They have unique habitat and they live in almost all the environments. They are the most abundant predator of insects of terrestrial ecosystem and consume large number of preys without damaging the plants.

Spiders are one of the dominant predatory groups found in ecosystems in India. They have special adaptations towards a predatory way of life. Their distensible abdomens enable them to consume large amounts of food in relatively short periods of time, while their rate of predation may greatly increase during short periods when plentiful supply of food is available (Sunderland

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and Samu, 2000; Jeyaparvathi et al., 2013, Platnik 2019, Rajeevan et al., 2019).

They have an exceeding high resistance to starvation, which enables them to survive and maintain normal reproduction during periods of low prey availability. Spiders, are the most common ubiquitous animals on land, constitute an essential portion of the predatory arthropods in several ecosystems. Spiders are known to occupying most of the terrestrial habitats. They are generalist predator, which can act against a broader range of insect pests (Sunderland and Samu, 2000). Spider species abundance in ecosystem can be high as undisturbed natural ecosystem. Spiders act as pest control creature, which feeds on crop destructive insects. Spiders are beneficial bio-control agents of insect pest in the ecosystem and are known to occupy most of the terrestrial habitats. They are general predators, which can act against a broader range of insect pests (Sebastian et al., 2006, Jeyaparvathi et al., 2013, Wankhade and Manwar 2016).

Spiders are considered to be of economic value to farmers as they play valuable role in pest management by consuming large number of preys in the agriculture fields without any damage to crops. In spite of their importance as a generalist predator, the role of spiders in ecosystems is usually ignored, mainly because spiders do not fit into the conventional profile of biological control agents. Spiders are among the most abundant insectivorous predators of terrestrial ecosystem. The current global list of spider fauna has approximately 44,057 of them, belonging to 3928 genera and 110 families. Spiders are an important but generally poorly studied group of arthropods that play a significant role in the regulation of insect pests and other invertebrate populations in most ecosystems, (Sebastian et al., 2006 Wankhade and Manwar, 2016 and Rajeevan et al., 2019).

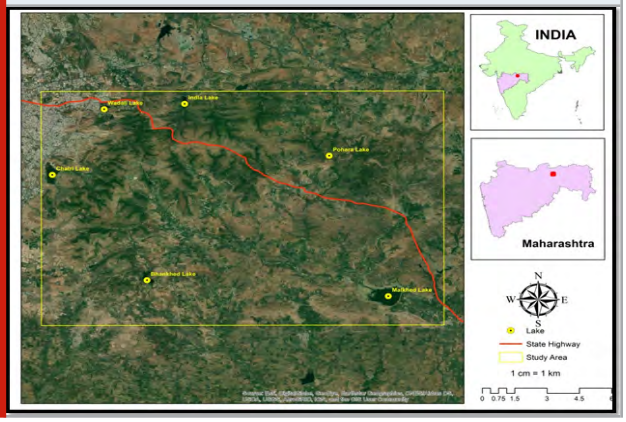
Recently in agricultural fields reduced pesticide use and ecological sustainability have led to increased interest in spiders as potential biological pest control agents. Spiders act as natural biological control agent in ecosystem. Some recent workers on Indian spiders include (Majumdar and Tikader, 1991, Reddy and Patel, 1992, Biswas and Biswas, 1992, Sadana and Goel, 1995, Biswas et al., 1996, Gajbe, 1999, Biswas and Majumdar, 2000, Biswas and Biswas, 2003, and Bastawade, 2005, Rajeevan et al., 2019). As per the literature cited very less work has been carried out in the tropical lying Pohra-Malkhed Reserve forest with respect to the spider diversity. Earlier 42 species of spiders were enlisted in and around Malkhed water body only of the Pohra-Malkhed Reserve Forest (Sebastian et al., 2006; Wankhade and Manwar, 2016). The study of Spiders was carried out in Pohra-Malkhed reserve forest of Amravati District during September 2017 – September 2019.

MATERIAL AND METHODS

The selected study area is of Pohra – Malkhed Reserve forest, which is the most diversity rich reserve forests of Amravati District. It is located between N 200 54' 229'' and E 770 51' 104'' with an elevation on 455 meter. Annual average rain fall is in between 1000 – 1600 mm. Total area under forest is 80 sq km. It is dry deciduous type and mixed type of forest with some grassland forest. More than 275 species of birds are reported form this forest. The other faunal species includes Mammals 17, Reptiles 26, Amphibians 04, Fishes 17, Butterflies 72, and numerous species of insects.

