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Continued Inside Cover

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Continued From Back Cover

Bioscience Biotechnology Research Communications
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About the Journal

Bioscience Biotechnology Research Communications, BBRC is a broad based internationally indexed official publication of Society for Science & Nature (SSN) since 2008. The international journal publishes peer reviewed original research papers, exciting reviews and short communications in basic and applied areas of life sciences and the upcoming state of the art technologies, including Biology and Medicine on a fast track. The young editorial team of BBRC tries hard to provide a high quality flawless format of scientific communication for the popularization and advancement of science, worldwide. During these 10 years more than 1000 peer reviewed research papers of very high quality have been published in BBRC and authors like Kiran Shaw Majumdar of Biocon, Bangalore have contributed to BBRC helping it achieve high readership in a short span of time. Reviewing the published research articles, it becomes evident that on an average, about 7 papers out of 10 are subjected to healthy revisions in BBRC making quality reading. We owe this achievement to our reverend reviewers! We hope the standards set by BBRC will improve further making this international journal unique and easily accessible to the scientific fraternity across the globe. In its tenth year of successful existence as a scholarly publication, BBRC has now become an open access Thomson Reuters ISI ESC/Clarivate Analytics USA Indexed journal also approved by University Grants Commission (UGC) Ministry of Human Resource Development, Government of India, New Delhi and has a NAAS-2017, Government of India, Indian Council of Agricultural Research (ICAR) New Delhi rating of 4.38 and SJIF (2017) 4.196.

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The journal offers an international peer reviewed fast track platform, encouraging contributions from research students, faculty members and academicians from developing countries. Manuscripts in the following areas of Bioscience and Biotechnology are considered for rapid publication:

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- Biotechnology, Bioinformatics, Proteomics and Nanotechnology
- Microbiology, Pathology, Immunology and Diagnostics
- Physiology, Endocrinology, Biochemistry and Biochemical Engineering
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Mini Review

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Ethics in Publishing: An Opinion on its Importance

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ABSTRACT

A research effort actually comes in to effect when it is published and becomes available to the scientific community; however, publishing it after following the required ethics is an equal challenge. Since last few years, a number of agencies specifically medical organizations and publishing editors have released several guidelines to resolve the issues regarding the conflicts related to publications and resolve the issues. In this communication we provide the required guidelines about publication ethics in a simple form so that they can be easily followed. This article may contribute towards spreading the awareness in the researchers regarding publication ethics may diminish the research misconduct.

INTRODUCTION

Assumption, interpretation and experimentation are the basic pillars of science. In the past, thousands of scientists worked in several fields without any social boundaries (Maqbool et al. 2014). Every person should have the authority to utilize scientific innovations for a favorable life style. A shared understanding between the government policies, scientists and industries is required for research upgrading. Innovative ideas should be largely shared among the scientific community to benefit the entire mankind. Publication is the best way for sharing ideas, innovations and views to the scientific world. It is last and most crucial phase of

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a successful research project. It is the outcome of long term efforts on careful preparations and experimental analysis (Derntl 2014). As most of the research projects are directly or indirectly supported through public investment, it should be developed for the benefits of entire society. A detailed description of the performed procedures should be universally accessible and easily acceptable. As a particular objective is responsible for raising so many scientific aims consequently affecting the entire world, publications should be comprehensive, truthful, correct, and fair and should not give deceptive information (Titus et al. 2008; Shaw and Erren 2015; Resnik and Shamoo 2017).

ROOTS OF A RESEARCH PUBLICATION

Consistency in Methodology

The researchers should follow all the ethical and legislative guidelines while accomplishing the research. Standard methodology should be employed for data investigation. Information provided in the publications should be liability of all the authors (Wager and Kleinert 2010). Researchers should systematically control and organize the research specifically for conclusions, statistical facts and data presentations (Wager and Kleinert 2013; Awais 2015).

Truthfulness in Data

Honesty is the best policy not only in life style but also in research. Data should not be fabricated or manipulated (Martinson et al. 2005). Related pictures should be presented in original form without any ambiguous modification (White 2007). All the efforts should be made to explain the methodology and results in a way so that it can be followed by other researchers. All the information including contradictory and strange outcomes against the expected results should be presented in their original form. It is responsibility of all the authors to convey with editor on finding any gaffe in submitted or published articles. All authors should help in corrections of manuscript whenever it is asked by editor or reviewers (Wager and Kleinert 2010; Wager and Kleinert 2013; Awais 2015).

All the previous relevant works should be included and cited accordingly. However, only those citations should be included that is related to the work and can guide the future researchers to get a complete scenario of the scientific problem (Ball 2002; Poulton 2007; Kamat and Schatz 2014; Damineni et al 2015).

Innovativeness

The work submitted to any journal should be innovative and not submitted at two platforms at a time. If co-submitted or co-published, it should be brought in to notice of editors and reader (Uzun 2013). Several publications developed from the same project should be differentially presented with different information and benefits for the researchers. Copyright laws should be considered before publishing or reproducing any data. Statistical or other informative data included from previous publications should be precisely acknowledged. Authors should adhere to publisher guidelines and send the manuscript to one journal at a time. All the queries and publishing issues of editors' and reviewers' should be handled effectively (COPE 2009; Wager et al. 2009; Wager and Kleinert 2013; Awais 2015). Actual outcomes should be reflected in the entire work and should not include any unconfirmed results.

Precision or Accuracy/ Clarity/ Unambiguity

Authors should reveal all the equipments, funding resources and other aids employed in the research. Role of funding agencies or external supports in performing the research should be clearly mentioned in the publication (Masic et al. 2014). Authors should obey journal and institutional regulations and specify all the conflicts of interest (Dhammi and Haq 2017).

Proper Authorship and Acknowledgement

This is one of the most crucial and sensitive phases reflecting team work efforts in solving scientific problem. Authorship ascertains the liability and praise for the people who contribute to bring a scientific issue to a publication level (Marusic et al. 2006; Wager 2009; Wager and Kleinert 2013; Awais 2015).

Most of the guidelines emphasize that authorship should be given to a candidate only if he/she made a significant contribution in following:

- designing the research
- collecting and inferring the data
- drafting or critically revising the manuscript

People who perform the work and take the accountability for the research should get the credit and listed as authors.

Authorship, types and the number of publications should be discussed at the time of planning the research project (Claxton et al. 2005 a, b). At the time of writing a project proposal, decide on the things to be done, what will be done by whom and in what duration. A transparency and confirmation about each role should be maintained. Views of all the participants should be considered on the basis of their contribution in the work. A proof of these views and decisions should be kept. Every team must have a written conformity on authorship before the article is written. This is to diminish the disagree-

ment at final stage, when all the efforts have been put by the deserved members. The acknowledgments can be utilized to pay gratitude towards those who indirectly added to the work like providing the experimental material or research facilities etc. Utmost care should be taken that a person deserves to hold an authorship should not be included in the acknowledgement section (Graf et al. 2007; Peh and Ng 2009; Tarkang et al. 2017).

Articles addressing broader audience and that are beneficial for major parts of the society are mostly preferred for publication. Manuscripts that are innovative, noteworthy, well-planned and well written are the main focus of all the reputed journals.

CONCLUSION

Generally, articles on publication ethics emphasize on the responsibilities of publishing agencies, editors, academic institutes and authors to diminish the possibilities of plagiarism and research misconduct. However, limited discussion has been made on the ethics in allocation of authorship in publication. As authorship assignment is the foundation that may strengthen the thought of publication ethics at every step, it should be prioritize in research and publication world. Although publication agencies confirm the contribution of authors, they are naturally unable to control the cases where authorships are not allocated to worthy candidates. Hence, scientific groups and team of authors need to be righteous, understandable and answerable on publication ethics. As concluding remarks, publication ethics should be deeply considered by different players involved in the scientific world specifically authors who make it available to the society.

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Biophotonics and machine learning model for the diagnosis and treatment of HIV

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ABSTRACT

All over the world the scientists working in biophotons and biophotonic therapy gave the hope to those who have struggled with a disease that have not been treated. This method is inexpensive and shows no side effects prove better in diagnosis and treatment such as HIV transmission. Some biomedical techniques of HIV detection need photons as source of light. These results have been obtained by CCD cameras or highly modified digital systems. The noisy background in these pictures gave the idea of implementing machine learning models. They can be extremely fast, offer high degree of picture quality and differentiation or classification of molecules of interest. In this paper libSVM (Support Vector Machines) models are applied to classify CD4+ cells from whole blood cells with great accuracy.

KEY WORDS: BIOPHOTONIC THERAPY, CCD CAMERAS, CD4+ CELLS, MACHINE LEARNING, SUPPORT VECTOR MACHINES

INTRODUCTION

Fritz-Albert Popp proposed the theory of Biophotons which is a single quantum that is transmitted by living systems in a continuous and repeated cycle Popp (1996). The scientists and experts that are working in this area referred the radiation biophotons and systematic fields as Biophotonics. Biophotonics allows scientists to view and detect diseases such as HIV (Human Immunodeficiency Virus) transmission. This has led to a revival of interest in BioPhotonic Therapy (BPT) among medical practitioners worldwide regarding the ability of using ultraviolet (UV) light rays in the treatment of such disorders. BioPhotonic Therapy (BPT) is a process of exposing blood to ultraviolet (UV) light rays to stimulate the immune system to destroy any and all pathogens, whether they are viral, bacterial or fungal. BioPhotonic Therapy (BPT) is also known by other names such as Ultraviolet Blood Irradiation (UBI), Photoluminescence, Photopheresis, Photodynamic Therapy and Hematologic Oxidative Therapy. There have been over 1 million BPT treatments. BPT has proved to be highly effective in treating bacterial infections, including septicaemias, pneumonia, wound infections, peritonitis and typhoid.

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BPT presents an interesting and relatively low cost alternative for patients willing to try this therapeutic modality. This is close to nature therapy which gives hope to those who have struggled with a disease Dillon et al (2003) and Scientia Press (2015).

Recent research in the field of HIV-AIDS diagnosis or treatment by Biophotonics threw light in some of the important work does is described as: Kufa (2017) presented his research findings on laser-driven label-free approaches of detecting HIV-1, which is the most widespread type worldwide, in living biological cells. While laser technology is used in the detection and treatment of cancer cells, it is rarely used as a technological tool to investigate HIV-infected cells also used for diagnosis. Lemboumba and Kufa (2017), have focussed on the novel tag-free detection of HIV-1 infected and uninfected cells via Raman and transmission spectroscopy coupled with optical trapping, which are techniques used to improve the reliability of data when cells are being analysed. Compared to gold-standard HIV-1 diagnostics such as the enzyme-linked immunosorbent assay and nucleic acid based tests that require the use of labels and substrates respectively for the first time in HIV research, coupling of laser trapping with Raman spectroscopy allows a non-invasive immobilisation and label-free analysis of single infected cells was also studied. The optical system, essentially allows grabbing single cells at will and analysing their chemical fingerprint by merely shining laser light of varying wavelengths and carefully structured beams on them are also for diagnosis for diseases like HIV/AIDS," says Ombinda-Lemboumba (2017).

According to him, this detection technique, compared to the labelled detection technique, is more likely to provide accurate results within a short period of time at a cost-effective rate. "Addressing issues of accuracy, cost and time were important for the purpose of the biophotonics research. In light of HIV/Aids being a serious disease claiming the lives of many in developing countries, particularly in Sub-Saharan Africa, it is important that all the research presently going on addresses the challenges of the day," Mthunzi-Kufa (2017). Present HIV detection or ART response techniques are [A] Flowcytometry where lasers are source of light in terms of photon. [B] Fluorochromes, and [C] Photonic crystal biosensors which have been described as:

{A}FLOWCYTOMETRY: HIV infected person go through flow cytometry for detection of HIV infection. Throughout the progression of disease, T-cell subsets are followed as the best surrogate marker for immune status Stein (1992). An AIDS definition was established based on a CD4 T-cell count of less than 200 cells/ml centers for disease control (1993). As T- cell count decreases from 350 to 200, millions of viruses have started reproducing into the body, which gives indication of start of antiretroviral therapy. So accurate and reproducible measurement of T-cell subset during therapy is an important part of managing treatment for HIV infected patients BergeronM(1998). Current therapies add years of quality life to patients living with HIV. Monitoring through FC is as follows: Fresh whole blood with anticoagulant is required by immunophenotyping of T-cell subsets (viral load is also important tool for HIV detection). Fluorescein isothiocynate (FITC) is the most universal fluorochrome for phenotyping and R-phycoerythrin (PE) are the dyes excited using 488 nm light.

Other dyes are also used & multicolour applications make better understanding of distinguished components. And laser light is passed through. Various components of T-cell subsets can be identified with lymphocytes CD4/CD8 T-cells have been reported as % of lymphocytes. The antigens most often monitored on CD8 T-cells are CD57, CD 28 and CD38. NK cells (CD16) decreases with HIV infection, particularly in later phase of disease Hardy (1992) & Lucia et al 1995. Additional uses of FC in the detection of HIV infection includes evaluation of cell function such as activation, in vitro proliferation,cytotoxic T-lymphocytes responses, detection of various cytokine producing cells and measurement of in-situ HIV antigens and apoptosis. All these results have been monitored through fully automated computer system linked with FACS (Fully Activated Cell Sorter).

{B}.Fluorochromes like GFP (green fluorescent protein): This is obtained from jellyfish is of much importance as a probe to track recombinant proteins movements, location and expression in living cells, The recent successful creation of a replication competent clone of the HIV-1 containing GFP (HIV Gag-Igfp) has enabled, for the first time the direct visualization of viral spread between live cells Hubner et al (2007) and Hubner et al (2009). When a HIV-1 infected T cell and an uninfected CD4+ T-cell engage each other through interactions of HIV Env and CD4 they can form a virological synapse Chen (2007), Jolly (2004) Jolly and Sattentau (2005 and 2007), Fackler (2007).

HIV-1 viral proteins are then observed to rapidly and focally assemble virus particles at the point of cell-cell contact. Virus is subsequently transferred to target cell, leading to productive infection. This process is highly efficient and very rapid. Manipulating microscopic objects by optical tweezers utilizes changes in the momentum of photons to drive a target with a high index of refraction to the centre of tightly focused laser beam Ashkin (1970 & 1986). Once the cell has trapped in this fashion it will follow the light focus when moved. The use of near infra-red laser sources for optical tweezers enables the manipulation of non-adherent cells such as germ cells, red blood cells, and T-lymphocytes because

cells have no to little absorption at these wavelengths and the process is virtually non-invasive Chen (2008), Ozkan (2003), Nilsson (2009) Anveri (2004), Rao (2009), Shi (2009). Another advantage is that optical tweezers do not require open access to the sample or the use of needle aspirators, both of which significantly escalate the risk of accidental exposure when handling infections like HIV-1.

{C}Photonic Crystal biosensors: Flow cytometry and reverse transcription quantitative polymerase chain reaction (RT-Qpcr) are sensitive standards methods for CD4 cell count and viral load measurements to monitor ART, but they require complex laboratory infrastructure, expensive reagents, and skilled operators Wang (2010), Fiscus et al (2006). Several technologies have been developed for virus detection utilizing optical, electrical, and acoustic sensing methods such as surface Plasmon resonance (SPR), localized surface Plasmon resonance etc. A nanoplasmonic-based platform was developed to detect intact HIV-1 using self-assembled gold nanoparticles conjugated with biotinylated anti-gp120 antibodies through impedance spectroscopy of viral lysate samples Shafiee et al (2013). Among these approaches, photonic crystal (PC) biosensors offer a rapid and sensitive optical detection method for biomolecules, cells, and viruses by monitoring the dielectric permittivity changes at the interface of a transducer substrate and liquid media Shamah (2011). A PC biosensing platform was also developed that captures and detects intact viruses (HIV-1) as well as bio molecules and antibodies. Multiple HIV-1 subtypes (A, B and D) were detected in spiked samples with viral loads ranging from 10 4 to 10 8 copies/ml and validated with gold standard method (i.e. RT-q PCR) Shafiee et al (2014).

These techniques are used as they are described but researches would improve the way of diagnosis. Hence machine learning techniques are in advance position to enhance the quality of these photonics methods by reducing the time, manpower and accurately predict the results. In view of this here a method of machine learning is presented which surely threw a light on biophotonics research of diagnosis of diseases.

IMPLEMENTATION OF MACHINE LEARNING

The result of experiments done in FC and other techniques are analyzed by CCD Cameras or by automated computer system. These digital imaging technologies have the advantage of regularly capture high-content images that may contain the location of thousands of moving particles or molecules in a single cell. Due to low contrast and noisy background of the images, sometimes the molecules appear more like a shapeless blob than a distinct feature. This traditional method shows the failure of automated detection system. Thus researchers have sorted this problem by implementing machine learning. Computers are trained by algorithms that are capable to distinguish a molecule from what is not a molecule through training data. Haar features are used o make the distinction. This method is time consuming to extract such a molecular data. Haar features were developed in part to ease the computational burden arising from image analysis using such as pixel intensity alone. Haar methods look at rectangular regions in an image and sum up the number of pixels per region.

The resulting value then is used to classify the image and to categorize an area as either having a particle (molecule) or not, for example: in the case of particles within a cell, Haar features are a combination of the intensity, shape and size of the objects. To get around fluorescence intensity fluctuations in classifying objects, the researchers relied upon signal-to-noise ratios to decide which automatically identified particles were valid. They discarded those too low ratios and kept those with a ratio above a threshold. Support vector machines with kernel functions are able to distinguish molecules of interest with whole cells. Here our interest of molecule is CD4+ cells in whole blood cells. Flow cytometry and other techniques are applied while using fluorochromes to clearly distinguish CD4+ cells to understand its morphology, interactions with other cells and the image is taken by different intensities of cameras. The combination of SVM (machine learning) algorithms with above discussed methods leads to classify our interest molecule in better intensity images. For making this analysis possible, the training set consisting of 10pixel subwindows. Some are selected because they did not contain molecule of interest, i.e. negative sample. Others are selected as positive samples, i.e. images that contain molecules of interest. Using these training and testing samples (sets) support vector machine based classifier is developed to finally find the molecule of interest without noise, i.e. molecule is being categorized as a molecule.

Datasets and Methods: The protein data of CD4+ cells and whole blood protein have been taken from Uniprot/Swissprot database Uniprot 2016.

Dataset: For support vector machines dataset is needed. Here in our study two datasets are required. Dataset (1): This consists of CD4+cells which is our interested molecule i.e. the positive sample, to be separated from whole blood (whole sequence), the other sample which is marked as negative.

Dataset(2): This consist of 100 whole blood sequences which marked as positive and other sequences of any animal marked as negative..

Amino acid compositions of these two datasets have been taken. The amino acid composition is the fraction of each amino acid type within a protein.

The fractions of all 20 natural amino acids were calculated by using Equation 1,

$$= \frac{Total\ Number\ of\ amino\ acid\ i}{Total\ number\ of\ amino\ acids\ in\ a\ protein}$$

Support Vector Machine (SVM) a machine learning technique that has a potential for learning separating functions in pattern recognition (classification) tasks and in performing functional estimation in regression problems. It originated from the statistical learning theory (Vapnik 1995) and represents the novel learning techniques that were introduced in the framework of structural risk minimization and in the theory of Vapnik Chervonenkis dimension (VC) (1995). SVMs are a set of related supervised learning methods used for classification and regression. A classification task usually involves separating data into training and testing sets. Each instance in the training test contains one "target value" (i.e. class labels) and several "attributes" (i.e. the features or observed variables). The goal of SVM is to produce a model (based on the training data) which predicts the target values of the test data given only the test data attributes. SVM is a supervised machine learning method which is based on the statistical learning theory Vapnik (1995) and Wang (2004). When used as a binary classifier, an SVM will construct a hyperplane, which acts as the decision surface between the two classes. This is achieved by maximizing the margin of separation between the hyperplane and those points nearest to it. The SVMs were implemented using freely downloadable software, lib SVM, (Cheng 2001). In this software there is a facility to define parameters and choose among various inbuilt kernels. They can be radial basis function (RBF) or a polynomial kernel (of given degree), linear, sigmoid. Simulations were performed using LIBSVM version 2.89 (a freely available software package). For our study RBF Kernel was found to be the best. The SVM training was carried out by the optimization of the value of the regularization parameter and the value of RBF kernel parameter.

RESULTS AND DISCUSSION

The values of these two datasets are fed into LibSVM and analysis done for linearly separable data. The present work describes the use of LibSVM models for the classification of CD4+ cells of HIV than other cells present in blood. The performance of classifier is checked by following:

$$Accuracy = \frac{tp + tn}{tp + tn + fp + fn}$$

True positives [TP] and True negatives [TN] were identified as the positive and negative samples, respectively. False positives [FP] were negative samples identified as positive. False negatives [FN] were positive samples identified as negative. The prediction performance was tested with sensitivity [TP/ [TP+FN]], specificity [TN/ [TN+FP]], and overall accuracy.