This reserve forest has more than 150 plants species (Wadatkar et al., 2014).The area receives rainfall during southwest monsoon. Average temperature of the district ranges from minimum of 10oC in winter to a maximum of 46oC in summer with the relative humidity varying from 10-15% to 60-95%. The spider inventory studies were conducted from September 2017 to September 2019 in the five different localities of Pohra-Malkhed reserve forest Amravati district from Maharashtra state. We have selected five microhabitats for observations in the study area viz; grassland, bush land, woodland, agricultural land and wetlands (Fig.1)

Figure 1: Map of Pohra-Malkhed Reserve Forest, District Amravati, Maharashtra (Study Area). Map of the study area was created by Shubham Wagh using Arch Map 10.5 & Arch GIS software.



For the sampling method, Spider Inventory work was conducted at the ecosystems by different groups of workers. Four surveys were conducted per season at all study sites. Five 30 x 30 m quadrates were taken for extensive surveys. All surveys were conducted in the morning hours between 6:00am to 10:00am Spiders were collected by adopting standard sampling techniques as described below. 1. Sweep netting: Spiders from herbaceous-shrub-small tree vegetation were collected using standardized insect-collecting net. This method is used to collect the foliage spider by this method from herbs and shrubs.2. Beating sheets: Spiders from trees and woody shrubs were dislodged and collected on a sheet by beating trees and shrubs with a standard stick. 10 beats per tree or shrub were employed in each quadrate. 3.

Active searching and hand picking: Spiders from all three layers were collected using this method. In this method

spider specimens were actively searched for 30 minutes per quadrat for searching under rocks, logs, ground debris, and loose dead barks of trees etc.4. Litter Sampling: Litter i.e. deciduate from the ground was collected by hand and was put in big tray. Litter samplings involved sorting of spiders from litter collection tray. Collected spiders were photographed in life and later preserved in 75% ethyl alcohol. Identification: Spiders were observed using stereo zoom microscopes for studying identification keys. All specimens were initially separated from other material and identified to the family level. Spiders were identified upto species level using the standard monographs (Majumder and Tikader, 1991).

Table 1. Checklist of Spider fauna from Pohra-Malkhed Reserve forest in Amravati district, Maharashtra State.

Sr. No.	Family	Species	Common Name of Spiders	Habitat	Status
1	Araneidae(34)	*Araneus cucurbitinus*	Orb Weaver	Grassland	UN
2		*Araneus mitifica* (Simon)	Orb Weaver	Grassland	C
3		*Araneus mitifica* (Simon)	Orb Weaver	Grassland	C
4		*Araneus pachganiensis*	Orb Weaver	Grassland	R
5		*Araneus pahalgaonensis*	Orb Weaver	Grassland	UN
6		*Argiope aemula*	Orb Weaver	Grassland	C
7		*Argiope aemula*	Orb Weaver	Grassland	C
8		*Chorizopes anjanes*	Orb Weaver	Shrubland	R
9		*Chorizopes calciope*	Orb Weaver	Shrubland	C
10		*Cyclosa bifida* (Doleschall)	Orb Weaver	Shrubland	C
11		*Cyclosa bifida* (Doleschall)	Orb Weaver	Shrubland	C
12		*Cyclosa confraga* (Thorell)	Orb Weaver	Shrubland	C
13		*Cyclosa fissicauda* Simon	Orb Weaver	Shrubland	R
14		*Cyclosa insulana* (Costa)	Orb Weaver	Shrubland	UN
15		*Cyclosa moonduensis*	Orb Weaver	Shrubland	C
16		*Cyclosa moonduensis*	Orb Weaver	Shrubland	C
17		*Cyclosa mulmeinensis*	Orb Weaver	Shrubland	UN
18		*Cyclosa neilensis* Tikader	Orb Weaver	Shrubland	R
19		*Cyclosa simoni*	Orb Weaver	Shrubland	C
20		*Cyrtophora bidenta*	Orb Weaver	Shrubland	C
21		*Cyrtophora cicatrosa*	Orb Weaver	Shrubland	C
22		*Cyrtophora citricola*	Orb Weaver	Shrubland	C
23		*Larinia chloris* (Audouin)	Orb Weaver	Shrubland	C
24		*Larinia chloris* (Audouin)	Orb Weaver	Shrubland	C
25		*Neoscona achine* (Simon)	Orb Weaver	Bushland	C
26		*Neoscona achine* (Simon)	Orb Weaver	Bushland	C
27		*Neoscona bengalensis*	Orb Weaver	Bushland	UN
28		*Neoscona bengalensis*	Orb Weaver	Bushland	UN
29		*Neoscona nautica*	Orb Weaver	Bushland	C
30		*Neoscona nautica*	Orb Weaver	Bushland	C
31		*Neoscona theis*	Orb Weaver	Bushland	C
32		*Neoscona theis*	Orb Weaver	Bushland	C
33		*Zygiella indica* Tikader	Orb Weaver	Bushland	C
34		*Zygeilla indica* Tikader	Orb Weaver	Bushland	C
35	CLUBIONIDAE(3)	*Clubiona acanthochemis*	Sac Spider	Shrubland	C
36		*Clubiona analis* Thorell	Sac Spider	Shrubland	C
37		*Clubiona analis* Thorell	Sac Spider	Shrubland	C
38	ERESIDAE(2)	*Stegodyphus sarasinorum*	Colonial Spider	On Trees	C
39		*Stegodyphus sarasinorum*	Colonial Spider	On Trees	C
40	GNAPHOSIDAE(8)	*Drassodes lubrica Simon*	Ground dwelling	Ground	R
41		*Drassodes sagarensis*	Ground dwelling	Ground	C
42		*Gnaphosa poonaensis*	Ground dwelling	Ground	UN

Continue Table 1

43		*Gnaphosa poonaensis*	Ground dwelling	Ground	UN
44		*Sosticus nainitalensis*	Ground dwelling	Ground	R
45		*Sosticus poonaensis*	Ground dwelling	Ground	C
46		*Zelotes poonaensis*	Ground dwelling	Ground	C
47		*Zelotes sajali* Tikader	Ground dwelling	Ground	R
48	LYCOSIDAE(22)	*Hippasa greenalliae*	Wolf Spider	Wetland	C
49		*Hippasagreenalliae*	Wolf Spider	Wetland	C
50		*Hippasa partita*	Wolf Spider	Wetland	C
51		*Hippasapartida*	Wolf Spider	Wetland	C
52		*Hippasapisaurina*	Wolf Spider	Wetland	C
53		*Hippasapisaurina*	Wolf Spider	Wetland	C
54		*Lycosa barnesi* Gravely	Wolf Spider	Wetland	C
55		*Lycosa bistriata* Gravely	Wolf Spider	Wetland	C
56		*Lycosa choudhuryi*	Wolf Spider	Wetland	R
57		*Lycosa fuscana* Pocock	Wolf Spider	Wetland	C
58		*Lycosa poonaensis*	Wolf Spider	Wetland	R
59		*Lycosa poonaensis*	Wolf Spider	Wetland	R
60		*Lycosa prolifica* Pocock	Wolf Spider	Wetland	C
61		*Pardosa annandalei*	Wolf Spider	Wetland	C
62		*Pardosa annandalei*	Wolf Spider	Wetland	C
63		*Pardosa birmanica*	Wolf Spider	Wetland	C
64		*Pardosa birmanica*	Wolf Spider	Wetland	C
65		*Pardosa timida* (Simon)	Wolf Spider	Wetland	C
66		*Pardosa timida* (Simon)	Wolf Spider	Wetland	C
67		*Pardosa minutus*	Wolf Spider	Wetland	C
68		*Pardosa minutus*	Wolf Spider	Wetland	C
69		*Pardosa timida* (Simon)	Wolf Spider	Wetland	C
70	OECOBIIDAE(2)	*Oecobius marathaus*	Tiny Spider	Bushland	C
71		*Oecobius marathaus*	Tiny Spider	Bushland	C
72	OXYOPIDAE(9)	*Oxyopes bharatae* Gajbe	Lynx Spider	Grassland	R
73		*Oxyopes biharensis*	Lynx Spider	Grassland	UN
74		*Oxyopes burmenicus*	Lynx Spider	Grassland	C
75		*Oxyopes chittrae*	Lynx Spider	Grassland	C
76		*Oxyopes elongates*	Lynx Spider	Grassland	C
77		*Oxyopes pankaji* Gajbe	Lynx Spider	Grassland	C
78		*Oxyopes pankaji* Gajbe	Lynx Spider	Grassland	C
79		*Peucetia viridana*	Lynx Spider	Grassland	C
80		*Peucetia viridana*	Lynx Spider	Grassland	C
81	PHOLCIDAE(2)	*Artemaatlenta*	Cellular Spider	On Wall	C
82		*Pholcus phalangioides*	Cellular Spider	On Wall	C
83	SALTICIDAE(11)	*Marpissa decorata*	Jumping	All habitats	C
84		*Marpissa dhakuriensis*	Jumping	All habitats	R
85		*Myrmarachne maratha*	Jumping	All habitats	UN
86		*Myrmarachne maratha*	Jumping	All habitats	UN
87		*Phidippus pateli* Tikader	Jumping	All habitats	C
88		*Phidippuspaykulli*	Jumping	All habitats	C
89		*Plexippus paykullii*	Jumping	All habitats	C
90		*Plexippus paykullii*	Jumping	All habitats	C
91		*Rhene indicus Tikader*	Jumping	All habitats	C
92		*Telamonia dimidiata*	Jumping	All habitats	C
93		*Telamonia dimidiata*	Jumping	All habitats	C
94	SPARASSIDAE(2)	*Heteropoda venatoria*	Giant Crab	Debris	C
95		*Heteropoda venatoria*	Giant Crab	Debris	C
96	TETRAGNATHIDAE(4)	*Leucauge decorata*	Water orb weaver	Pond area	C
97		*Leucauge fastigata*	Water orb weaver	Pond area	C
98		*Tetragnatha mandibulata*	Water orb weaver	Pond area	C