Total Number of Instances in dataset: CD4+cells are 94. Which are correctly identified as positive out of 100.

SVM has the ability to separate between positives and negative instances. The table 1 shows FP is 0 then TP is 0.933 and if FP is 0.067 then TP is 1. The ROC is found to be 0.942 which correctly predicts the good quality of classifier. As it is inbuilt that if ROC > 0.5 then good classification as of FP or TP values lies between 0 and 1 respectively. Here ROC depicts the classification of CD4+ cells with an average of 0.942. Statistical analysis showed that blue points are marked as CD4+ cells as they are separated by other cells present in blood lymphocytes. The false positive rate was 4.44%. This technique is cost effective automated solution to detecting molecules of interest within living cells.

ROC curves depict the performance of a classifier without regard to class distribution or error costs. They plot the number of positives included in the samples on the vertical axis, expressed as a percentage of the total number of positives, against the total number of negatives on the horizontal axis. For each fold of a 10 fold cross validation ,weight the instances for a selection of different cost ratios train the scheme on each weighted set ,count the true positives and false positives in the test set, and plot the resulting point on the ROC axes. In diagnostic "accuracy" as a measure of decision performance require introduction of the concepts of the "sensitivity" and "specificity" of a diagnostic test. These measures and the related indices, "true positive fraction" and "false positive fraction," are more meaningful than "accuracy," yet do not provide a unique description of diagnostic performance because they depend on the arbitrary selection of a decision threshold. As a final

Table 1. Detailed Accuracy by Class

TP Rate	FP Rate	Precision	Recall	F-Measure	ROC Area	Class
0.933	0	1	0.933	0.966	0.942	Whole sequence
1	0.067	0.988	1	0.994	0.942	CD4+cells
0.989	0.056	0.989	0.989	0.989	0.942	Weighted avg.

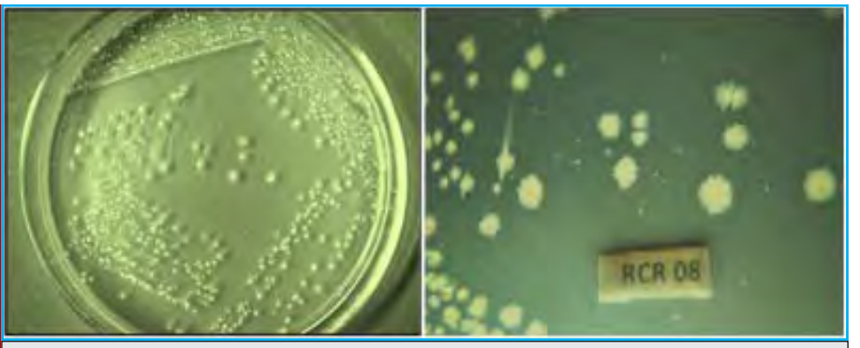

FIGURE 1. ROC for CD4+cells from other lymphocytes by LivSVM (WEKA3.7)

step, after molecule of interest (CD4+ cells) detection the data is further processed to extract the boundary and area of the particles for accurate tracking. Now this picture is compared with the results of manual approach to determine the true positive and false positive rates. Researchers found that the true positive rate averaged more than 98 %. Thus, more than 98 out of 100 times the algorithm successfully identified a molecule (CD4+cells) as a molecule.

CD4 cells are white blood cells that play an important role in the immune system. The CD4 cell count gives us an indication of the health of immune system. This is our body's natural defence system against pathogens, infections and illnesses. Several cohort studies and clinical trials have shown that the CD4 count is the strongest predictor of subsequent disease progression and survival Egger et al (2002) & Mellors et al (1997). The use of the CD4 count as an independent and reliable marker for treatment outcome is attractive from various aspects. First, CD4 counts are already the most important factor in deciding whether to initiate antiretroviral therapy and opportunistic prophylaxis. All HIV-positive patients in high-income countries and an increasing number of patients in low-income countries have a baseline CD4 count at entry into care Panel of Antiretroviral guidelines for Adult and Adolescent (2008). Second, the CD4 count is a relatively objective and simple marker to follow. Finally, the cost of CD4 counts has become more affordable, including in developing countries Lutwama (2008) & Mac Lennan (2007). This article further evaluates the use of the CD4 count in assessing the clinical status of HIV-infected individuals, in making informed decisions regarding the initiation of antiretroviral therapy and in monitoring by the use of machine learning in jointly working with biophotonics

CONCLUSION & FUTURE ASPECTS

The study shows the handshake of biophotonics and machine learning techniques for diagnosis and treatment of diseases like HIV-AIDS in a better way with high speed and great accuracy. The future of biophotonics research and development is very bright. It will be necessary to bring together the wide spectrum possibilities offered by biophotonics and machine learning methods in conjunction with other biomedical techniques to develop solutions to existing clinical problems. Laser systems and fluorochromes are the possible form of light or photons that are implemented as light source for diagnosis or therapy. Nature always shows the path of light in constructive way, similarly if we will be able to utilize the constructive properties of light, the further progress is surely seen. The demand of todays is biophotonics with machine learning brings high speed, low cost and non-invasiveness in diagnosis and treatment of diseases. The advances in technology are being coupled with biology and medicine to revolutionize healthcare.

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Hand hygiene practice among laboratory workers in selected hospitals in Saudi Arabia

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ABSTRACT

One of the major challenges facing medical institutions is how to reduce healthcare-associated infections. The World Health Organization (WHO) (2009) observes that ignoring hand hygiene is the leading cause of nosocomial infections in various hospitals. Therefore, it is essential to address hand hygiene techniques that can be used to reduce incidences of healthcare-associated infections. This study aims to evaluate hand hygiene practices in Saudi Arabia as well as how it can be improved to reduce nosocomial infections within the institutions. This study finds that education has effectively improve the hospital laboratory workers hand hygiene practices. The study also holds that the leadership of the medical organizations also plays an essential role in supporting as well as promoting hand hygiene practices. As such, it is the finding of the current study that improving hand hygiene through the management is critical in facilitating reduction of hospital-acquired infections.

KEY WORDS: HAND HYGIENE, LABORATORY WORKERS, LEADERSHIP, MANAGEMENT, NOSOCOMIAL INFECTIONS

INTRODUCTION

Increasing nosocomial infections is one of the major challenges facing the medical community. Magill et al. (2014) reported that hand contamination comprises of about forty percent of nosocomial infections among immunocompromised patients. WHO estimates that close to 1.5 million individuals around the world suffer from infections acquired from medical institutions. Nosocomial infection is believed to be the leading cause of morbidity and mortality among hospitalized patients (Zingg et al. 2015). The most vulnerable of this group

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is the neonates and patients with severe illness because of their intrinsic susceptibility to infections. Hospital laboratories stand as one of the main sources of cross contamination of pathogenic microbes. It is essential to realize that various common laboratory operations produce microbe-filled droplets, thus contaminating the working surface, workers hands, fingers as well as wrist (Sickbert-Bennett et al. 2016, Jones et al., 2017).

Once the hands have been contaminated in the laboratory, the worker can inadvertently carry the pathogens outside the laboratory. They may also accidentally transfer pathogens to other parts of their body such as eyes, mouth or nose. In as much as there are a number of safety standards proposed for laboratory procedures to help prevent infections, an effective engineering control preventing hand contamination is yet to be realized. Ellingson et al. (2014) observed that in the past, gloves have been used in laboratories to limit skin contact. However, the gloves are known to leak and in some cases, workers are likely to spread contamination to their hands as they remove the gloves. Smiddy, O'Connell and Creedon (2015) argue that there are categories of microorganism that can be found in the hands of a medical professional including transient flora and the resident flora. Transient flora is a microorganism that health care workers get from the external environment. According to Abdella et al. (2014), the microorganisms have the capability to survive on the human body or in some cases reproduce themselves. On the other hand, the resident flora is a microorganism that is represented by permanent microorganisms living on the skin surface or immediately under the skin surface of an individual (Al-Asmari and Nooh 2017).

Such microorganisms can survive on the skin of an individual and freely grow on it. It is also essential to observe that the resident flora have low pathogenicity as well as infection rate. Kapil, Bhavsar and Madan (2015) observes that they are some sort of protection from the colonization of other more pathogenic bacteria. Among such microorganisms is the Micrococci, Staphylococcus Pittosporum among many other microorganisms. It is essential to note that the objective of observing hand hygiene in laboratory settings is to remove the transient flora with effective and careful hand washing practices. Sansam (2016) argues that this can be achieved through the use of various kinds of soap such as antiseptics as well as alcohol-based gels. Whether hand washing practices meet their intended objective significantly depends on the worker's quality of hand washing performed.

According to Sakihama et al. (2016), the factors that influence the quality of the hand washing done include the scrubbing duration, amount of friction applied as well as the lathering of soap. It is essential to observe that among these factors the duration taken to scrub the hands is the most essential given that the longer one takes to scrub their hands the higher the likelihood of them getting rid of a significant portion of the bacterial count from their hands. It is the objective of the current study to evaluate hand hygiene practices among hospital laboratory workers in various medical institutions in Saudi Arabia.

MATERIAL AND METHODS

Questionnaire design: A list of questions designed to study a category. It is one of the most common search tools, also called poll. There are several common patterns of questions, including:• "Yes" or "No" questions, which were accompanied with the "maybe" option or the "do not know" option.• Optional questions: which include either one or several possible answers.• Evaluation questions of different scales. We designed our hand hygiene questionnaire from two work from the World Health Organization and Minnesota hospital association. We summarized our questionnaire objectives into four parts as follows:

Questions on hand hygiene education and practice using alcohol-based hand rub.

Questions on the medical laboratory workers' hand hygiene practices.

Questions on the workers' opinion on how effective various actions would be to improve hand hygiene permanently in their institution. The preference of workers regarding reminding them to perform hand hygiene. The first part contained two questions ("Yes" or "No" questions), the second part contained nine questions (Evaluation questions), the third part contained seven questions (Evaluation questions) and the fourth part contained one Optional question.

Data analysis: Figure 1 shows the methodology flowchart, we divided it into three stages, Stage I focused on development our questionnaire, which had reliability analysis for selected questions. Cronbach's Alpha was 0.757 after we delete third question which belong to the second objective, then we applied correlation test between each objective and their questions. Stage II focused on sample selection and survey administration, we applied our questionnaire on 53 on medical laboratory specialist and technologist who worked in different hospital in Saudi Arabia. Stage III focused on data analysis and reporting, statistical analysis was performed using IBM SPSS Statistics for Windows, Version 22.0 software was used for statistical analyses. We applied Likert Scale analysis for all evaluation questions and the association between demographic characteristics and questionnaire results were analyzed using the Chi-square test of association with confidence interval 95%.

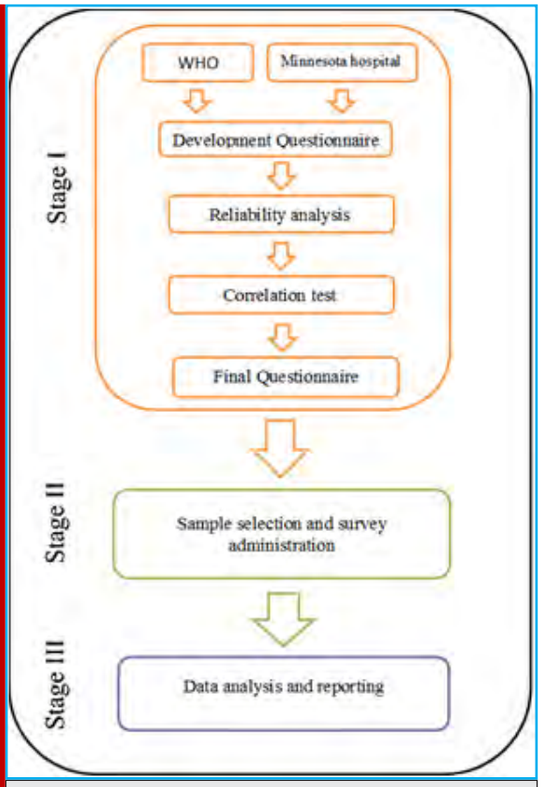

FIGURE 1. Methodology flowchart

RESULTS

Based on the objective of the current study, the instrument used for data collection was divided into three segments. The participant's socio-demographic composition was first determined. This was followed by evaluating the participant's personal experience with hand sanitation as well as the factors that influence their hand hygiene practices. Finally, the study evaluated the management influence in facilitating hand hygiene among their laboratory workers. As such, the current study utilized 53 workers drawn from various hospital laboratories in Saudi Arabia as participants to inform the study. Of the selected workers, 33 were laboratory technologist while 20 were laboratory specialist making up 62.3% and 37.7 percent of the study participants respectively. It is also critical to note that the study participants were made up mostly of male occupying 71.7% of the participants involved in the research. Of the 53 workers, a majority of them were observed to be between the ages of 30-40 make up for about 56.6% of the study participants. Participants in the age group of 20-30 who made up about 38% of the total participants followed this closely (Table 1).

An analysis of the participant's response reveals that a majority of the participants have received education in hand hygiene in the last three years of their profession. Satisfactorily, the study realized that all the participants involved in the study utilized alcohol-based hand rub for hand hygiene routinely (Table 2). Using the Linkert scale of 1-5, the study also realized that slightly over half the participants (56.6%) required limited effort to perform good hand hygiene. A majority of the participants also observed that hospital-associated infections were a major concern within their hospital as it negatively influenced the outcome of the patients. As such, 64.2% of the participants composed of 34 workers agree that hand hygiene is essential in preventing healthcare-associated infections. Hand hygiene among the institutions of the study participants is therefore considered as a primary priority that every employee within the institutions should is required to observe. Over half of those who participated in the study argue that hand hygiene is automatic to them and thus need to reminder to it. It was also pointed out by a majority of the participants that their patients, head of departments as well as colleagues placed significant importance on their hand hygiene practices (Table 3).

An analysis of the leader's influence on hand hygiene among the participants reveals that their senior managers supported and openly promoted hand hygiene practices. This was achieved through various means such as the provision of alcohol-based hand rubs as well as the use of posters to remind the hospital personnel of

Table 1. Socio-demographic analysis

Parameter	Characteristics	Frequency	Percentage
Lab Position	Laboratory specialist	20	37.7%
	Laboratory technologist	33	62.3%
Gender	Female	15	28.3%
	Male	38	71.7%
Age	from 20 - 30	20	37.7%
	from 30 - 40	30	56.6%
	from 40 -50	2	3.8%
	from 50 -60	1	1.9%

Table 2. Questions on hand hygiene education and practice using alcohol-based hand rub.

Question	Yes (%)	No (%)
Have you received education in hand hygiene during the last three years?	51 (96.2%)	2 (3.8%)
Do you routinely use an alcohol-based hand rub for hand hygiene?	53 (100%)	0 (0%)

the significance of hand hygiene. The study also realized that the participant's medical institutions provided training on hand hygiene as well as had clear instructions on the practice instilled in their employees. Despite the strides taken by the hospital management to enhance hand hygiene within their institution, the study realized that a number of the institutions rarely provide their employees with feedback on their hand hygiene performances. On the other hand, patients are rarely invited to remind medical professionals to perform hand hygiene. This the study can attribute to the fact that over half of the participants preferred to be reminded about hand hygiene by their managers followed closely by their peers (Table 4 and 5).

DISCUSSION

In the current study, the frequency of the participant's education regarding hand hygiene was evaluated. The results show that a majority of the study participants regularly received education regarding hand hygiene. Rosenbluth et al. (2016) argue that educating medical professionals on the significance of hand hygiene plays an essential role in influencing them to observe and practice hand sanitation. As such, it becomes essential for every health practitioner to receive regular education on hand sanitation to avert the spread of hospital-acquired infections (Deyneko et al. 2016). From the study's results, it was also realized that all the participants used alcohol-based hand rubs for hand hygiene. According to Li, Wang, Yan and Rao (2015), alcohol-based hand rubs is considered as the most effective form of maintaining hand hygiene within medical institutions.

From the findings of the present study, it is apparent that a majority of the participants are aware of the impact of hospital-acquired infections on the outcome of their patients. This is in line with Luangasanatip et al. (2015) assertion that hospital-acquired infections

Table 3. Questions on the medical laboratory workers' hand hygiene practices.

Question	1	2	3	4	5
What effort is required for you to perform good hand hygiene?	30 (56.6%)	11 (20.8)	5 (9.4)	3 (5.7)	4 (7.5)
What is the impact of a health care-associated infection on a patient's clinical outcome?	1 (1.9%)	0 (0%)	7 (13.2%)	16 (30.2%)	29 (54.7%)
What is the effectiveness of hand hygiene in preventing health care-associated infection?	1 (1.9%)	0 (0%)	4 (7.5%)	14 (26.4%)	34 (64.2%)
Among all patient safety issues, how much of a priority is hand hygiene at your institute?	2 (3.8%)	1 (1.9%)	6 (11.3%)	16 (30.2%)	28 (52.8%)
What importance does the head of your department attach to the fact that you perform optimal hand hygiene?	2 (3.8%)	1 (1.9%)	3 (5.7%)	20 (37.7%)	27 (50.9%)
What importance to your colleagues attach to the fact that you perform optimal hand hygiene?	1 (1.9%)	3 (5.7%)	6 (11.3%)	14 (26.4%)	29 (54.7%)
What importance do patients attach to the fact that you perform optimal hand hygiene?	3 (5.7%)	5 (9.4%)	5 (9.4%)	9 (17.0%)	31 (58.5%)
Is hand hygiene automatic or do you need to remember or be reminded to do it?	5 (9.4%)	3 (5.7%)	7 (13.2%)	10 (18.9%)	28 (52.8%)

Table 4. Question on the workers' opinion on how effective would various actions be to improve hand hygiene permanently in their institution.

Action	1	2	3	4	5
Leaders and senior managers at your institution support and openly promote hand hygiene	0 (0%)	0 (0%)	9 (17%)	12 (22.6%)	32 (60.4%)
The health care facility makes alcohol-based hand rub always available at each point of care	0 (0%)	1 (1.9%)	7 (13.2%)	13 (24.5%)	32 (60.4%)
Hand hygiene posters are displayed at point of care as reminders	0 (0%)	1 (1.9%)	10 (18.9%)	17 (32.1%)	25 (47.2%)
Each health care worker receives education on hand hygiene	1 (1.9%)	1 (1.9%)	3 (5.7%)	11 (20.8%)	37 (69.8%)
Clear and simple instructions for hand hygiene are made visible for every health care worker	0 (0%)	0 (0%)	10 (18.9%)	16 (30.2%)	27 (50.9%)
Health care workers regularly receive feedback on their hand hygiene performance	0 (0%)	1 (1.9%)	5 (9.4%)	14 (26.4%)	33 (62.3%)
You always perform hand hygiene as recommended (being a good example for your colleagues)	1 (1.9%)	1 (1.9%)	3 (5.7%)	23 (43.4%)	25 (47.2%)
Patients are invited to remind health care workers to perform hand hygiene	12 (22.6%)	7 (13.2%)	16 (30.2%)	7 (13.2%)	11 (20.8%)

also referred to as nosocomial infection is one of the major challenges facing medical institutions. Yokoe et al. (2014) argue that a majority of healthcare-associated infections are as a result of medical professionals ignoring hand hygiene practices. Their observation is backed by the current study as over 64.2% of the participants agree that hand hygiene plays an essential role in preventing healthcare-associated infections. As such, hand hygiene is the leading priority among medical institutions looking to avert cases of nosocomial infections.

With a majority of the participants observing that hand, hygiene is a leading priority in their institutions, it is apparent that their heads of departments consider it as an essential component in enhancing patient safety. Brewster et al., (2016) argues that departmental heads play a critical role in facilitating hand hygiene in medical institutions as they are the ones that influence and guide the operations of their departments. Apart from the departmental heads, it is also critical for patients as well as every other medical professional to observe adherence to optimal hand hygiene within the hospital (Septimus et al., 2014). Based on the findings, of the study, it is apparent that the participant's colleagues, as well as patients, placed significance on optimal hand hygiene to reduce healthcare-associated infections. Having everyone involved in observing hand hygiene within the medical institution is critical as it helps reduce carriers and infection rate within the medical institutions (Jones et al., 2017).

Table 5. The preference of workers in regards to reminding them to perform hand hygiene.

Who do you want to remind you to perform hand hygiene? (Multiple answers selected)		
Manager	Peer	Patients
46	40	24

In as much as every individual should be involved in ensuring hand safety within the medical institution, the management and senior executives within the hospitals also play an essential role in facilitating hand hygiene in their institutions. Banach et al., (2015) argue that leadership plays a critical role in enhancing hand hygiene given that they are the ones that determine and implement the policies used to guide the operations of the organizations. As such, the leadership of every medical institution should openly promote and support hand hygiene within their institutions to avert the increasing cases of nosocomial infections, (Smiddy et al., 2015). This assertion is proven in the present study as its findings indicate that the medical institutions have leaders and senior managers that support and promote hand hygiene.