Continue Table 1

99		*Tetragnatha mandibulata*	Water orb weaver	Pond area	C
100	THERIDIIDAE(4)	*Argyrodes gouri*	Cob web Spider	Pond area	R
101		*Argyrodes gouri*	Cob web Spider	Pond area	R
102		*Theridion manjithar*	Cob web Spider	Pond area	UN
103		*Theridion manjithar*	Cob web Spider	Pond area	UN
104	THOMISIDAE(14)	*Thomisuspugillis*	Crab Spider	Garden	C
105		*Thomisus pugillis*	Crab Spider	Garden	C
106		*Thomisus whitakeri*	Crab Spider	Garden	C
107		*Tmaruspachpediensis*	Crab Spider	Garden	R
108		*Xysticus jayantius*	Crab Spider	Garden	C
109		*Xysticus minutes*	Crab Spider	Garden	C
110		*Xysticus minutes*	Crab Spider	Garden	C
111		*Synaema decorata*	Crab Spider	Garden	C
112		*Synaema decorata*	Crab Spider	Garden	C
113		*Thomisuselongates*	Crab Spider	Garden	C
114		*Thomisus memae*	Crab Spider	Garden	R
115		*Thomisus beautifularis*	Crab Spider	Garden	UN
116		*Thomisuspooneus*	Crab Spider	Garden	C
117		*Thomisus projectus*	Crab Spider	Garden	C
118	ULOBORIDAE(3)	*Uloborus danolius*	Feather leg Spider	On Wall	C
119		*Uloborus danolius*	Feather leg Spider	On Wall	C
120		*Uloborus khasiensis*	Feather leg Spider	On Wall	R

C=Common; UN=Uncommon; R=Rare; =Female spider,=Male spider

RESULTS AND DISCUSSION

During the study we had reported 120 species of Spiders belonging to 14 Families and 37 genera from the different habitats of the Pohra-Malkhed reserve forest. Spiders' species were recorded from different 14 families viz Araneidae, Clubionidae, Eresidae, Gnaphosidae, Lycosidae, Oecobiidae, Oxyopidae, Pholcidae, Salticidae, Sparassidae, Tetragnathidae, Theridiidae, Thomisidae and Uloboridae. Abundance of the spiders species are arranged family wise with descending order. Orb Weaver (Araneidae) >Wolf Spider (Lycosidae) > Crab Spider (Thomisidae)> Jumping Spider (Salticidae) > Lynx Spider (Oxyopidae) (Fig.2, 3, 4&5).In this study two categories of spiders were observed, one was web weaver and another one was non web weaver. The web weaving spiders were belonging to the family Araneidae, Eresidae, Oecobiidae, Pholcidae, Tetragnathidae, Theridiidae, and Uloboridae.