To effectively support and promote hand hygiene in medical institutions, it is essential for the management of the institutions to ensure that alcohol-based hand rubs are available in almost every point of care (Nair et al., 2014). This is apparent in the study as the participants

indicate that the provision of such facilities plays an essential role in facilitating hand hygiene permanently in their institutions. Apart from providing alcohol-based hand rubs at each point of care, Kirk et al. (2015) also note that hand hygiene can be promoted through the use of posters displayed at points of care. Such posters act as reminders not only to medical professionals but to their patients as well to observe and maintain hand hygiene to avert the spread of nosocomial infections, (Thoa et al. 2015). This assertion is proven in the study as over 60% of those who participated in the study agree that posters at points of care play a critical role in reminding them to maintain and encourage hand hygiene in their institutions.

Chassin et al., (2015) state that it is also essential to have clear and simple instructions for hand hygiene made visible for every healthcare worker. Like the posters, the instructions are meant to remind and encourage the medical professionals to observe and maintain hand hygiene. The current study affirms this as a majority of the participants agree that having clear instructions visible for them assist them in improving hand hygiene permanently in their institutions. Shlomai et al., (2015) also demonstrate that for executives to enhance hand hygiene in their institutions, their employees need to receive feedback on their hand hygiene performance regularly. This helps them realize the areas that they need to improve on as well as those that they need to maintain. From the findings of the current study, it is apparent that feedback helps the participants realize their performance regarding hand hygiene thus proving why it is essential in promoting the practice.

CONCLUSION

One of the major challenges facing the medical industry is nosocomial infections also referred to as healthcare-associated infections. The primary cause of these infections has been observed to be as a result of poor hand hygiene practices found in various medical institutions. As such, the objective of the current study was to evaluate the hand hygiene practices found in medical institutions in Saudi Arabia as well as how to improve hand sanitation permanently. It is essential to note that the study observes that education plays an essential role in facilitating hand hygiene among medical practitioners. As such, it becomes essential that medical professionals are frequently trained and educated on the significance of hand hygiene in reducing healthcare-associated infections. The findings of the study also indicate that the institution's management also plays an essential role in facilitating hand hygiene within their medical institutions. As such, the study holds that to improve hand hygiene in various medical institutions in Saudi Arabia permanently, it is essential for the management of every medical institution within the country to be actively involved in promoting and supporting the practice.

AUTHOR CONTRIBUTION

MA has collected the data and designed the manuscript. MIW has made the statistical data analysis. All authors wrote the manuscript.

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Review on bacterial production of alkaline pectinase with special emphasis on *Bacillus* species

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ABSTRACT

Pectinases consist of an exclusive group of enzymes which catalyze the degradation of pectic polymers present in the plant cell walls. Today, pectinases are the upcoming industrially important enzyme having major industrial importance and they hold a leading position among the commercially produced industrial enzymes. Microorganisms including yeast, bacteria, actinomycetes and a large number of filamentous fungi are commonly recognized as the best natural sources for the production of pectinase enzyme. The chief source of acidic pectinases is fungi but alkaline pectinases are produced from alkalophilic bacteria, primarily *Bacillus* spp. The alkaline pectinase has developed as important commercial enzymes with far-flung applications mainly in textile processing, bio-scouring of cotton fibers, degumming and retting of fiber crops, pretreatment of pectic wastewater etc. This review discusses the microbial production of pectinases with special emphasis on bacterial pectinase from *Bacillus* spp.

KEY WORDS: PECTINASE, *BACILLUS*, POLYGALACTURONASE, PECTATE LYASE, PECTIN LYASE

INTRODUCTION

Pectinases comprises of a unique group of enzymes which catalyze the degradation of pectic polymers present in the plant cell wall. They belong to the family of polysaccharidases that contribute to the breakdown of pectins from a variety of plants and are also known as pectolytic or pectic enzymes (Prathyusha & Suneetha, 2011). In the current biotechnological era, pectinase are one of the forthcoming enzymes showing progressive increase in their market. They maintained the average annual growth rate of 2.86% from 27.6 million $ in 2013 to 30.0 million $ in 2016 and it is estimated that by 2021, the market size of the pectinase will reach 35.5 million $ (Global Pectinase Market Research Report, 2017).

The pectinase enzymes are classified based on their mode of action against the galacturonan backbone of pectins. Primarily, there are two groups of pectic enzyme; the de-esterifying enzymes which catalyses the deesterification of pectins and the depolymeriz-

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ing enzymes which break the glycosidic α-(1-4) bonds between GalA residues either by hydrolysis (hydrolases) or by transelimination (lyases). Moreover, the latter two types of enzymes are classified on the basis of whether they exhibit a preferential hydrolytic /transelimination power against pectin, pectic acid or oligogalacturonate as the substrate and whether the pattern of action is random (endo-) or terminal (exo-). Another group of the pectic enzyme is protopectinase (PPase) which convert insoluble native protopectin into soluble pectins (Alkorta *et al*., 1998; Kashyap *et al*., 2001; Sharma *et al*., 2013; Garg *et al*., 2016; Hassan & Ali, 2016). The classifications of pectic enzymes acting on pectic substances are given in Figure 1.

Based on the optimum pH for enzyme activity, pectic enzymes are also classified into two, acidic and alkaline pectinase. Research and application of acidic pectinase are more established compared with alkaline pectinase. The chief source of acidic pectinase is from fungus *Aspergillus niger*. Acidic pectinases are used mainly in fruit juice industry for extraction and clarification of fruit juices, improvement of chromaticity and stability of red wines etc. They are also having application in maceration of plant tissue, liquefaction and saccharification of biomass, isolation of protoplasts. Whereas, alkaline pectinase are mostly used in the degumming and retting of fiber crops, textile processing and bio-scouring of cotton fibers, pretreatment of pectic wastewater from fruit juice industries, paper making, coffee and tea fermentation, enzyme based oil extraction etc. The important applications of pectinase are shown in Figure 2. Bacteria, mainly *Bacillus* spp. are used for the production of alkaline pectinase (Kashyap *et al*., 2001; Jayani *et al*., 2005). Information regarding pectinase types, structure, applications, substrate etc. has been reviewed previously by many authors (Alkorta *et al*., 1998; Kashyap *et al*., 2001; Hoondal *et al*., 2002; Jayani *et al*., 2005; Prathyusha & Suneetha, 2011; Sharma *et al*., 2013; Kohli *et al*., 2015; Hassan & Ali, 2016). This review emphasizes reports on pectinase production by *Bacillus* spp.

Microbial production of pectinase: It is a well-known fact that, microbes are the prominent source of enzymes because they allow an economical technology with truncated resource consumption and low emission involving no social and political issues, as in the case of animal and plant sources (Chaplin & Bucke, 1990). It is reported that, fifty percent of accessible enzymes are initiated from fungi and yeast; 35 % from bacteria, while the remaining 15 % are either of plant or animal origin. Filamentous microorganisms are most widely used for pectinase production (Soares *et al*., 1999). Microbes are chosen as a source of enzyme production compared to plants and animals because; (a) they produce a wide

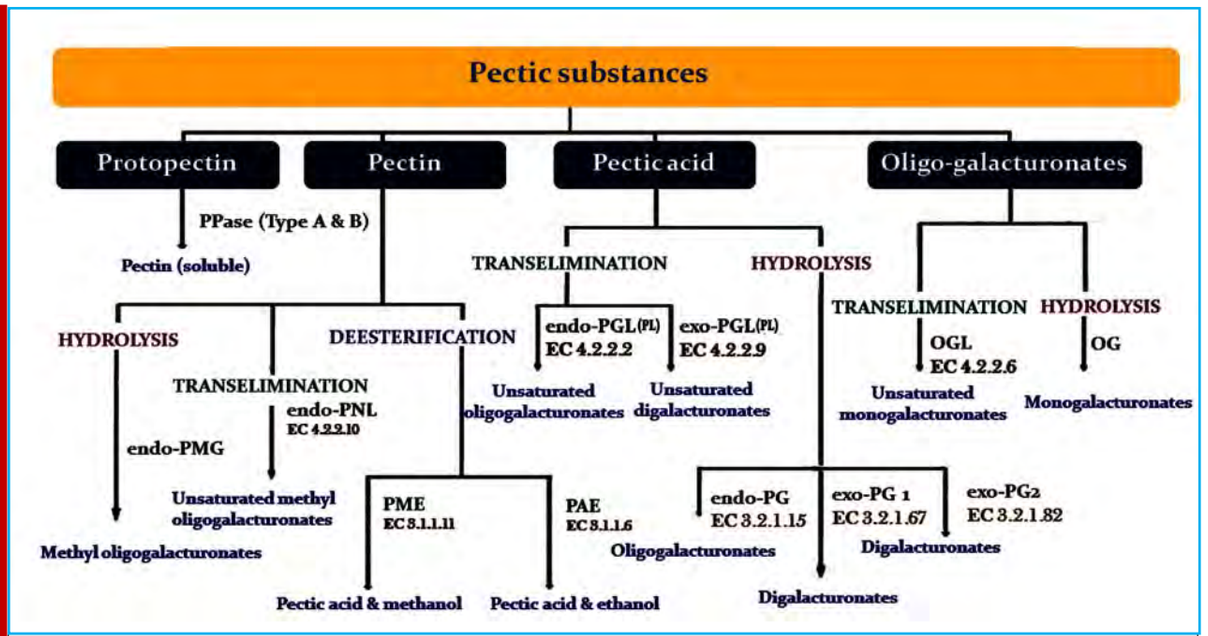

FIGURE 1. Classification of pectic enzymes acting on pectic substances – data modified from (Alkorta *et al*., 1998; Jayani *et al*., 2005) PMG: Polymethylgalacturonase, PNL: Pectinlyase, PGL: Polygalacturonatelyase, PN: Pectate lyase PME: Pectin methylesterase, PAE: Pectin acetyl esterase, OGL: Oligogalacturonidelyase, OG: Oligogalacturonase

FIGURE 2. Applications of pectinases in various industries – a schematic representation

variety of enzymes and their enzyme contents are more predictable and controllable, (b) generally economical in bulk production and dependable provisions for raw material of constant composition, (c) high productivity rate and enzymes obtained via microbial source are greater in volume, (d) microbes are easy to manipulate to derive enzymes of desired nature and they can be cultured in large quantities in a relatively short period of time by the established method of fermentation using sophisticated tools, (e) they can be made to produce enzymes over wide range of environmental condition and (f) plant and animal tissues contain more potentially harmful materials than microbes, including phenolic compounds (from plants), endogenous enzyme inhibitors and proteases (Chaplin & Bucke, 1990).

The production of pectin degrading enzymes has been extensively reported and meticulously studied in bacteria and filamentous fungi because they play an essential role in the phytopathogenesis (Blanco *et al.*, 1999). Several reports are available regarding pectinase enzymes by the microorganism such as bacteria, fungi, yeast and actinomycetes. They are also distributed in higher plants and in some protozoa, nematodes and insects but they are not found in higher animals (Jayani *et al.*, 2005; Pedrolli *et al.*, 2009; Sharma *et al.*, 2013).

Pectinase production by fungi: Filamentous fungi have been used for more than 50 years in the production of industrial enzymes (Dalboge, 1997). Many extracellular enzymes are produced by fungi which are capable of decomposing organic matter and one such enzyme is pectinolytic enzymes. Filamentous fungi are considered as one of the most potent producers of pectinases and they can be employed extensively in SSF for the economic production process. Various types of fungal species have been reported to be employed for the production of pectinases. *Aspergillus niger* is the most commonly used fungal species for industrial production of pectinolytic enzymes (Gummadi & Panda, 2003). Findings on the isolation, characterization, selection, properties and fermen-

tation of *A. niger* strains for the production of pectinolytic enzymes using different substrates was made by different workers (Finkler *et al*., 2017). Other species of *Aspergillus* were also reported to produce pectinase including *A. oryzae A. fumigatus, A. terreus, A. soje, A. awamori* etc (Pedrolli *et al*., 2009; Garg *et al*., 2016). Production of endo-PGL was firstly reported in *A. giganteus* (Pedrolli & Carmona, 2014). Further, species of *Penicillium, Fusarium, Mucor, Neurospora crassa*, *Sclerotinia sclerotiorum* etc also have role in pectinase production (Pedrolli *et al*., 2009; Pedrolli & Carmona, 2014; Garg *et al*., 2016). The fungus produces these enzymes to break down the middle lamella in plants so that it can extract nutrients from the plant tissues and insert fungal hyphae.

Pectic enzyme production in yeasts: The pectinolytic enzyme production in yeasts has received less consideration and only a few yeast species show this ability (Blanco *et al*., 1999). The first reports of pectinolytic (endo-PG) production by yeasts were described by Luh and Phaff in 1951 in *Saccharomyces fragilis*. Among different yeast species representing all yeast genera, they found that only six (*S. fragilis, S. fragilis* var. no. 351, *S. thermantitonum, Torulopsis kefyr, Candida pseudotropicalis* var. *lactosa*, and *Candida pseudotropicalis*) were capable of causing a noticeable change in pectin (Luh & Phaff, 1951). Recent reports are also added to describe the ability of Yeast species for pectinase production these includes; *Saccharomyces sp., Cryptococcus sp., Aureobasidium pullulans, Rhodotorula dairenensis, Kluyveromyces marxianus, Geotrichum klebahnii, Wickerhanomyces anomalus* etc. (Alimardani Theuil *et al*., 2011; Merin *et al*., 2015; Hassan & Ali, 2016; Naumov *et al*., 2016).

Bacterial production of pectinase: A review of the currently available literature reveals little quantitative information about the diversity of bacterial genera having pectinolytic properties. Bacterial pectinase is produced mainly by bacteria belonging to genera *Bacillus* and *Erwinia*. Elyrod in 1942 reported that the bacterium *Erwinia* sp. can degrade pectin with the aid of pectin degrading enzymes (Elrod, 1942) and in the past decade, bacterial pectinase biosynthesis has been extensively studied in phytopathogens, especially in the soft-rotting *Erwinia* species *E. carotovora* and *E. chrysanthemi* which are reported to produce a set of pectinolytic enzymes such as PL, PG, PME, and PAE (Matsumoto *et al*., 2003).

Chessen *et al*., 1980 reviewed that bacteria like *Bacillus*, *Pseudomonas* and *Micrococcus* isolated from retting flax, jute, sisal and coir, and *Erwinia* from coffee fruits have shown to possess the ability to degrade pectin by producing pectinolytic enzymes (Chesson, 1980). Other bacterial genera reported to have pectinolytic properties include species of *Pseudomonas* (Sohail & Latif, 2016), *Streptomyces* (Ramirez-Tapias *et al*., 2015) *Lactobacillus* (Karam & Belarbi, 1995) etc.

Importance of bacterial production of pectinase: *Aspergillus niger*, a GRAS microorganism is the major organism used for the industrial production of pectinase. However, this mold also secretes several other enzymes which may trigger collateral reactions such as the release of volatile phenols less desirable for the production of wine or fruit juices, for instance arabinofuranosidase, which can cause turbidity (Whitaker, 1990). Pectinases from fungal sources are produced best under acidic pH and low temperature conditions and can therefore not be used in various industrial bioprocesses which utilize neutral to alkaline pH with high temperatures exceeding 45°C. It has been shown that bacteria produce pectinase that withstands high pH and temperature (Hoondal *et al*., 2002; Andrade *et al*., 2011). Also, it is easy to harvest pectinase than fungus as it is an extracellular product in bacterial culture (Sohail & Latif, 2016). Bacterial strains producing commercial enzymes are always preferred over fungal strains because of ease of fermentation process (for production) and implementation of strain improvement techniques or any modern technique to increase the production yield (Prathyusha & Suneetha, 2011). Moreover, bacterial pectinases with novel properties have the added advantage that enzyme production is achieved in less time as compared to fungal sources (Joshi *et al*., 2015).

***Bacillus* species for industrial enzyme production:** Among the diverse types of microorganisms inhabiting in the soil, bacteria are the amplest and major organism. Considering the bacterial genera of soil, Bacilli are most abundant followed by Cocci and Spirilla. The genus *Bacillus* and *Cocci* comprises several varieties of industrially important species contributing approximately half of the existing commercial production of bulk enzymes (Priest, 1977; Aaisha & Barate, 2016). *Bacillus* species have been the imperative industrial enzyme producers with roles in applied microbiology for over a millennium.

Because of several reasons *Bacillus* species continue to be the predominant bacterial workhorses in microbial fermentations (Schallmey *et al*., 2004; Satyanarayana.T *et al*., 2005). They produce more than two dozen of biologically active molecules generating a high potential for biotechnological and biopharmaceutical applications (Stein, 2005). Also, it is estimated that enzymes from *Bacillus* spp. makeup about 50 % of the total enzyme market (Schallmey *et al*., 2004). Another major feature that makes these groups predominant is that most of them are environmental friendly, don't have fastidious nutritional requirements and are easy to grow and handle (Sohail & Latif, 2016). *Bacillus* species such as *B. subtilis* and *B. licheniformis* are on the Food and Drug

Administration's GRAS (generally regarded as safe) status (Food and Drug Administration, 1999; Schallmey *et al*., 2004). Moreover, the biochemistry, physiology, and genetics of *B. subtilis* and other species are well studied and the complete genome sequence of *B. subtilis* 168 comprising of 4100 protein-coding genes has been published in 1997 (Kunst *et al*., 1997).

Various properties of *Bacillus* strains are reported which make them superior in industrial biotechnology, including their high growth rates leading to short fermentation cycle times, ability to secrete proteins into the extracellular medium (Acton, 2012; Barros *et al*., 2013) and their ability to adapt with changing environmental and nutritional conditions (Buescher *et al*., 2012; Nicolas *et al*., 2012). The capacity of selected *Bacillus* strains to produce and secrete large quantities (20–25 g/L) of extracellular enzymes has placed them among the most important industrial enzyme producers (Satyanarayana. *et al*., 2005). Moreover, many researchers used strains belonging to *Bacillus*, because this genus includes strains (such as *B. subtilis*) that can grow on cheap substrates such as agro-wastes (Sakai *et al*., 1989).

Pectinase production by *Bacillus* spp.: Pectic enzymes are of functional relevance in the retting process and evidence regarding pectinolytic properties of *Bacillus* spp. was recorded years ago. Different species of the genus *Bacillus* have been reported to be retting agents and active against pectic materials (Potter & McCoy, 1955). Nortje and Vaughn, 1953 tested the pectinolytic activity of *B. subtilis* and *B. pumilus* in relation to the softening of olives and pickles (Nortje & Vaughn, 1953). The first *in vitro* fermentation studies of pectin and pectic acid was reported in 1955 using *B. polymyxa* strain 30 (Potter & McCoy, 1955). Over the past few years, pectinolytic properties have been described in several *Bacillus* species.

It is evident from many research works that, among different bacterial isolates screened for pectinolytic properties *Bacillus* strains were selected as the most potent enzyme producers (Soares *et al*., 1999; Jayani *et al*., 2010; Rehman *et al*., 2012; Kavuthodi *et al*., 2015; Sohail & Latif, 2016). As mentioned in the introduction, alkaline pectinases have a wide variety of industrial applications and, bacteria mainly *Bacillus* spp. are the chief producers. Apart from this fact, there are also some other reasons for researchers to focus on pectinase from *Bacillus* spp. These include; (i) they produce all class of pectic enzymes, (ii) have short fermentation period for enzyme production, (iii) can produce enzymes very economically by using different agro-wastes as cheap substrates, (iv) fermentation can be attained by either SSF, Smf and (v) genetic information regarding pectinase genes of many *Bacillus* spp. are available in various nucleotide sequence databases. Thus it supports successful cloning and expression of pectinase gene in other organisms.

Pectic enzymes from *Bacillus* sp.: *Bacillus* spp. are renowned producers of all classes of pectinases including hydrolases, lyases, esterases and protopectinases. The important strains of *Bacillus* reported for different groups of pectinases are listed under;

Hydrolases: The pectic hydrolases are a type of depolymerases that split the α-(1,4)-glycosidic bonds between galacturonic monomers by hydrolytic cleavage releasing *mono-*, *di-*, *oligo* galacturonates. These enzymes have been divided into four groups; those acts on pectate were called polygalacturonases (PG), while those preferentially hydrolyze pectin were called polymethylgalacturonases (PMG). In connection with their mode of action on the galacturonic acid backbone, the prefixes *endo-* and *exo-* used (Alkorta *et al*., 1998). It is a member of glycosyl-hydrolases family 28 (Pedrolli *et al*., 2009). These are one of the most studied and widely used pectinases which has high potential in commercial applications especially in fruit juice industry.