Figure 2: Showing family wise spider species and their number

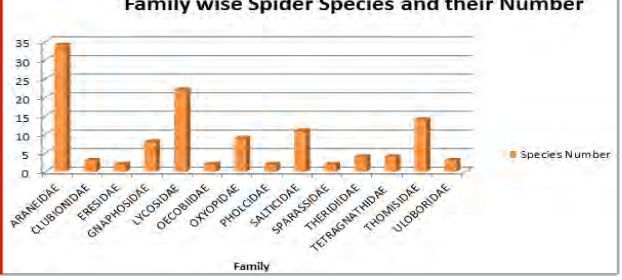


Figure 3: Showing common name wise spider –species with percentage

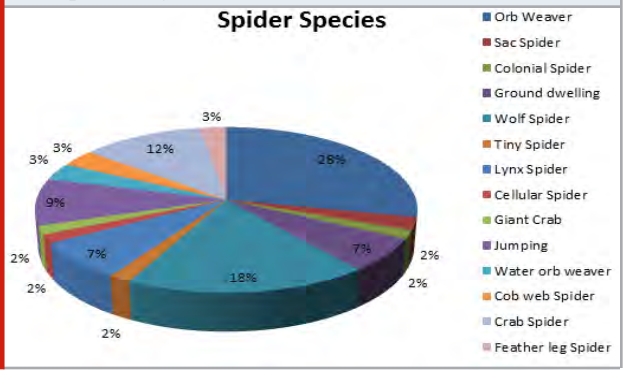


Figure 4: Showing habitat wise spider species and their number

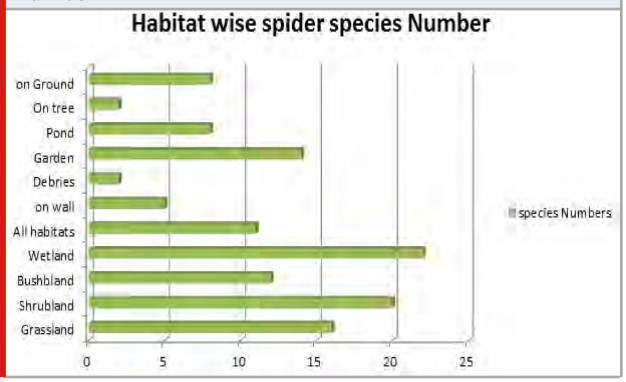
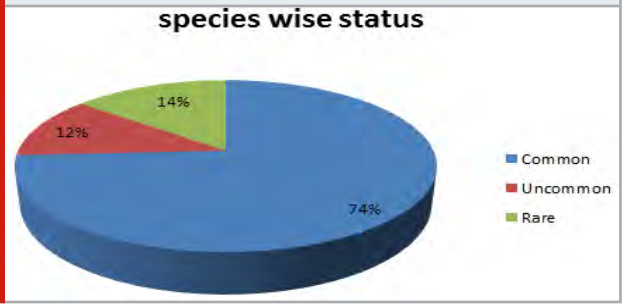


Figure 5: Showing encounter status of Spiders in the study Area



The non web weaving spiders were belonging to the family Clubionidae, Gnaphosidae, Lycosidae, Oxyopidae, Salticidae, Sparassidae and Thomisidae. The increase in the spider's density suggested that spider's density was influenced by the increase in prey density. In particular area, the interaction of prey and predator showed a constant numerical interaction about these relationships which was fundamental to biological control. Spiders are considered as the favorable biological control agents in the forest ecosystem (Rajeevan et al., 2019).

Wetland spiders are in large number than after Shrubland and below grassland spider species. Due to grazing habitat the grassland spider species are comparatively lower than wetland and Shrubland. The spider species in debris are very much low and in wetland spiders species are more in numbers. In Western Ghats, Wayanad region, Kerala, India survey total 150 species belonging 73 genera under 20 families were recorded, where spiders of Family Salticidae (44) are dominant where as in our study of Pohra-malkhed reserve forest we were observed total 120Species belonging 37 genera under 14 families, where spiders of Family Araneidae (34) are dominant (Rajeevan et al., 2019).

CONCLUSION

During investigation we have studied 120 species belonging to 37 genera of 14 spider Families. The present work includes the Taxonomic position and list of diversified species of spiders. The major families abundant in this forest are Araneidae 34, Lycosidae 22, Thomisidae 14, Salticidae 11, and Oxyopidae 09. The study shows information related to the species distribution in a particular habitat with response to the environment and availability of food. On the above result and discussion, it is clear that the Spiders are very much important creature and beneficial bio-control agent in the Forest ecosystem. Spiders are an integrated part of all ecosystems and contribute to the balanced ecosystem evidently due to their predatory potential. This study provides updated checklist and base-line data of spider diversity from Pohra-Malkhed Reserve forest in Amravati district of Maharashtra State. Study area shows great diversity of spiders.

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