Bacillus strains are inferred to be the potent sources of exo-PG (Nagel & Vaughn, 1961; Kobayashi *et al*., 2001; Jahan *et al*., 2017). The ability of *B.polymyxa* in PG production was reported in 1961 (Nagel & Vaughn, 1961). A number of *Bacillus* species including *B. subtilis, B. pumilus* and *B. licheniformis* were reported to have PG production ability. A novel, alkaline and thermostable PG produced from an environmental isolate *Bacillus* sp. MG-cp-2 was reported by Kapoor *et al*., in 2000 and its application in degumming of ramie and sunn hemp bast fibres was also revealed in the next year (Kapoor *et al*., 2000; Kapoor *et al*., 2001). Further, the ability of this isolate to produce PG by both SmF and SSF was also confirmed (Kapoor & Kuhad, 2002). Reports regarding a high molecular weight (115000 Da) alkaline exo-PG in a culture of *Bacillus* sp. strain KSM-P576 was publicized in 2001(Kobayashi *et al*., 2001). The ability of *B. sphaericus* for PG production was initially reported by Jayani *et al*., 2010 (Jayani *et al*., 2010).

Among several bacterial strains isolated from soil and rotten vegetables, the strain which produced maximum PG was identified as *B. licheniformis* KIBGE IB-2 (Rehman *et al*., 2012). Also, the PG produced from this isolate was immobilized in chitosan for continuous degradation of pectin polymers (Rehman *et al*., 2014). Further, properties of the enzyme were characterized and reported that the PG is having thermal stability and is able to perform its catalytic activity in a diversified environment (Rehman *et al*., 2015). Pectinolytic (PG) properties of a new soil isolate *B. subtilis* C4 was reported in 2014 by Kusuma and reddy (Kusuma & Reddy, 2014a). Further, after purification and characterization of the enzyme,

they suggested that there was a threefold increase in the specific activity of PG produced by this isolate (Kusuma & Reddy, 2014b). PMG activity from *Bacillus* sp. strain BR1390, a novel environmental isolate was presented in the same year (Rastegari & Karbalaei, 2014).

Bacillus spp. were also reported for endo PG production. Endo-PG, exo-PG and PNL activities of five *Bacillus* strains isolated from decaying vegetable material was studied by Sores *et al*., 2001 and these enzymatic solutions resulted in maximal reduction of the solution of citrus pectin viscosity, between 80 and 97 % (Soares *et al*., 2001). The molecular weight of endo-PG produced by *B.subtilis* was found around the range of 67 kDa (Munir & Haidri, 2015).

Lyases/trans-eliminases: Lyases or trans-eliminases cleave α (1, 4)-glycosidic linkages by transelimination resulting in galacturonide with a double bond between C-4 and C-5 at the non-reducing end. They split the glycosidic bonds of either pectate (endo- and exopolygalacturonate lyase -PGL) or pectin (polymethylgalacturonate lyase - PMGL). Up to the recent times, all described pectin lyases were endo-PGL (Alkorta *et al*., 1998). The first report of an exo-pectin lyase was in 2014, in the fungus *A. giganteus* (Pedrolli & Carmona, 2014). But the enzyme has not yet been reported in *Bacillus* sp. These enzymes are classified into the polysaccharide lyase family 1 (CAZy database). PGL has an absolute requirement of Ca2+ ions whereas PMGL requires Ca2+ and other cations only for its stimulation (Jayani *et al*., 2005).

Production, applications, biochemical and molecular characteristics of lyases acting on pectic polymers have been reviewed previously (Dubey *et al*., 2016). Kelly and Fogarty, 1978 reported PL production from an isolated soil bacterium *Bacillus* sp. RK9 and noted that hydrolysis of substrate occurred in a random fashion and the enzyme was 50% more active towards acid soluble pectic acid than towards sodium polypectate (Kelly & Fogarty, 1978). *B. stearothermophilus* with pectinolytic activity has been isolated by Karbassi and Vaughn, 1980 and found that it is producing a considerable amount of endo-polygalacturonic acid *trans*-eliminase (*endo*-PATE) (Karbassi & Vaughn, 1980). They also noted that enzyme produced by this organism was much more heat stable than previously reported similar enzymes from the mesophilic *B. polymyxa* (Nagel & Vaughn, 1961) and the thermotolerant *B. pumilus* (Dave *et al*., 1976).

Details regarding a bacterial strain (*Bacillus* sp. PN33) producing large amounts of extracellular PNL was revealed in 1998 where, the maximum activity was found at acidic pH of 6 and is an unusual example for bacterial PNL (Kim *et al*., 1998). Another high yielding pectinase strain, *Bacillus* sp. DT7 producing alkalothermophilic PNL with a shorter incubation period of 24 hr was reported in 2000 (Kashyap *et al*., 2000). A low-molecular-weight (20,300 Da), high-alkaline PL was found in an alkaline culture of *Bacillus* sp. strain KSM-P15 by Kobayashi *et al*., 1999 and suggested that this may be a novel enzyme that belongs to a new family (Kobayashi *et al*., 1999). Later, it was also reported that the strain KSM-P15 produces high alkaline PL with high molecular weight (70,000 Da) (Ogawa *et al*., 2000).

A thermophilic bacterial strain of *Bacillus* sp. with endo-PL activity has been isolated by Tako *et al*., 2000 and noted that it had PPase activity, besides PL activity on lemon protopectin and cotton fibers (Takao *et al*., 2000). A novel alkalophilic strain of *B. pumilus* BK2 producing a new type of extracellular endo- PL with high pI and a high pH optimum was reported in 2006 (Klug-Santner *et al*., 2006). The PL producing ability tested from a group of six *Bacillus* species (*B. subtilis*, *B. pumilus*, *B. sphaericus*, *B. cereus*, *B. thuringiensis*, and *B. fusiformis*) isolated from cocoa fermentation, it was revealed that *B. fusiformis, B. subtilis*, and *B. pumilus* species were the best PL producers compared to other species (Ouattara *et al*., 2011).

Esterases: The major component of pectic polysaccharides is homogalacturonan (HG), constituting about 65% of the pectin and contains (1→4) linked α-D-galacturonic acids (1,4-d-GalpA) which can be acetylated or methyl esterified, called smooth regions of pectin. The group pectin esterases comprises of pectin methylesterase (PME) and pectin acetyl esterase (PAE). The enzyme PME catalyzes reactions according to the double-displacement mechanism and its types, mode of action, structure etc. were reviewed previously (Kohli *et al*., 2015) whereas PAE hydrolyzes the acetyl ester from the HG region of pectin forming pectic acid and acetate (Remoroza *et al*., 2014). These enzymes act before the action of PG and PL which need non-esterified substrates (Kashyap *et al*., 2001). These are a well-studied group of enzymes, which belong to carbohydrate esterase (CE) family 8 of CAZy database (Remoroza *et al*., 2015).

The PME gene of *E. crysanthemi* B374 was successfully cloned in *B. subtilis* in1991 (Heikinheimo *et al*., 1991) and its purification and characterization were also reported (Pitkanen *et al*., 1992). A recombinant acetylesterase from *B. licheniformis* DSM13 was purified and biochemically characterized by Remoroza *et al*., 2014 and reported to deacetylates a wide range of acetyl-rich pectins (Remoroza *et al*., 2014). Later they also reported PME from the same organism that de-methylesterifies lemon pectin and sugar beet pectin in a stepwise manner (Remoroza *et al*., 2015).

Protopectinases: *Bacillus* sp. was also reported to produce PPase, an enzyme that liberates water-soluble pec-

tic substances by restricted hydrolysis from water-insoluble protopectin in plant cell walls (Sakai *et al*., 1989; Sakai & Sakamoto, 1990; Sakamoto *et al*., 1994; Takao *et al*., 2000). There are two types of protopetinases based on their reaction mechanism; A-type and B-type. A-type PPases react reacts with the polygalacturonic acid region of protopectin (inner site) and the B-type PPases react with the polysaccharide chains that may connect the polygalacturonic acid chain and cell wall constituents (outer site) (Sakai *et al*., 1993). A-type PPases are again classified in to PPase-F, -L and –S based on the organism they isolated and all these types are having an approximate molecular weight of 30 kDa. B-type PPases are also in different types such as PPase- B, -C and –T. PPase-B, -C and -T have molecular weights of 45, 30, and 55 kDa, respectively. PPase-B and -C have an isoelectric point (pI) of around 9.0 whereas PPase-T has a pI of 8.1 (Gundala & Chinthala, 2017).

Saki *et al*., (1989) used some strains belonging to the genus *Bacillus* such as; *B. subtilis, B. amyloliquefaciens, B. cereus, B. circulans, B. coagulans, B. firmus, B. licheniformis, B. macerans*, and *B. pumilus*, to study the production of PPase and concluded that *B.subtilis* IFO 12113 produce a new type of 'PPase -B' in respect to its reaction mechanism of the solubilization of protopectin, since it does not catalyze the degradation of polygalacturonic acid (Sakai *et al*., 1989). Further, they also reported 'PPase-C' (does not react with rabbit antiserum against PPase -B) from *B. subtilis* IFO 3134, (Sakai & Sakamoto, 1990). Later, from the culture filtrate of *B.subtilis* IFO 3134 Sakamoto *et al*., 1994 discovered two pectinolytic enzymes PPase-N and PPase-R. But, according to their substrate specificities and modes of action, these could be respectively classified as endo-PL and endo - PNL (Sakamoto *et al*., 1994).

The major strains of *Bacillus* spp. that has been reported recently for pectinase production with their characteristic temperature and pH are shown in Table 1.

Shorter fermentation period for enzyme production: The time required for the production of pectinase by *Bacillus* spp. are remarkably less when compared to other microbial pectinases reported in the literature (Kashyap *et al*., 2000). Literature review indicates that maximum pectinase production through many strains of *Bacillus* can be attained within 48 h of fermentation period. The optimum condition for pectinase production by *B. firmus* was recorded at a fermentation time for 18 h and the enzyme production was declined after 30 h (Roosdiana *et al*., 2013). Whereas, pectinase production by *B. subtilis* SS started after 18 h of incubation and the production reached highest at 24 h (Ahlawat *et al*., 2009). Alkaline pectinase production by *B. subtilis* WSHB04-02 showed an optimum fermentation time of 25 h (Wang *et al*., 2007). Maximum pectinase production by *B. pumilus* dcsr1 was attained in 30 h through SmF using fermenter (Sharma & Satyanarayana, 2006) while enzyme production by *B. gibsonii* S-2 reached highest in 48h (at 35 °C) through SSF (Li *et al*., 2005).

Economical enzyme production utilizing agro-wastes as substrates: Pectinase production from *Bacillus* spp. can be also achieved economically by utilizing different agro-waste as substrate. It is proved that orange peel waste can be used as a sole carbon source for pectinase production by various strains of *Bacillus* (Kapoor *et al*., 2000; Embaby *et al*., 2014; Tepe & Dursun, 2014; Kaur & Gupta, 2017). Wheat bran is another substrate, proven as a cheap and easily available source throughout the year for higher pectinase production and many researchers used wheat bran as an economical carbon source for pectinase production by *Bacillus* spp. Among various agro-byproducts studied for PG production by *Bacillus* sp. MG-cp-2, it is found that PG production level was boosted significantly by using wheat bran and ramie fibre in the production media (Kapoor *et al*., 2000). *Bacillus* strains cultivated on wheat bran produced endo-PG, exo-PG and PNL in the crude enzymatic solution (Soares *et al*., 2001). A high yield of pectinase (PG) was attained from *B. licheniformis* KIBGE IB-21 (Rehman *et al*., 2012) and *B. licheniformis* KIBE-IB3 using wheat bran as substrate (Jahan *et al*., 2017). Various other agro-industrial wastes such as; rice bran, cassava bagasse, sugar beet pulp, carrot peels etc. are also exploited for pectinase production by *Bacillus* spp. (Ghazala *et al*., 2015; Nawawi *et al*., 2017).

Mode of fermentation by either SmF or SSF: Pectinase production using *Bacillus* sp. can be attained by either SSF, SmF or by both. High quantities of PG is produced by *Bacillus* strains cultivated by SmF and semi-SSF (Soares *et al*., 1999). Multiple pectinase enzymes activities such as endo-PG, exo-PG and PNL was showed by SSF culture filtrate of *Bacillus* strains (Soares *et al*., 2001). Improved PG from *Bacillus* sp.MG-cp-2 under SmF and SSF and effect of amino acids and their analogues, vitamins and surfactants in fermentation was reported in 2002 (Kapoor & Kuhad, 2002). Medium optimization was found to effective in both SSF and Smf for pectinase production using *Bacillus* strains (Ghazala *et al*., 2015; Bibi *et al*., 2016; Kaur & Gupta, 2017). Zou et al., 2014 developed a new Fed-Batch fermentation for enhanced production of alkaline PGL using *B. subtilis* 7-3-3. The process combines the enzymatic pretreatment of the carbon source with controlled pH of the fermentative broth to enhance enzyme production in a cheap manner (Zou *et al*., 2014).

Cloning and expression of pectinase gene: Genetic information regarding pectinase genes of many *Bacillus*

Table 1. Recently reported *Bacillus* spp. producing different types of pectinase

No.	*Bacillus* species	Type of pectinase	Characteristics		References
			pH	Temp (°C)	
1	*B. subtilis*	Pectinase	8.0	50	(Torimiro & Okonji, 2013)
		Endo -PG	5	60	(Munir & Haidri, 2015)
2	*B. subtilis* KSM-P358	Exo -PG	8	55	(Sawada *et al*., 2001)
3	*B. subtilis* EFRL 01	PG	8	45	(Qureshi *et al*., 2012)
4	*B. subtilis* (TCCC11286)	PL	9	50	(Liu *et al*., 2012)
5	*B. subtilis168*	PL	9.5	50	(Zhang *et al*., 2013)
6	*B. subtilis C4*	PG	9	60	(Kusuma & Reddy, 2014b)
7	*B. subtilis 7-3-3*	PGL	6.5	34	(Zou *et al*., 2014)
8	*B .subtilis* BKDS1	PG, PNL,PL	8	40	(Kavuthodi *et al*., 2015)
9	*B. subtilis* AD11	Pectinase	8.42	30	(Nawawi *et al*., 2017)
10	*B. subtilis* SAV-21	Pectinase, PNL			(Kaur & Gupta, 2017)
11	*B.subtilis* Btk27	Pectinase	7.5	50	(Oumer & Abate, 2017)
12	*Bacillus sp.* N16-5	PL	11.5	50	(Li *et al*., 2010)
13	*Bacillus sp.* SMIA-2		10	60-70	(Andrade *et al*., 2011)
14	*Bacillus sp.* strain BR1390	PMG	6	60	(Rastegari & Karbalaei, 2014)
15	*Bacillus sp.* ZGL14	pectinase	8.6	50	(Yu *et al*., 2017)
16	*Bacillus sp* ZJ1407	pectinase	5	37	(Yu & Xu, 2018)
17	*B. pumilus* (NRRL B-212)	Exo-pectinase	8	30	(Tepe & Dursun, 2014)
18	*B. pumilus*	Exo-pectinase	8	30	(Tepe & Dursun, 2014)
19	*B. pumilus* (ATCC 7061)	PL	8	65	(Liang *et al*., 2015)
20	*B. licheniformis* KIBGE IB-21	PG	7	37	(Rehman *et al*., 2012)
			8-10	45	(Rehman *et al*., 2015)
21	*B. licheniformis* SHG10	PG	8	37.8	(Embaby *et al*., 2014)
22	*B. licheniformis* DSM-13	PAE	8	50	(Remoroza *et al*., 2014)
		PME	8	50	(Remoroza *et al*., 2015)
23	*B. licheniformis* KIBGE IB-3	PG	7	37	(Jahan *et al*., 2017)
24	*B. stearothermophilus*	Pectinase	7.5	60	(Torimiro & Okonji, 2013)
25	*B. cereus*	Pectinase	8.5	37	(Namasivayam *et al*., 2011)
26	*B. cereus*	Pectinase	8	50	(Torimiro & Okonji, 2013)
27	*B. sphaericus* (MTCC 7542)	PG	6.8	30	(Jayani *et al*., 2010)
28	*B. megaterium AK2*	PL	8.5	50	(Mukhopadhyay *et al*., 2012)
29	*B. clausii*	PNL	10	60	(Li *et al*., 2012)
30	*B. firmus*	PG	7	50	(Roosdiana *et al*., 2013)
31	*B. halodurans* M29	Pectinase	10	80	(Mei *et al*., 2013)
32	*B. tequilensis* SV11	PL	9	60	(Chiliveri & Linga, 2014)
33	*B. mojavensis* I4	Pectinase	8	60	(Ghazala *et al*., 2015)
34	*B. vallismortis* (JQ990307)	PG			(Sohail & Latif, 2016)

spp. are available in various nucleotide sequence databases. Thus it supports successful cloning and expression of pectinase gene in other organisms. The most cloned pectinase gene from *Bacillus* sp. is PL gene '*pel*'. The first cloning and characterization of a *pel* gene from the *Bucillus* genus was reported in *B.subtilis*. The cloned gene indicated a 1,260 bp open reading frame (ORF) encoding a 420 amino acid polypeptide which includes a 21 amino acid (aa) signal sequence. Molecular weight is found to be 45,605 Da and the purified enzyme had similar properties to the PL isolated from extracellular media of the organism (Nasser *et al.,* 1993). Since

then many researchers reported the successful cloning, expression, sequencing and characterization of pectinase genes (especially PL) from several *Bacillus* strains (Li *et al*., 2010; Dubey *et al*., 2016).

The gene encoding the PNL of *B. subtilis* IFO 3134, has been cloned, sequenced, and characterized in 1996 and it consists of a coding sequence of 345 aa and expression of the PNL in *E.coli* was also reported (Sakamoto *et al*., 1996). The gene encoding the exo-PG (pehK) of *Bacillus* isolate was cloned and characterized in 2001. The cloned gene revealed a 2940 ORF consist of 980 aa (signal sequence -27 aa and mature protein -953 aa) (Sawada *et al*., 2001). A highly alkaline thermostable pectinase from *B. halodurans* M29 was cloned and expressed in *E.coli*. The expressed enzyme showed high thermostability and long half-life and only 54 % sequence similarity to known enzymes and thus considered novel (Mei *et al*., 2013). The gene encoding *Bacillus* PAE was also cloned and expressed in *E. coli* (Bolvig *et al*., 2003; Remoroza *et al*., 2014).

PRESENT SCENARIO

The reports on pectinase production by *Bacillus* spp. are still continuing with latest findings. Kavuthodi *et al*., 2015 isolated and identified the most potent pectinolytic bacterial strain as *B.subtilis* BKDS1 and found to produce pectinases (PG, PL & PNL) also, coproduction of biosurfactant along with pectinase was reported (Kavuthodi *et al*., 2015). Pectinase production by *B. mojavensis* I4 using carrot peels and its application in sesame seeds oil extraction was reported in 2016 (Ghazala *et al*., 2015). In the same year, it was reported that among different microbial species screened for pectinase production, most prominent pectinase producing isolates were *Bacillus* sp. and identified as *B. firmus, B. coagulans, B. endophyticus* and *B. vietnamensis* (Aaisha & Barate, 2016). Extracellular pectinase production and its purification from a new strain of isolated *B.subtilis* were also published in 2016 (Mercimek Takcı & Turkmen, 2016).

The results of a recent study indicated that out of 20 isolates screened for xylanopectinolytic enzyme activity, the most prominent strain was identified as *B. subtilis* ADI1 (Nawawi *et al*., 2017). Also, pectinolytic enzyme production by *B. subtilis* SAV-21 was also reported in 2017 (Kaur & Gupta, 2017). The study conducted by Jahan *et al*., in 2017 revealed that *B. licheniformis* KIBGE IB-3 has potential to produce a high amount of pectinase by utilizing different agro-wastes (Jahan *et al*., 2017). Thermo acidic pectinase production from Ba*cillus* sp. ZJ1407 has a good acidic and thermal stability within a pH range of 3.0-5.0 and at 80–90 °C (Yu & Xu, 2018).

CONCLUSION

As revealed in this review, *Bacillus* spp. are highly efficient for pectinase production mainly by alkaline pectinase and upholds their position as dominant pectinase producer among bacterial genera. They have proved to produce almost all classes of pectinase enzymes based on the substrate provided. It is quite evident from the literature that *B. subtilis* is the leading bacterium reported to have pectinolytic property followed by *B. pumilus*. So, considering the importance of alkaline pectinase, new bacterial strains especially from the genus *Bacillus* need to be identified for the industrial production of pectinase.

Conflict of Interests: There are no conflicts of interest.

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A regenerative protocol and SEM study for *in vitro* propagation of *Anthurium* crossed lines via indirect somatic embryogenesis

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ABSTRACT

A reproducible protocol for indirect somatic embryogenesis was established in the crosses of *Anthurium*. Two new lines namely - A1P and A2W were formed from the crosses of *Anthurium ornatum* and *A. andraeanum*. Leaf explants were selected based on coloured spathe having fragrances spadix (pink - A1P and white - A2W). Half-strength modified MS (lowering ammonium nitrate to 250 mg/l and for the first time - 0.1% EDTA Ferric Sodium) medium supplemented with 2, 4-D (1.4 µM) and 6-BAP (4.4 µM) was found to induce high percentage (82.6±0.57) of callus after 30 days in dark conditions (A2W). Later, MS basal medium having 6-BAP (0.2 – 4.4 µM) was found to initiate shoot proliferation from the calluses of both the lines. The highest number of adventitious shoots were obtained in A2W(29.1±0.88) compared to A1P (26.8±0.33), at 1.3 µM6-BAP after 6 weeks in 16/8 h light and dark cycle under a photoperiod of 50 µmol/m2/s. Separate rooting media was not required, and the best rooting occurred with 0.4 µM and 1.3 µM6-BAP shooting media. The results showed that A2W line had different responses compared to A1P line, both for callus induction as well as number of shoots-per-explants. Shoots for both the lines increased in the multiplication stages and this increase in the number of shoots-per-explants was found significant ($p<0.01$). The scanning electron microscopic (SEM) study also confirmed the stages of somatic embryogenesis from the leaf explants.

KEY WORDS: *ANTHURIUM*, 2, 4-D, 6-BAP, CALLUS, SEM, SOMATIC EMBRYOS(SE'S), ACCLIMATIZATION

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INTRODUCTION

Anthurium andraeanum Linden, the herbaceous, evergreen and perennial flower, belonging to the family Araceae, is popular and one of the most important cut flowers produced in the tropical and sub-tropical countries. Numerous species of these economically important genera are grown as potted cultivars and landscape plants.Species and hybrids within this monocotyledonous genus *Anthurium*, are highly prized as ornamentals for their beautiful showy flowers and exotic foliage, (Adelheid *et al*., 1992). In the global market, the *Anthurium* is valued only next to Orchids which rank first among the tropical cut flowers and the world import market size for *Anthurium* is estimated to exceed US$ 20 million annually (Dufourand Guerin 2003; Chen *et al.,* 2003; Atak and Celik 2009).

Traditionally, *Anthurium*is propagated through seeds and offshoots or nodal cuttings. However these traditional methods have posed challenges for large-scale production as seeds are highly heterozygous. Also, the traditional methods of propagation involve a high end-to-end timeline of around 3 years. On the other hand, plant tissue culture techniques have proved a better alternative for large-scale production of ornamental plants (Rout *et al.,* 2006, Thokchom and Maitra, 2017 and Thaneshwari and Aswath, 2018).

Micropropagtion in particular, using bulking up via callus stage, followed by adventitious bud formation, has been proposed as an interesting possibility for commercial viability (Pierik *et al.,* 1974). As an alternative, most commercial tissue culture laboratories now favour axillary bud proliferation techniques for *Anthurium*. Nevertheless, neither of these techniques are free from the associated de-merits. While the micropropagtion technique results in formation of off-types (Geier, 1988), axillary bud proliferation technique has serious problems with weaning of material, not withstanding a low propagation rate (Hamidahn *et al.,* 1997, Thokchom and Maitra, 2017).

Because of all the afore mentioned problems with conventional method - *in vitro* and *in vivo* propagation techniques,the possibility of producing micro shoots via somatic embryogenesis was considered the best for mass propagation because, 1) the high multiplication rate, 2) the ease of use of liquid medium, 3) the handling of enormous numbers of embryos at one time, 4) and the possible use of bioreactors (Merkle *et al*., 1990). Though a good number of conventional protocols have been developed for various explants (Pierik *et al.,*1974; Kunisaki 1980; Kuehnle and Sugii 1991; Matsumoto and Kuehnle 1997; Martin *et al.,*2003; Viégas *et al.,* 2007; Beyramizade *et al.,* 2008), somatic embryogenesis has been less commonly reported in *A. andraeanum* (Teixeira da Silva *et al.,* 2015). Kuehnle *et al.,* (1992) firstly reported somatic embryogenesis from leaf blade derived embryogenic calli, and the histological evidence for bipolar structure of somatic embryos (SEs) and their origin was put forward by Matsumoto *et al.,* (1996) (Bhattacharya *et al.,* 2016, Thaneshwari and Aswath, 2018).

An embryogenic-like callus of *A. andraeanum*, cultured on medium containing 2,4-D and BA was described by Kuehnle and Sugii (1991). Plants were readily obtained from that callus but regeneration from somatic embryos was not demonstrated. Later, somatic embryos and plant regeneration was reported using an induction medium containing 2,4-D and kinetin (Kuehnle *et al.,* 1992). Embryo-like structures were observed by Geier (1982), using spadix explants of *A.scherzerianum*, but plants were not recovered. Recently, somatic embryogenesis was reported using nodal segments from *in vitro* established plantlets that were cultured in Pierik medium supplemented with 10 µM NAA (Marcos *et al.,* 2014). In this study, we are reporting a novel *in vitro* protocol for plant regeneration of the lines of crosses between *A. ornatum*and *A. andraeanum*,through indirect somatic embryogenesis from leaf explants of field grown plants, and their acclimatization to field conditions. The stages of embryo developmental process have been elaborated through morphological analysis. Finally somatic embryos were confirmed through scanning electron microscopic (SEM) studies.

MATERIAL AND METHODS

Plant materials and explant preparation: The study was conducted at Centre for Post-Graduation Studies, Jain University, Karnataka, India. Lines were obtained by crossing *A. ornatum* and *A. andraeanum* and are selected based on the combination of colour and fragrance of the flowers. For culturing callus, young *ex vitro* leaf explants (2 – 4 days old) were collected from these lines. For surface sterilization, the folded young leaves were thoroughly washed with running tap water for 5 min. All the further steps of surface sterilization were followed inside the laminar air flow chamber. Initially explants were swabbed with 70% ethanol,and later cut into 5 cm squares. Cut pieces were then treated with sterile double-distilled (DD) water for 5 min, soaked in 1% bavistin for 15 min, 70%(V/V) ethanol for 30 sec, antibiotic gentamycin for 30 min, and finally followed by mixture of 1% (w/v) sodium hypochlorite [5.0% (w/v) available chlorine, (Nice – Kochi) solution] and Twin 20 (Nice – Kochi) (1-2 drops) for 12 min. Each chemical treatment was followed by washing with sterilized DD water for five times. Before inoculation, leaves were cut into 1cm squares, pricked few times with a sterilized scalpel and placed on culture media with the adaxial

surface down. The cultures after inoculation were incubated under cool and dark conditions at 25±2.0°C and 70% relative humidity (RH) (Bhattacharya *et al.,* 2016).

Culture media for induction of SE's: The basal MS medium (Murashige and Skoog's, 1962) supplemented-with 3% (w/v) sucrose and 0.8% (w/v) agar (Bacteriological grade, *Hi-media*) was used for one set of treatment. Half-strength Modified MS (MMS), first time for callus induction, was used for the other set of treatment. This Half-strength MMS medium was prepared, by modifying the basal MS medium for lower concentrations of ammonium nitrate (250mg/l) (Atak and Celik, 2009; Nhut *et al.,* 2006) and for the first time reducing iron content to 0.1% - EDTA Ferric Sodium (Duchefa biochemie). Both the sets were supplementing with different combinations of 2,4-Dichlorophenoxyacetic acid (2,4-D, *Hi-media*) concentrations ranging from 0- 2.3 µM and 6-Benzyladenine (6-BAP, *Hi-media*) concentrations of 2.2 and 4.4 µM. The pH was then adjusted to 5.8 - 6.0 with 1N NaOH before autoclaving at 1.1 kg/cm^2 pressure (121°C) for 15 min.

Culture media and condition for shoot and root induction: For the shoot and root induction, only Basal MS medium was supplemented with different concentrationsof 6-6-BAP (0-2.2µM), 3% (w/v) sucrose and 0.8% (w/v) agar was used. The pH was adjusted to same level of 5.8 - 6.0 with 1N NaOH before autoclaving at 1.1 kg/cm^2 pressure (121°C) for 15 min. The incubation of cultures after inoculation in controlled condition was under cool, white fluorescent lights (16 h photoperiod; 55 µmol m^{-2}s^{-1}, Philips, India) at 25±2.0°C and 70% relative humidity (RH).

Acclimatization: A well-developed 50 plantlets (25 plantlets from each line of both the media) were selected and planted for primary hardening. Prior to this plantlets were first washed with tap water to remove traces of media followed by soaking in bavistin (0.2%) solution for 2 minutes and then transferred to trays containing jiffy plugs in the plant growth chambers. After 30 days of primary hardening under artificial light, the plantlets were transferred to pots containing cocopeat in the humidity chamber of poly house. To maintain humidity, the seedlings were covered with 100 gauge thick polythene covers. After 30 days of secondary hardening, seedlings were transferred to individual pots with potting mixture containing soil, dung manure, cocopeat 1:1:1. The data was analysed for survival rate.

SEM studies: Hitachi Table top Scanning Electron Microscope (TM3030 plus) with Variable Pressure (VPSEM) detectors was used. Both Scattered Electron (SE) and Back Scattered Electron 9BSE) detectors were used in 80:20 mix, at 15 KVA. The samples were imaged without any dehydration, and were not coated. Magnification was in the range of 80-1800X.

Statistical analysis: The results are presented as mean values ± standard errors. All experiments were repeated three times.The data on callus induction rate and number of shoot-per-explant were subjected to analysis of variance (ANOVA) with the means separation ($p<0.01$) by Web Based Agricultural Statistics Software Package (WASP.2) - Central coastal Agricultural Research Institute Goa available at www.icargoa.res.in - (Shawaf *et al.,* 2012).

RESULTS AND DISCUSSION

The results from the present study demonstrated that indirect somatic embryogenesis from leaf explants of crosses between two monocotyledonous plants *A. ornatum* and *A. andraeanum*. Lines produced from the cross between the two species gave coloured and fragrant flowers, the combination which is not available in its natural form/habitat. Owing to the monocotyledonous nature of the plants, the embryogenic potentiality is generally restricted to cells of meristems or embryogenic origin such as shoot apices, lateral buds, immature embryos, and seeds (Fehér, 2005). In our study, cut pieces of young leaves were used as explants procured from field grown plants. The leaf tissue has been widely used for organogenesis (Kuehnle and Sugii1991; Kumari *et al.* 2011) and indirect somatic embryogenesis (Kuehnle *et al.* 1992; Beyramizade *et al.* 2008) of *A. andraeanum*. Medium composition and PGR concentrations have a key role in the formation and differentiation of calli (Thaneshwari and Aswath, 2018).

Geier in 1986 clearly mentioned that lowering of NH_4NO_3 had a significant effect on callus and shoot formation especially from leaf tissue. A low level of NH_4NO_3 (200mg/l) proved beneficial for the induction and regeneration in all genotypes of *Anthurium*. Faris *et al.,* (2012) and Atak and Celik (2009) reported that, (250mg/l) NH_4NO_3 helps for shoot regeneration. Iron (NaFeEDTA) is one of the major micro elements in MS media, helps for the growth and development of plants. Lower concentration of Na Fe EDTA (30-25 mg/l) helps in induction and maturation of both zygotic and somatic embryos (Matsumoto *et al.,* 1998 and Beyramizade *et al.,* 2008). In the present study, for the first time half strength MMS having (250mg/l) $NH_4NO_3$and (0.1%) EDTA Ferric Sodium was used for callus induction and maturation.

Generally, auxin is used for the conversion of somatic cells to embryogenic cells. Pinheiro *et al.* (2014) demonstrated that in *A. andraeanum*, Pierik basal medium (Pierik *et al.* 1974) supplemented either with NAA, or Picloram or 2, 4-D produced highest number of SEs from

Table 1. Effect of different concentrations of growth hormones for callus induction in *Anthurium* lines

Full MS				Half MMS			
6-BAP (µM)	2,4-D (µM)	% of callus		6-BAP (µM)	2,4-D (µM)	% of callus	
		(A1P)	(A2W)			(A1P)	(A2W)
0.0	0.0	1.013±0b	1.013±0b	0.0	0.0	1.013±0b	1.013±0b
2.2	0.5	1.013±0b	1.013±0b	2.2	0.5	1.013±0b	1.013±0b
2.2	0.9	1.013±0b	1.013±0b	2.2	0.9	1.013±0b	1.013±0b
2.2	1.4	1.013±0b	1.013±0b	2.2	1.4	1.013±0b	1.013±0b
2.2	1.8	1.013±0b	1.013±0b	2.2	1.8	1.013±0b	1.013±0b
2.2	2.3	1.013±0b	1.013±0b	2.2	2.3	1.013±0b	1.013±0b
4.4	0.5	1.013±0b	1.013±0b	4.4	0.5	1.013±0b	1.013±0b
4.4	0.9	40.16±0.35a	42.8±0.52a	4.4	0.9	1.013±0b	1.013±0b
4.4	1.4	1.013±0b	1.013±0b	4.4	1.4	76.9±0.35a	82.59±0.56a
4.4	1.8	1.013±0b	1.013±0b	4.4	1.8	1.013±0b	1.013±0b
4.4	2.3	1.013±0b	1.013±0b	4.4	2.3	1.013±0b	1.013±0b

Values are mean ± standard error of three replicated experiments, each with eighty explants per treatment. Means followed by the same letter are not significantly different at P<0.01. Data were scored after 30 d of culture

callus. Atak and Celik 2009 reported that, Half-strength MS basal salt with 0.6 mg/l 2,4-D, 1 mg/l BA were used for callus induction.Later, MS media containing (1.0 mg/l) BA and (0.1 mg/l) 2,4-D resulted in 2.08 shoots from the callus clumps was reported by Bakhsi-Khaniki *et al.,* (2011) (Cardoso and Habermann, 2014). However, for the first it was observed that, indirect somatic embryogenesis using leaf explants of crossed *Anthurium* lines requires both 2, 4-D (1.4µM) and 6-BAP (4.4µM) in Half strength modified MS medium.

Callus was initiated at the four corners of each leaf explant within 15 days of inoculation in the MS media containing 2, 4-D and 6-BAP. The leaf explants cultured on MS basal medium without growth hormone did not show any morphogenetic changes. Callus formation (over a period of 30 days) was observed in both the lines on leaf explants, with Half-strength MMS as well as FullMS media. However, only a particular concentrationof hormones formed callus - Half MMS containing 1.4µM 2, 4,-Dand 4.4 µM 6-BAP,and Full MS medium containing 0.9 µM 2, 4,-D and 4.4 µM 6-BAP. In this study, it was clear that Half-strength modified MS medium, 6-BAP with 2,4-D was required for SE induction but the presence of only low concentrations of 2,4-D (1.4µM) for initiation of callus was adequate for maturation and germination of embryos.Callus induction percentage of explants at different concentrations of 2, 4-D (0-2.3 µM) and 6-BAP (2.2 and 4.4 µM) for both Full MS and Half-strength MMS is given in Table 1. In our observations, irrespective of the lines, Half-MMS induced higher percentage of callus as well as matured embryos-per-explant compared to Full-MS media. With Half-MMS, white line showed better response than pink line. Callus initiation for (82.6±0.57) in A2W compared to (76.9±0.35) A1P, and number of advanced stage of embryos-per explant of (4.27±0.12) in A2W compared to (3.13±0.088) A1P. Similar pattern was observed in Full-MS media as well where, white lines showing higher response compared to pink line for callus initiation (42.8±0.52) in A2W compared to (40.16±0.35) A1P, and number of advanced stage of embryos-per-explant of (2.16±0.88) in A2W as compared to (1.23±0.14) A1P. The SEM studies also confirmed the indirect somatic embryogenesis stages which are formed during maturation (Fig. 2) and finally initiation of adventitious shoot development were also observed (Fig. 2f).

Table 2. Matured somatic embryos number and percentage forming explants of *Anthurium* lines

Media	6-BAP (µM)	2,4–D (µM)	% of embryo forming explants (A1P)	% of embryo forming explants (A2W)	No. Of SE's/ explant (A1P)	No. Of SE's/ explant (A2W)
Full MS	4.4	0.9	27.305±2.1b	36.934±1.47b	1.233±0.15b	2.167±0.89b
Half MMS	4.4	1.4	55.528±4.93a	66.955±3.1a	3.133±0.09a	4.267±0.12a

Values are mean±standard error of three replicated experiments each with 6 matured embryos generated in medium containing Full MS+0.9µM2,4-D+BA 4.4µM; Half strength MMS+1.4µM2,4-D+BA 4.4µM. Means followed by the same letter are not significantly different at P<0.01. Number of SE's was counted after 30 d of culture.

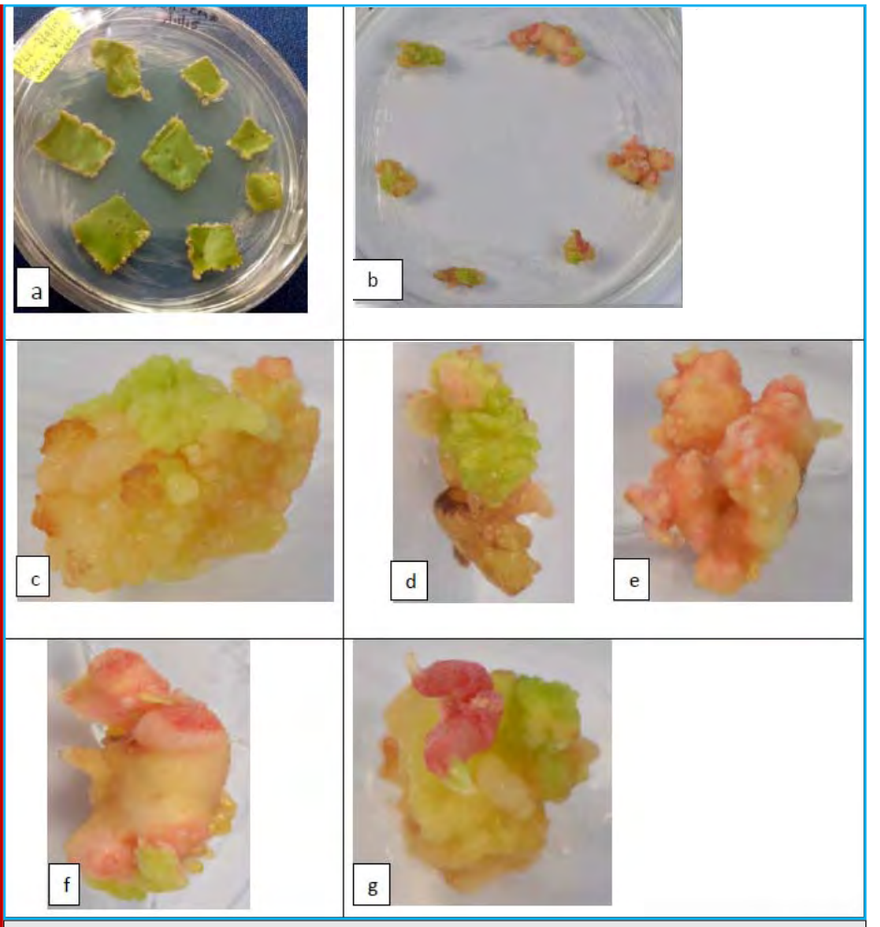

FIGURE 1. Somatic embryogenesis in leaf of *Anthurium* crossed lines (A1P and A2W). (a) *Exvitro* leaf segment cultured in Half MMS medium with 1.4µM 2, 4, - D and 4.4 µM 6-BAP; all four edges of leaf shows callus formation, after 30 days of *In vitro* culture. (b) Leaf segment forming embryogenic calli on Half MMS medium with 1.4µM 2, 4, - D and 4.4 µM 6-BAP, after 30 days of culture *In vitro*. (c) (d) (e) and (f) stages of somatic embryos – globular, torpedo, heart and cotyledonary stages. (g) matured embryos showing the shoot initiation

Table 3. Effect of different concentrations of 6-BAP on number of germinated somatic embryos of *Anthurium* lines

Media – Full MS 6-BAP (µM)	No. of Germinated embryos into plants (A1P)	No. of Germinated embryos into plants (A2W)
0.0	4.61±0.17d	5.18±0.17c
0.4	9.39±0.08b	10.61±0.21a
0.9	7.33±0.33c	8.09±0.29b
1.3	11.44±0.2a	11.44±0.16a
1.8	6.62±0.08c	7.96±0.38b
2.2	6.97±0.28c	7.29±0.44b

Values are mean ±standard error of three replicated experiments each with 4 matured embryos in germination medium containing 6-BAP(0- 2.2µM). Means followed by the same letter are not significantly different at P<0.01.

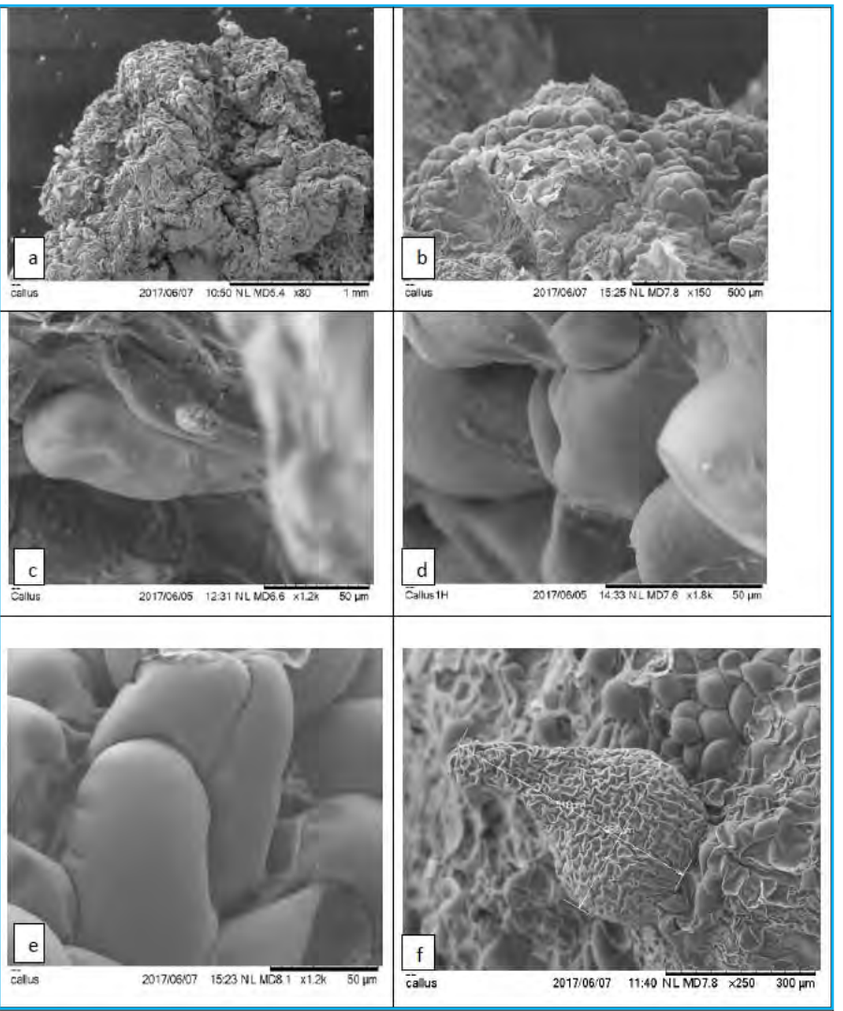

FIGURE 2. SEM analysis of indirect somatic embryogenesis from leaf explants of *Anthurium* lines.The value following x is the magnification ranges from 80-1800 X. (a) Tightly packed cells of outer surface and large number of somatic cells with definite plane of divisions. (b) Globular protrusions. (c) (d) and (e) Torpedo, Heart and cotyledonary stages of somatic embryos. (f) Shoot tip emergence from the surface of matured embryos.

When the same media was used for second subculture of undifferentiated callus, Half-strength MMS showed higher percentage and number of SEs compared to Full MS medium (Fig.1b). As shown in Table 2, Half-strength MMS at 67% for A2W (4.26±0.120) and 55.5% for A1P (3.13±0.088) vis-à-vis Full MS at 36.9% for A2W (2.17±0.88) and 27.3% for A1P (1.23±0.15). Stages of somatic embryos were morphologically identified (Fig. 1c, d,e, and f). The different concentrations of 6-BAP (0 – 2.2 µM) had a noticeable effect on embryo germination. Matured somatic embryos showed highest number of germination (11.43±0.067) in Full MS 6-BAP at 1.3µM for both the lines (Table 3). Hence our result suggested that less amount of 2,4 – D and 6-BA 4.44 µM gives increased number of matured somatic embryos (Pinheiro *et al.,* 2013, Yu Yi-xun *et al.,* 2009).

Shoot induction using 6-BAP at 1 mg L^{-1} has been reported for this genus by Pierik (1976). Liendo and Mogollon (2009) obtained similar results for *A. andreanum;* in which 4.17 number of shoots were produced in a medium with 1 mg L^{-1} of 6-BAP. Later Paola *et al.,* (2014) obtained in a ½ MS (Half-salt content) medium with 1 mg L^{-1} of BAP, which attained 23.7 shoots / explant per month multiplication rate; In our study, we were able to achieve higher levels of shoot induction at 1.3 µM 6-BAP (0.3 mg L^{-1}). The highest

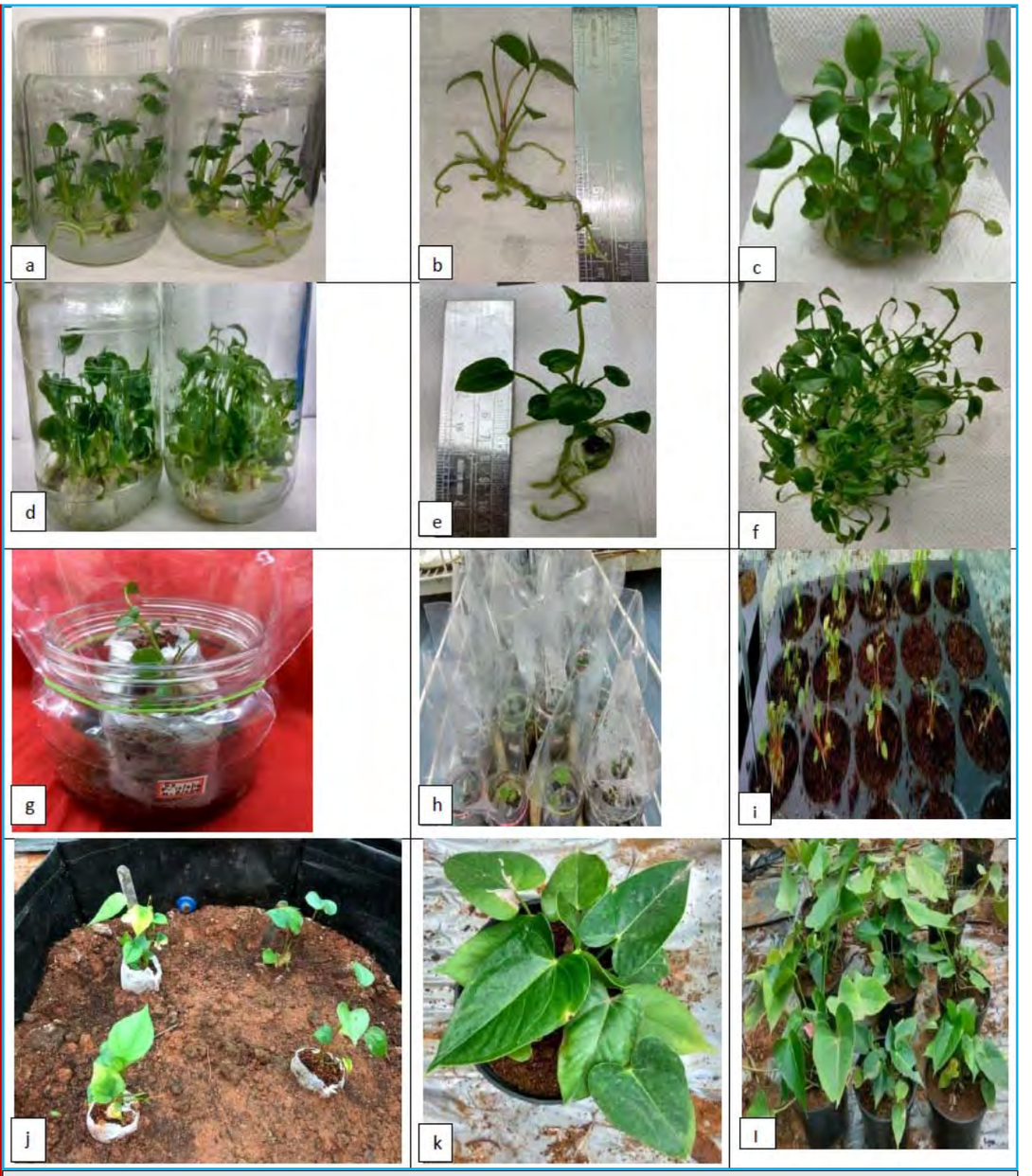

FIGURE 3. (a) And (d) A1P; A2W - Shoot multiplication occurring on the multiplication medium (1.3µM - 6-BAP). (b) and (e) individual plantlets shoot and root length of A1P; A2W (c) and (f) multiple shoots of A1P; A2W lines (g) and (h) Plants acclimatized under artificial light and room temperature at growth chamber - primary hardening (i) Secondary hardening plants - autoclave cocopeat, humidity chamber of poly house. (j) *Anthurium* – grow bags (k) individual potted plant and (l) plant from culture 3 months after potting

number of shoots were observed in A2W (29.1±0.89) compared to A1P (26.8±0.33). Rooting for *anthurium* does not require any phytohormones; the occurrence of spontaneous roots has already been reported by other authors for this genus (Viegas*et al.,* 2007; Liendo and Mogollon, 2009, Paola *et al*., 2014); therefore, one specific medium for forming this organ is not necessary. Recently, Farsi *et al.* (2012) reported rooting without the addition of phytohormones for the micropropagation of *A. Andreanum cv.* Terra. Furthermore, different researchers have reported rooting in 84 to 100% of the shoots of different varieties of *A. andreanum* using

Table 4. Effect of different concentrations of 6-BAP for shoot induction – (A1P)

Media Full MS+ 6-BAP(µM)	No. of shoots	Length of shoots	No. of roots	Length of roots
0.0	9.36±0.33d	9.26±0.21e	9.36±0.33d	5.12±0.17f
0.4	24.34±0.57b	14.57±0.08b	13.34±0.33b	6.192±0.18e
0.9	16.77±3.84c	10.51±0.08d	12.46±0.33bc	8.65±0.15c
1.3	26.80±0.33a	16.70±0.15a	18.11±0.33a	14.17±0.25a
1.8	17.78±0.33c	13.96±0.33b	11.016±0.33cd	10.35±0.15b
2.2	16.41±0.57c	12.29±0.15c	9.88±0.57d	6.95±0.033d

Values are mean±standard error of three replicated experiments in the shoot induction medium containing 6-BAP(0- 2.2 µM). Means followed followed by the same letter are not significantly different at P<0.01. Data were scored after 60 d of culture.

growth regulators (Martin *et al*., 2003; Bejoy *et al*., 2008; Islam *et al*., 2010; Paola *et al*., 2014).

The results are in accordance with, though separate rooting media was not used for root initiation, the highest level of rooting (A2W: 20.0±0.33 and A1P: 18.1±0.33) was found in with 1.3µM 6-BAP in the shooting media itself. The root initiation was lower (A2W: 10.5±0.33 and A1P: 9.4±0.33) in the absence of 6-BAP. Root formation takes place in the 6-BAP media along with shooting (Fig 3a, b, c, d, e, and f). The different concentration of 6-BAP ensures difference in the number and length of shoots and roots. Full MS 6-BAP (1.3µM) showed highest number of shoots (29.1±0.88) and roots (19.96±0.33) in A2W (Table 5) than in A1P – shoots (26.8±0.33) and roots (16.7±0.15) (Table 4). Overall, the callus induction was significantly high in A2W, the number of shoots-per-explants were more-or-less comparable across both the lines.

For transplantation of *Anthurium* plants, peat and sand mixture, (Geier, 1986) peat compost (Finnie and van Staden1986), burned-rice husk, raw rice husk and organic manure (2:2:1 v/v/v) (Winarto and da Silva 2012), etc. were used. In the present work, for the first time, jiffy plugs were used for primary hardening. The embryo-generated plants from germination medium were directly harvested (Fig. 3a and 3d) and planted into jiffy plugs (Fig. 3g). The highest percentages (80%) of the SE derived plantlets were successfully hardened in plant growth chambers (Fig. 3h) within 30 days. Based on the experiment by Sinta, Riyadi, & Sumaryono (2011), the usage of plastic wrap as culture closures provides aeration for *in vitro* cultured oil palm. It helps to exchange the gas from inside to the outside of culture vessels to be more intense, which leads to the reduction in moisture. However, to compensate with the high evaporation, double layer medium was used for hardening (Saptari *et al.,* 2017) so that plantlets were not completely dried. After 60days of secondary hardening in the cocopeat (Fig. 3i) at poly house, shows 78.3% of plants survived and were transferred to grow bags (Fig. 3j). *Anthurium* after 3 months in pots (Fig. 3k) results in flowering. Fig. 3l shows emergence of flower bud.The SEM study also confirmed the occurrence of somatic embryogenesis from epidermal layer of leaf tissue. The stages clearly indicated the embryo maturation leading to the formation of shoots (Fig. 2f) (Bhattacharya *et al*., 2016).

All stages in the somatic embryos were clearly identified in our studies which will leads to the conformation of somatic embryogenesis.Through the experiment, the objective was to find a suitable method of indirect somatic embryogenesis for the crossed lines. Different genotypes within the two lines were chosen; it was observed that there was a different response for callus induction and shoot formation among the lines. However, the present investigation demonstrated that the regeneration protocol shows good responses for both the *Anthurium* lines.

Table 5. Effect of different concentrations of 6-BAP for shoot induction – (A2W)

Media Full MS+ 6-BAP µM	No. of shoots	Length of shoots	No. of roots	Length of roots
0.0	8.94±0.29f	11.30±0.29d	10.49±0.33e	4.40±0.21c
0.4	22.95±0.2b	14.66±0.70b	16.07±0.33b	12.92±0.26a
0.9	11.99±0.19e	12.03±0.54d	12.87±0.57cd	9.92±0.31b
1.3	29.10±0.88a	16.37±0.24a	19.96±0.33a	14.29±0.37a
1.8	16.49±0.39c	13.99±0.19bc	14.15±0.57c	9.54±0.27b
2.2	13.59±0.11d	12.74±0.35cd	11.99±0.33de	9.98±0.28b

Values are mean±standard error of three replicated experiments in the shoot induction medium containing 6-BAP(0- 2.2µM). Means followed by the same letter are not significantly different at P<0.01. Data were scored after 60 d of culture.

CONCLUSION

This three-step (induction, maturation, and germination) protocol takes only 4-5 months to obtain plantlets from *Anthurium* crossed lines,via indirect somatic embryogenesis, using Half-strength modified MS medium supplemented with 2,4-D and6-BAP. SEM studies revealed the characteristic development of indirect SEs from leaf to complete plantlets. The embryo-generated plants successfully flowered in the field. The promising protocol developed here for regeneration of crossed *Anthurium* through indirect somatic embryogenesis may be used in future experiments aiming at genetic transformation and large scale propagation of true to type of plants of *Anthurium*.

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Clinical uses and toxicity of *Atropa belladonna*; an evidence based comprehensive retrospective review (2003-2017)

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ABSTRACT

Atropa belladonna (AB), commonly known as deadly nightshade is a poisonous plant which contains several alkaloids such as atropine and scopolamine etc. Any deliberate as well as unintentional ingestion of AB fruits, roots or leaves may result toxicity. Aim of the study: This study aims to present the clinical uses of atropine alongwith the adverse effects and toxicity associated with the use of atropine in the form of conventional or as herbal medication. A retrospective (2003-2017) literature was searched in various databases such as web of science, PubMed, google scholar, Scopus, E-Resource Portal of Imam Abdulrahman bin Faisal University etc. using the keywords i.e. AB, clinical uses of AB, adverse effects and cases reported with AB. Ten (10) cases met the eligibility criteria where the toxicity was mostly associated with the use of AB fruit, tablets and AB contaminated drinks. Major symptoms observed in these cases were related to Anticholinergic syndrome (ACS); tachycardia, dilated pupils, confusion, flushed dry skin, hallucinations and seizures in some cases alongwith a toxic megacolon. Furthermore, the treatment protocol used widely for ACS consisted of; gastric lavage and the use of Physostigmine as an antidote (antagonizes the central as well as peripheral toxicity of atropine). AB fruit revealed a vital role as anticholinergic drug, mydriatic agent and as an antidote for OP poisoning, however, its use deprived of medical supervision or in a large quantity, it may cause severe toxicity.

KEY WORDS: *ATROPA BELLADONNA*, CLINICAL USES, TOXICITY, ANTICHOLINERGIC, CASES REPORTED

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INTRODUCTION

Solanaceae, an alkaloidal containing plants family, is well known for its applications in cosmetics, traditional medicines and as a poison since ancient times. *Atropa belladonna,* synonym; deadly nightshade, belongs to the family solanaceae and is known for its effects on "increase pupil size" which imparted it the name of "beautiful lady" (Atropa; Greek word means "goddess" & Belladonna; Italian words means "beautiful lady"). It is a tall plant that can reach up to five feet and it's abundantly found in waste ground and quarries. AB has oval shaped leaves, greenish to purple color flowers and black, globular shape, sweet berries. Though leaves and roots are poisonous and ingestion of a single leaf can cause severe ACS which may be fatal, however the berries have been reported with more poisonous cases in children due to its attractive look and sweet taste. The major components in the leaves, fruits and roots are mostly alkaloids such as Hyoscyamine, scopolamine and more abundantly; atropine, which is responsible for the plant's Anti-cholinergic toxic effects, (Berdai et al., 2017; Ahmad et al., 2017a).

Atropa belladonna plant is surrounded by fear, awe, and myth. The Romans and Greeks believed the fact of AB containing a deadly poison due to more wide use by professional poisoners, sorcerors, and witches. Later on, Linnaeus codified its remarkable properties such as dilating the pupils and it was 1830s, when I-atropine, the pure alkaloid, was extracted from AB plant. It was pure I-atropine that helped as a substantial tool in understanding the physiology of autonomic nervous system and lead to identification of the important neurotransmitters in mammalian biological system which is acetylcholine, (Lee, 2007; Ahmad et al,. 2017b).

Deadly nightshade exerts clinical as well as toxic effects through an anticholinergic mechanism in human body, which can affect the central and the peripheral nervous system. It is responsible for many symptoms such as tachycardia, dry skin, hallucination, mydriasis and choreoathetoid movements. On the other side it may be clinically useful when used to do anamneses in clinical examination as well as detailed imaging in cases which are confused with acute cerebrovascular events. *Atropa belladonna* poisoning should be considered when diagnosing geriatric patients experiencing hallucination, meaningless hand gestures and consciousness. (Saritas et al., 2014; Ahmad et al., 2017c)

Atropa belladonna poisoning has been reported in different age groups however the most dangerous part of AB to children is its berries especially younger than six years' children, as they have a sweet taste and look attractive to them. (Milanlioglu, 2011).Toxicity may develop as a result of the ingestion of two to three bays of AB in a child and ten bays in an adult. The most common cause of AB intoxication in children is confusion with some other berries such as blueberries and black-currant.(Berdai et al., 2012).

Atropa belladonna toxicity can be classified as mild, moderate and severe intoxication. Various treatment plan, depending the severity and condition of the patients, are used for treatment of *Atropa belladonna* intoxication including gastric lavage and Neostigmine and Physostigmine, as an antidote. (Caksen et al., 2003; Ahmad et al., 2017c)

Numerous literature is available regarding the clinical uses, adverse effects and toxicity cases of AB; however none of the studies have reported a complete and updated review about the comparative effects of AB in treating various conditions and the adverse effects of toxicity thus reported. Current study highlight evidence-based clinical uses alongwith toxicity observed with AB use and the most common reason of toxicity i.e. due to intentional or unintentional use of atropine tablets, barriers, leaves or plant as a whole.

MATERIAL AND METHODS

DATABASES SEARCHED

The relevant literature was searched using different databases such as Central library Imam Abdulrahman bin Faisal University, PubMed, Google Scalar, Science Direct and Google scholar, Web of science. In addition, Journals (The American journal of therapeutics, Bali medical journal, Human and experimental toxicology, and journal of the Royal college of physicians of Edinburgh) and books (Lippincott's pharmacology and Katzung's basic, clinical pharmacology and Neuropathy of Drug Addictions and Substance Misuse) were also utilized as tools for gathering related information.

Keywords Searched

Atropa belladonna, cases reported of *Atropa belladonna* toxicity, Atropine intoxication, *Atropa belladonna* intoxication, Deadly nightshade intoxication, Anticholinergic toxic syndrome cases and cases of *Atropa belladonna* poisoning.

Review Period

This article used a retrospective fourteen (14) years review from year 2003 to 2017.

Inclusion Criteria

Clinical cases included in the review are only related to/reported in human subjects only. Any adverse effect or toxicity resulted due to use of atropine in conventional

system or herbal system as well as any use related to intentional or unintentional ingestion was included in the study. Furthermore, the toxicity or overdose associated with the use of any part of AB i.e. leaves, fruit and berries as well as contamination/adulteration of herbs with atropine, were also included.

Exclusion Criteria

Any clinical cases reported in animal subjects and *in vitro* studies were excluded from the study. Similarly, any case reporting traditional or general uses of AB rather than evidence-based i.e. any clinical case reported to hospital emergencies or clinics were also excluded from the study.

Search Results

The literature search was refined and a total of eleven (11) articles matched the inclusion criteria and they are reported in the literature review section as below.

LITERATURE REVIEW

All the relevant cases were collected, studied, analyzed and reported after a broad literature review. The cases are presented below with respect to the year of publication.

CLINICAL CASES REPORTED IN 2017

AB causing ACS due to adulteration with marshmallow roots

Oerlemans *et al.*, reported a case for a patient visiting ER with ACS. The history revealed ingestion of hot chocolate drink with marshmallow root (*Althaea officinalis*) for treating cold symptoms. However the lab reports showed ingestion of 20-200 mg of atropine as the herb was contaminated with Ab. The patient after transferring to ICU was discharged in a healthy state. (Oerlemans et al., 2017)

ACS due to use of AB berries

Ibrahim karagoz *et al*, in a study reported a patient case from ER with anticholinergic toxic syndrome (ATS) symptoms (tachycardia, dilated pupils and hot reddish extremities and trunk). Upon investigation it was revealed that the patient used 5 to 6 blackberry like berries i.e. AB fruit. Symptoms resolved after application of urine catheter and inhibition of oral intake except for tachycardia. Redness also diminished leaving rashes on his arms. Sinus tachycardia disappeared during follow-up after oral intake resumed. Patient was discharged healthy from the hospital after the follow-up period. (Karagoz et al., 2017)

Toxic Megacolon devolved secondary to OP poisoning treatment

An old man was admitted to ER with loss of consciousness. The patient consumed pesticides containing organophosphate. The treatment plan consisted of atropine and pralidoxime intravenously however the patient developed a megacolon following 4 days of atropine administration. Atropine and pralidoxime tapering was done and patient was put on a maintenance therapy. The serum cholinesterase reached 3209 microkat/1, after 33 days. (Mostafazadeh et al., 2017).

CLINICAL CASE REPORTED IN 2016

AB in homeopathic remedies and ACS

A 20-days old infant was presented in ER with signs of seizures and fever, after swallowing a homeopathic agent containing AB for an infantile colic treatment. Detailed clinical examination revealed and was treated with Benzodiazepines. In addition i/v antibiotics were administered due to septicemia. Several hours following the monitoring, no signs of fever were found and the infant recovered from neurological abnormalities was thus discharged healthy. (Glatstein et al., 2016).

CLINICAL CASE REPORTED IN 2014

Jimsonweed as a source of suicide

Glatstein *et al*, (2016) reported a study of ten adolescent cases with serious ACS and two with a history of suicidal attempt. Detailed investigation revealed the use of *Jimson weed* (atropine containing plant) in half of the patients whereas remaining half of the patients used different atropine tablets. Treatment plan included Physostigmine, activated charcoal, benzodiazepines and haloperidol and all the patients were discharged healthy. (Glatstein et al., 2014; Ahmad et al., 2017a).

CLINICAL CASE REPORTED IN 2013

Wild fruit of AB vs consciousness

Abdullah Demirhan *et al.*, reported an emergency case for a patient presented with symptoms; loss of consciousness and fatigue, red neck and face, dry mouth and dilated pupils. The care taker for patient revealed the use of large amount of a forest fruit AB and the start of symptoms after four hours of ingestion. Gastric lavage followed by administration of activated charcoal (1mg/kg) diminished the symptoms within 24 hours and the patient was discharged healthy. (Demirhan et al., 2013).

Table 1. Clinical cases reported about *Atropa belladonna* (nightshade)

Case No.	Form of Atropine Ingested	Age	Gender	Clinical Manifestation	Treatment Protocol	Cause of Ingestion
1	Althaea officinalis	28 / 27	Female / Male	ACS	-	Ingestion of contaminated hot drink
2	AB fruit	71	Male	-Tachycardia -Dilated pupils -Redness of extremities and trunk	-	Confusion with blackberry fruit
3	IV Atropine	52	Male	Toxic megacolon	Tapering of Atropine and Pralidoxime	Treatment for OP poisoning
4	Homeopathic agent containing AB	20 days	-	-Seizures -Fever	-	Treatment of infantile colic
5	Jimsonweed / Atropine Tablets	Adolescents	-	ACS	-Physostigmine -Activated charcoal -Benzodiazepine -Haloperidol	Two of patients had them as suicide attempts
6	AB fruit	49	Female	-Loss of consciousness -Fatigue -Redness of face and neck -Dilated and isochoric pupils -Dryness of mouth	-Gastric Lavage -Activated Charcoal -Supportive therapy	Confusion with forest fruit
7		11	Female	-Confusion -visual and hearing hallucinations -uncontrollable vomiting -incoherent speech	Diazepam	Treatment of jaundice
8	AB Leaves	40	Male	-Flushed warm skin -Dry tongue -Sudden psychomotor agitation -Disorientation -Dilated pupils -Tachycardia	-Activated charcoal -Diazepam -Supportive therapy	Treatment of chronic peptic ulcer
9	AB Fruit	2	-	Central and Peripheral ACS		Confusion with wild berries
10	-	48	Male	-Tachycardia -severe disorientation -Aggressiveness	-Activated charcoal -Diazepam -IV Physostigmine	-
11	-	Children	-	-Tachycardia -Flushed skin -Meaningless speech -Mydriasis	Neostigmine	-

CLINICAL CASE REPORTED IN 2012

ATS developed from jaundice treatment

An eleven-year-old girl was administered with AB for the sake of treating jaundice developed due to use of rifampicin and isoniazid for lymph node treatment. The patient developed ATS with symptoms; confusion, visual and hearing hallucinations, uncontrollable vomiting, and incoherent speech. The treatment plan consisted of diazepam (5 mg) for sedation purpose whereas charcoal wasn't administered as AB was ingested a day before. For treating jaundice the tuberculosis treatment was stopped. (Berdai et al., 2012)

CLINICAL CASE REPORTED IN 2011

Chronic peptic ulcer treatment causing ACS

A patient was admitted to ER with symptoms; warm and flushed skin, dry tongue, sudden psychomotor agitation, disorientation, dilated pupils and tachycardia. Patient history revealed the ingestion of AB leaves for relieving peptic ulcer. Treatment was successful after administra-

tion of activated charcoal, diazepam (10mg) and gastric decontamination. (Milanlioglu, 2011).

AB wild barriers causing central and peripheral Atropine poisoning symptoms

A two year old child after eating wild berries was brought to ER. The plant as presented by his mother was identified to be AB whereas the symptoms observed were both peripheral and central atropine poisoning. Following a proper treatment, the patient was discharged healthy. (Laffargue et al., 2011).

CLINICAL CASE REPORTED IN 2009

AB Vs ATC

A forty eight year man ingested three handful of AB and developed symptoms of tachycardia, severe disorientation and aggressiveness, in the first six hours of ingestion. He was treated with diazepam, activated charcoal and Physostigmine (continuous therapy) and was recovered within two days. (Zimmermann et al., 2009)

CLINICAL CASE REPORTED IN 2003

Severe to mild AB toxicity symptoms in 49 children.

AB toxicity was reported in forty nine children's in a study. They were divided into two groups i.e. sever intoxication symptoms group (6 child's) and mild to moderate symptoms group (43 child's). The most commonly observed symptoms were; tachycardia, flushed skin, meaningless speech, and mydriasis. Due to lack of Physostigmine availability, Neostigmine was used as an antidote for treatment. No death cases were reported, (Caksen et al., 2003).

DISCUSSION

Atropa belladonna, known as deadly nightshade, can be very toxic. It has an anticholinergic effect on human body which can affect the central as well as the peripheral nervous systems. The symptoms and its severity may vary among age groups and gender depending upon the ingested dose and patients experience anticholinergic toxic syndrome with different symptoms, (Glatstein et al., 2014; Ahmad et al., 2017a; Ahmad et al., 2017b).

However, the most common symptoms reported in the cases founded in current literature i.e. from 2003-2017 includes; tachycardia, seizures, dryness of mouth, dilated pupils, hallucinations and confusion. This review underlines the clinical uses as well as any clinical manifestations associated with or any toxicity resulted due to use of atropine in any of its form i.e. plant extract, berries, tablets, powder etc. Generally, the plant AB is considered as a toxic one and using the plant without proper knowledge and guidance may be harmful.

Toxic dose of *Atropa belladonna: Atropa belladonna* was commonly used by professional poisoners, sorcerors and witches since ages. Mature fruits and green leaves have the highest concentration of atropine whereas the mature fruit is claimed to contain 2 mg of atropine, (Passos et al., 2016) As per Martindale, the mortal dose of atropine is 10mg (equal to 5 AB fruits) and 100mg (equal to 50 AB fruits) or less for children and adults, respectively. Duration of action*:* ACS symptoms start usually within one hour after ingestion and Sub-mortal clinical symptoms may continue for 2 days, (Spina et al., 2007). The severity of ACS/ATS, however, depends upon whether and when medical intervention was taken. (Adamse et al., 2015).

Atropine induced psychosis: as mentioned in the literature review, *Atropa belladonna* intoxications cause central ATS symptoms which include hallucinations, confusion and psychosis. Psychosis is induced as a result of decrease in acetylcholine transmission, accompanied with an increase in dopamine transmission in the Central nervous system, as their levels are influenced by each other. (Gaudreau et al., 2005). Atropine induced psychosis is explained by the ability of Tropane alkaloids to rapidly cross the blood brain barrier and affect the CNS functions. Having cholinergic blocking effects, atropine can block M_1 receptors (Ardila et al., 1991) in the brain causing psychosis by the mentioned mechanism. Although central and peripheral ATS have been reported in cases of overdose, rare cases are founded to cause such symptoms at pharmacological doses in sensitive subjects. Baker and Farley reported toxic psychosis caused by atropine eye-drops. The patient was prescribed with 1% atropine eye-drops in her right eye for the treatment of retinal detachment. Following a treatment of three and half weeks, she developed toxic psychosis and peripheral symptoms of ATS, (Baker, 2010).

Toxic megacolon developed form atropine intoxication: though incidence of atropine-associated toxic megacolon is very rare, however still few cases have reported such complication. These case reports suggested an involvement of atropine in the manifestation of toxic megacolon, (Mostafazadeh et al., 2017; Scharer et al., 1964; Mann et al., 1977). In the first two cases, toxic megacolon developed after the administration of anticholinergic agent and was reversed after the discontinuation of the anticholinergic agent. Although the pathophysiological bases of atropine involvement in toxic megacolon remain unclear, some theories suggest that anticholinergic agents, such as atropine, lead to gastrointestinal peristalsis inactivity and delay intestinal transit time which in turn may have caused toxic megacolon. (Mostafazadeh et al., 2017).

Memory defects caused by Hyoscine: Hyoscine, also known as scopolamine (an active drug in AB plant), has been widely used in clinical practice for years. However, many cases of hyoscine toxicities have been reported. Hyoscine toxicity has been associated with ATS as atropine and amnesia. An analysis done after days of hyoscine intoxication, by Ardila and Moreno in 1991, showed significant decrease in logical memory, visual reproduction, digits, associative learning which suggest defects in residual memory after hyoscine intoxications, (Ardila et al., 1991). Although hyoscine induced amnesia has been reported as an intoxication complication, hyoscine helped in inducing amnesia for testing of the anti-amnesic effect in medications and plants such as *Ficus religiosa*, (Kaur et al., 2010).

In our study we reported AB intoxication in retrospective years i.e. 2003-2017, in adults, children and adolescents. Almost half of the subjects were adults whereas the other half was children and adolescents. Furthermore, intoxication was produced irrespective of gender however the subjects mostly affected were male. In addition, few of the cases highlighted the use of AB fruit instead of forest fruit by mistake due to similar appearance. One of the cases reported the use of atropine tablets by adolescent subjects however majority of the case reports suggests the fact; ACS syndrome was associated with the use of AB fruit/plant for treating various diseases such as Jaundice, peptic ulcer, organophosphate poisoning and infantile colic. The literature review and eleven cases observed during the last fifteen years may not be sufficient evidence to claim a plant as a toxic one and disqualify it. As evident in the previously reported cases, ACS was one of the major drawback observed with the use of AB plant however lack of any causality or death still favor its use as a medicinal plant. The reported cases showed the use of Ab/atropine for various ailments, hence AB plays a major role in various conditions till date, as discussed.

Atropine as antidote in Organophosphate poisoning: Atropine has a huge therapeutic value and is widely used as an antidote in cases of organophosphate poisoning. Organophosphates are one of the long acting Ach esterase inhibitors used mainly as war gases. (Katzung et al., 2012) Symptoms of OP poisoning manifest as a result of accumulation of Ach at the nerve junction causing acute cholinergic crisis. Atropine is considered to be an important agent that reverses central and peripheral symptoms of organophosphate poisoning for its central and peripheral anticholinergic affects. (Dong et al., 2017; Kumar et al., 2001; Mustafa et al., 2016).

Atropine in the management of myopia; Atropine is a nonselective anticholinergic agent that can be administrated locally for controlling mild to moderate myopia, which is one of refractive disorders. In the last decade, atropine was used as an evidence-based treatment but the mechanism of action is unknown yet. It is considered to be well effective and tolerated at low doses in the management of low and moderate cases. Atropine has been also used to slow the progression of ocular axial elongation and myopia. Audrey *et al.,* studied the efficacy and safety of atropine in Asian children with myopia. Atropine in this study was very effective and well tolerated in slowing down the advancement of myopia and ocular axial elongation. (Chuang, 2017; Chia et al., 2012; Chua et al., 2006).

Managing Smooth muscle spasms hyoscine is an active constituent in the Buscopan formulation as Hyoscine-n-butyl bromide. Intestinal spasms result from the cholinergic stimulation of smooth muscle in the GIT, which increases its motility and secretions. (Whalen, 2015; Ardila et al., 1991; Zhu et al., 2017). Hyoscine can comfort the pain of bowl and stomach cramps by blocking the M_1 receptors therefore, blocking the cholinergic effects on the intestinal muscles. In addition, hyoscine is used as cervical antispasmodic agent in labor whereby exert effects via decreasing the duration of first stage in labor, owing to its anticholinergic effects of the smooth muscles of the uterus. (Sirohiwal et al., 2005).

Antiemetic effect of hyoscine: hyoscine also blocks the motility of the smooth muscles as well as their secretions by its anti-muscarinic action. Having these effects, hyoscine can be used to control the vomiting and nausea. A case reported by Brown *et al*., presented a breast cancer patient having nausea and vomiting which were resistant to the widely available anti-emetics, who was treated successfully with hyoscine transdermal patch. (Fay and Llio, 2016).

To conclude; AB ingestion may induce ACS however not all of the characteristics of ACS may be found in few of the AB poisoning cases. The fact is supported by the presence of hybrid form within AB plant, (Cikla et al., 2011). The major aim of current review was to search and report any recently observed cases related to AB use and explore the most widely observed and common symptoms of AB toxicity. The aforementioned cases suggest two types of clinical manifestation i. central nervous system effects (CNS) and ii. Peripheral nervous system effects (PNS). The symptoms presented with CNS includes; short-term memory loss, ataxia, hallucinations, agitated delirium, disorientation, seizures, confusion, psychosis, coma leading towards respiratory failure as well as cardiovascular collapse. PNS symptoms includes; dry mucous membranes, hyperreflexia, mydriasis with cyclopedia, diminished bowel sounds or ileus, flushed skin, hypertension or hypotension, tachycardia and urinary retention. Furthermore, the symptoms in children consisted of; lethargy, meaningless speech, absence of tachycardia and coma. The ACS

reported in most of the cases was due to use of AB for different treatment purposes including myopia, smooth muscle spasm, jaundice, peptic ulcer and organophosphorus poisoning however all the cases were properly managed with charcoal, gastric lavage and Physostigmine/neostigmine without any death.

CONCLUSION

Atropa belladonna, as a medicinal plant possesses a huge respect in patient treatment. Every plant, its extract or active drug as well as any conventional medication may produce specific side effects or adverse effects which is commonly associated with the use of these agents. AB produces ACS and the symptoms are properly treated with the available treatments or antidote. However, despite its wide therapeutic uses, AB if used inappropriately or without any proper medical supervision may be toxic. In addition, lack of proper treatment protocol as well as the antidote i.e. Physostigmine in most of the cases, may exaggerate the toxicity symptoms and lead towards fatal condition.

CONFLICT OF INTERESTS

No conflict of interest exists among the authors.

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Simultaneous degradation of organochlorine pesticides by microbial consortium

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ABSTRACT

Organochlorine pesticides (OCPs) primarily 1, 1, 1-trichloro-2,2-bis(4-chlorophenyl) ethane (*p,p*'-DDT) and γ-hexachlorocyclohexane (lindane) pose adverse health effects to the environment and community. Lindane and DDT (dichlorodiphenyltrichloroethane) have been broadly used for agricultural purposes. DDT is still the "sought after" for public health care programs to control vector-borne diseases like malaria in developing nations. Even though both these compounds are prohibited in many parts of the world, their residues are still found in various environmental, food, human and animal samples. In this study, the microbial population isolated from aquatic systems, rivers from Yamuna (North India) and Godavari (South India) was enriched until a Lindane and DDT tolerant population was established. Screening of the population for understanding bioremediation potential was done using 5ppm of DDT and Lindane. The populated microbial cells formed the consortium that was used subjected to metagenomic analysis to identify the organisms till species level. The 16S amplicon sequences were analysed using Kaiju online tool for establishing the identity of individual bacteria present in the mixed population. This consortium was shown to consist of 138 species with 11 uncultured bacteria with *Taylorella asinigenitalis* holding the maximum numbers in its population. The microbial consortium was found simultaneously degrading DDT and Lindane in shake flasks. The enriched consortium could degrade 95% of 5 ppm lindane and 57.8% of 5 ppm DDT in the supplied mixture by the end of 72 h. This is a promising observation and the consortium shows high efficiency in degrading the OCPs. The consortium can become a vital tool for biodegradation of organochlorine pesticides to eradicate the pesticide residues in water and soil ecosystems.

KEY WORDS: MICROBIAL CONSORTIUM, BIODEGRADATION, KAIJU, DDT, LINDANE

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INTRODUCTION

1,1,1-trichlorγo-2,2-bis(4-chlorophenyl) ethane (*p,p'*-DDT) and -hexachlorocyclohexane (lindane) are the two most extensively applied pesticides in the later part of the 20th century by many nations globally (Aktar et al. 2009, Donald, 2001). Even though both these compounds are prohibited in many parts of the world, their residues are still found in environment, food, human and animal samples. Lindane has been enlisted under carcinogenic compounds while DDT is a suspected oncogenic agent. The water bodies, agricultural soil and the glaciers in the polar regions of the earth show the presence of these synthetic pesticides. It has become a priority issue for the scientific fraternity to clear off these residues from the environment ultimately leading to pesticide-free food, water, fruits, beverages, vegetables, etc. and providing the future generations a conducive condition to thrive, (Pandey et al. 2011, Villa et al. 2003, Wilson et al. 2013, Loomis et al. 2015, Muinck, et al. 2017 and Jayaraj et al. 2017).

Lot of research has been carried out to answer this problem, but not much clearance of the pesticide residues is documented in the field applications (María et al. 2010). Even though there are many processes that have been protected under patents (Bidlan et.al. 2009) often no efforts are made to create awareness among the government agencies, farmers and others to exploit them. Most of the research in bioremediation revolves around a few microbes and a single compound at the laboratory level. Only the effluent treatment plants use the natural microbial flora in their systems. Still no organochlorine pesticide residues are eliminated from the nature leading to their persistence and causing major health issues among the population. In this paper, we describe a diverse population of microbes in a consortium isolated from Indian rivers Yamuna and Godavari and characterised using 16S metagenomics through illumina platform (Muinck, et al. 2017) that demonstrates the potential to degrade the organochlorine insecticides DDT and lindane provided as a mixture in shake flasks

MATERIAL AND METHODS

CHEMICALS

99.4% pure p,p'-DDT was donated for research by Hindustan Insecticides Ltd., India while 97% pure lindane was purchased from Sigma Aldrich, USA. HPLC grade acetone and ethyl acetate was procured from Merck. Other chemicals used were of analytical grades and purchased from standard manufacturers.

Microbial culture and enrichment: Water samples were collected from highly contaminated rivers Yamuna and Godavari. These samples were subjected to increasing concentrations of two organochlorine pesticides (OCP) 1,1,1-trichloro-2,2-bis-(4-chloro) ethane (DDT) and γ-hexachlorocyclohexane (lindane) over a period of 6 months. Periodically it was tested for the viability of the population through streaking on to nutrient agar (Bidlan, 2003).

Screening for the OCP degradation potential: The microbial population developed through enrichment was screened for its potential to degrade DDT and lindane mixture simultaneously. 25 mL of sterile water through reverse osmosis and autoclaving was spiked with a mixture of 5 ppm DDT and Lindane. The enriched microbial population was inoculated to this and incubated at ambient condition in a rotary shaker set at 150 rpm. Samples were drawn periodically and analysed for residual OCPs (Bidlan and Manonmani 2004).

Extraction of OCPs: Samples drawn at every 24 h were acidified and extracted with equal volume of dichloromethane twice and organic layers pooled after passing through anhydrous sodium sulphate and fluorisil. The residual OCPs were transferred into microfuge tubes after completely drying the dichloromethane and dissolving in a small volume of acetone.

Thin Layer Chromatography: TLC was performed on 0.25 mm thick silica gel G layers. Residual OCPs were dissolved in a known volume of acetone. Equal volumes of this solution carrying 10 micrograms of each pesticide at 0 h were loaded and developed in a saturated environment of cyclohexane. The air-dried plates were sprayed with 2% *o*-tolidine solution in acetone under bright sunlight to detect the individual OCP spots. The intensity and area under each spot was considered to quantify the residual insecticides by using the standard curve plotted with *vs* log (Concentration).

Gas Chromatography-Mass Spectrometry Fingerprinting: Qualitative and quantitative analyses were confirmed with GCMS/MS fingerprinting using Agilent 7000D equipment with a triple quad (Gawad, 2016). The column HP-5ms (Agilent 19091S EPC) was programmed with pressure 30.797 psi, the flow of 3.1793 mL.min-1, average Velocity of 54.506 cm.s-1 and temperature 70°C to 280°C, the sample was electron ionized (EI) with a source temperature of 300°C. The quantification was done using a standard curve prepared for different amounts of the two OCPs (DDT and Lindane) under the same conditions (Muir, 2006).

Genomic DNA extraction: DNA was isolated using Xcelgen Bacterial gDNA kit with few modifications. Quality of gDNA was checked on 0.8 % agarose gel and quantification was done on Nanodrop 8000.

Microbial Identification: The microbial population was tested for the presence of various bacteria using 16S rRNA gene analyses through metagenomic approach. Illumina platform (Blomquist TM et al. 2013) was used to carry out next-generation sequencing of ~460 bp amplicons obtained using Prokaryotic V3 Forward primer 5' CCTACGGGNBGCASCAG3' and Prokaryotic V4 Reverse primer 5' GACTACNVGGGTATCTAATCC3'. The obtained 1,94,544 sequences were subjected to online tool Kaiju (http://kaiju.binf.ku.dk/server) for identification purpose.

RESULTS AND DISCUSSION

The long-term enrichment of the water sample with the mixture of DDT and Lindane resulted in a microbial population that contained many types of microorganisms, including bacteria and fungi. Some of the strains were isolated by repeated streaking. The degradation of mixture of DDT and Lindane, both at 5 ppm concentrations was reflected in the TLC plate (Figure. 1) and confirmed by GCMS/MS fingerprinting. There was a marked reduction in the residual concentration of Lindane in three days of incubation. The amount of DDT also reduced from 0 h to 72 h. The visual/apparent concentrations in the spots for both the insecticides is a clear indication that the microbial population was effectively working against the OCPs supplied and catabolising these compound for its growth and energy. Since the only source of carbon in the medium was the mixture of DDT and Lindane, the other source of carbon can be the atmospheric carbon in the form of carbon dioxide and few cell masses that must have died. There was an increase in the biomass with incubation, indicating the efficiency of the consortium to resist the OCP and grow in sole supply of these compounds as carbon source. However, the TLC clearly indicated the degradation of DDT and Lindane in presence of the consortium.

Gas Liquid Chromatograms show that the retention time of Lindane was 13.3 min while that of *p,p'*-DDT was 26.9 min under the conditions of analyses. Fig. 3 gives the chromatograms of the screening experiment with 5 ppm of each DDT and Lindane in the broth. There is a gradual decrease in the peak heights and area under them for both DDT and Lindane from 0 h to72 h. The degradation pattern of the two pesticides is given in Fig. 4. The enriched consortium could degrade 95% of 5 ppm Lindane and 57.8% of 5 ppm DDT in the supplied mixture by the end of 72 h. This is a promising observation and the consortium shows high efficiency in degrading the OCPs. Other research groups have also shown the degradation of DDT and Lindane by various individual microorganisms or a defined consortium having only a few strains (Bidlan, 2002). Simultaneous degradation of organochlorine and organophosphate pesticides was demonstrated by researchers (Abraham et al. 2014).

This study is novel as it attempts to demonstrate the degradation of the mixture of DDT and Lindane (mixed organochlorines) by the bacterial consortium. This hold a great promise in future bioremediation applications.

Identification and relative abundance of various bacteria in the consortium

The consortium was subjected to metagenomic analysis through 16S rDNA gene sequencing using the set of prokaryotic primers. The amplified sequences of

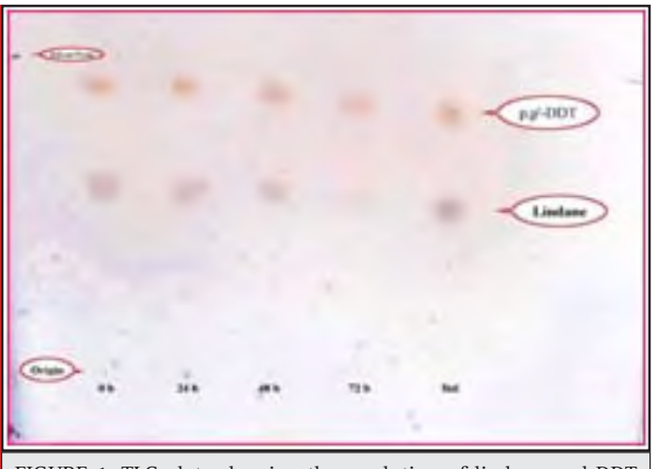

FIGURE 1. TLC plate showing the resolution of lindane and DDT from the residual mixture of samples at various intervals of incubation and standard. Spots appeared after the developed plate was sprayed with *o*-tolidine and exposed to sunlight.

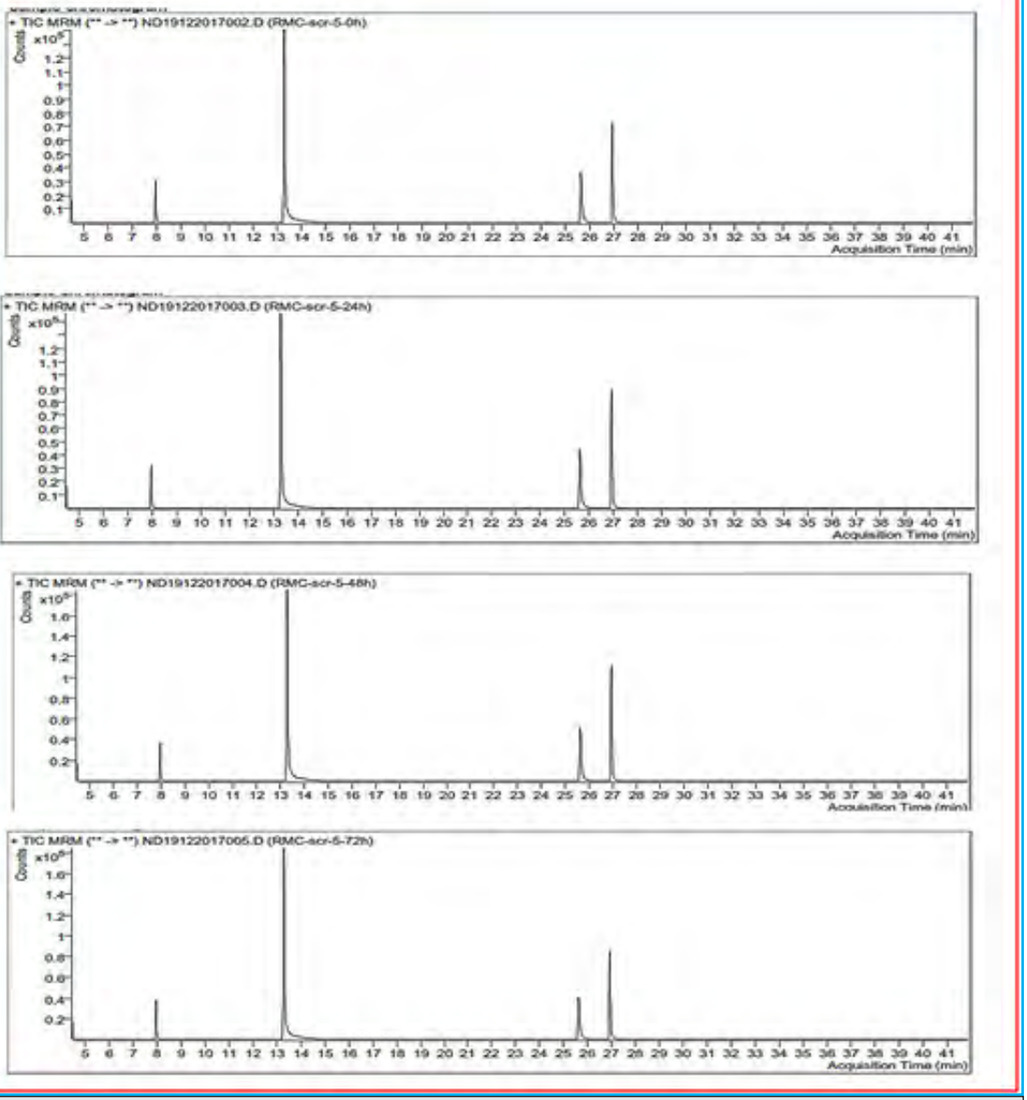

FIGURE 2. Gas Chromatograms of DDT and Lindane Residual Concentrations at different incubation periods at 0 Hr, 24 Hr, 48hr and 72 hr

approximately 146 bp were analysed after removing the chimeric reads. A total of 194544 reads were analysed by the webserver tool "Kaiju". The program could identify 138 different species, few of which were uncultured bacteria. We have represented the top 63 species distribution in Fig. 5.

Taylorella asinigenitalis with 52.51 % relative abundance was the highest number of reads (128267). Second highest reads with a relative abundance of 8.13% was from *Enterococcus faecalis*. One uncultured bacterium was occupying the third position among the consortium population (Handelsman, 2004). A total of eleven uncultured bacteria/archaea were detected in the consortium out of which seven are present in the top 63. This report shows more than 100 various species of bacteria / archaea that might play a significant role in the bioremediation of OCPs. With more studies and analyses, the consortium can be further trained to degrade higher concentrations of these OCPs and other environmental threats caused by human activities. Complete DNA sequences obtained have been deposited at NCBI under the bioproject ID PRJNA420925.

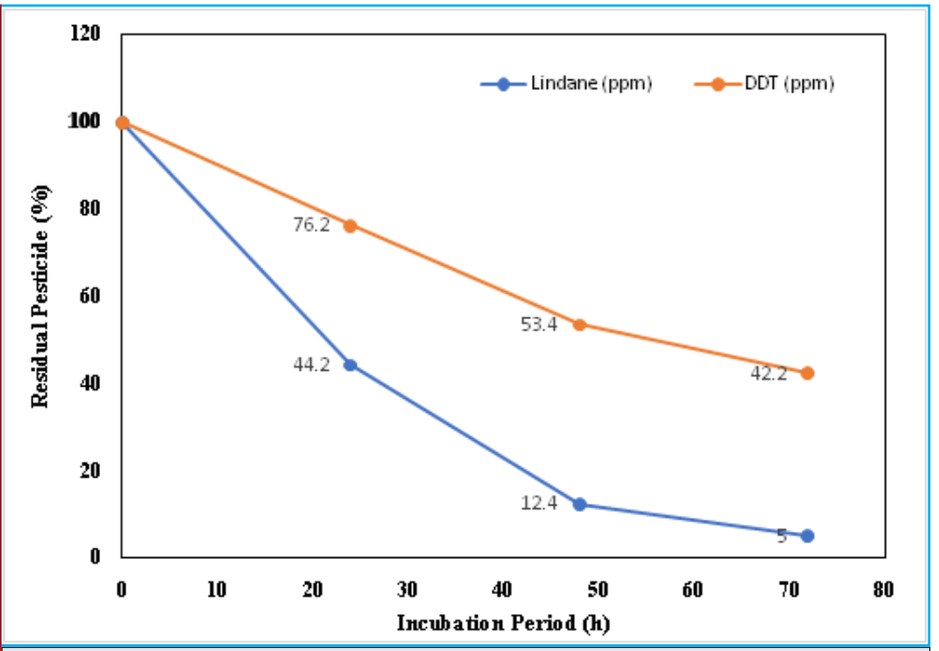

FIGURE 3. Degradation of DDT and Lindane by Consortium

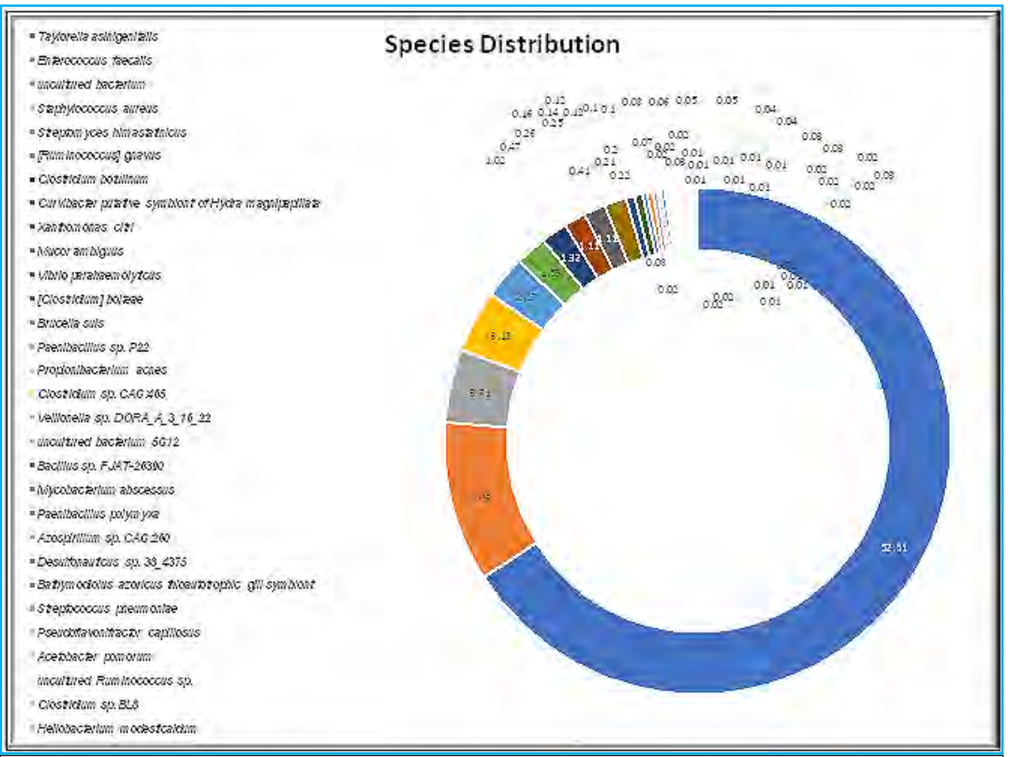

FIGURE 4. Pie chart of species distribution of bacteria characterized using 16S metagenomics and Kaiju tool with relative abundance in the consortium.

CONCLUSION

Many reports have appeared in the last 6-7 decades regarding the use of microbes in cleaning of environmental contaminants. Ours is also an attempt with a view that the single axenic culture or just a few membered consortia might not suffice the need of the hour. Hence, a consortium developed from the highly contaminated river sources in India was demonstrated to have a great potential in remediating the environment off OCPs. This consortium was shown to consist of 138 species with 11 uncultured bacteria and *Taylorella asinigenitalis* holding the maximum numbers in its population. This consortium not only comprises of bacteria but also have various fungi that have not been considered in the present study. The complete analyses of this consortium and further studies to confirm its efficacy in treating higher amounts of OCPs and other compounds need to be taken up. This is just the beginning of a new era in combating the environmental and health issues, new avenues are yet to be explored. The consortium can become a promising solution for biodegradation of organochlorine pesticides to eradicate the pesticide residues in water and soil ecosystems.

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Evidence of biological activity of *Pulicaria crispa* on *Biomphalaria pfeifferi* host snails of *Schistosoma mansoni*

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ABSTRACT

The present study was carried out to investigate the molluscicidal activity of the herb *Pulicaria crispa* against the freshwater snails *Biomphalaria pfeifferi*, the intermediate host of *Schistosoma mansoni* parasites that cause intestinal schistosomiasis. It is an attempt to search for potential molluscicidal plants indigenous to Republic of Sudan. The molluscicidal activity of different parts of *Pulicaria crispa* (leaves, stems, and roots) on *Biomphalaria pfeifferi* snails in different periods of exposure *(24, 48, and 96 hours)* was studied. The assessment of toxicity was based on calculations of the lethal concentrations that killed 50% and 90% (LC_{50} and LC_{90}) of the snail populations. Results revealed that leaves were the most potent against the snails (having the least LC_{50} and LC_{90} values) for all exposure periods (24, 48, and 96 hours), followed by flowers, and finally the stems. With regard to the effect of exposure time, it was evident that the prolongation of exposure period resulted in more potency of plant leaves against the snails as shown by the decreased LC_{50} and LC_{90} values for 96 hours exposure period, followed by 48 hours, and finally 24 hours. During the first five hours of exposure, snails showed sluggish activity, meanwhile dead snail remained inside their shells with blood around the shells. Accordingly, the herb *Pulicaria crispa* could be considered as one of the promising plant molluscicides in schistosomiasis control programs.

KEY WORDS: *PULICARIA CRISPA*; *BIOMPHALARIA PFEIFFERI*; PLANT MOLLUSCICIDE; SCHISTOSOMIASIS

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INTRODUCTION

Schistosomiasis is a freshwater-borne disease caused by the worm parasites of the genus Schistosoma. Aquatic snails of several genera including *Biomphalaria, Bulinus*, and *Oncomelania* are the intermediate hosts of the parasites (Gryseels *et al.* ; 2006 Steinmann *et al.* 2006). Human schistosomiasis is ranked second after malaria in terms of public health significance (Michaud *et al.* 2003; Hamed, 2010; WHO, 2013). About 70-78 countries are schistosomiasis-endemic, and almost 240 million people are infected and require preventive chemotherapy; while over half a billion others are at risk of infection. Human schistosomiasis is endemic in tropical and sub-tropical areas including Africa (with more than 90% of infection occurring in sub-Saharan Africa), the Americas, the Eastern Mediterranean region, the South-east Asian region and the Western Pacific (Michaud *et al.* 2003; WHO, 2013). Schistosomiasis has profound negative effects on child development, outcome of pregnancy, and agricultural productivity. Various factors are responsible for the continuous and persistent transmission of schistosomiasis in sub-Saharan Africa. These include climatic changes and global warming, proximity to water bodies, irrigation and dam construction as well as socio-economic factors such as occupational activities and poverty. Domestic activities such as washing clothes and fetching water in infected water expose women and children to infection. Recreational activities like swimming and poor hygiene also make children vulnerable to schistosomiasis. The disease remains a major public health problem in many parts of the world causing a global cost of 3.3–4.5 million disability-adjusted life years, (Madsen and Frandsen, 1989; WHO, 2002; Adenowo *et al.* 2015, Deol *et al.* 2016 Antonio and Garba 2017).

The best method to control trematode infection is to control the population of vector snail by the use of molluscicide either synthetic or plant origin. Chemical molluscicides have been extensively used to control snail populations in water bodies used by humans around many schistosomiasis-endemic countries. However, these chemicals, such as niclosamide, have demonstrated severe toxicity to many aquatic non-target organisms. Moreover, their high cost and evidence of acquiring resistance by some species of snails stimulated many countries to abandon use of chemical molluscicides, and consequently began exploiting indigenous plants as sources of molluscicides in snail control programs, (Schall *et al.* 2001; Singh *et al.* 2005; (Soni and Singh, 2017, Faria *et al.* 2018).

Plant-derived molluscicides have many advantages over chemical ones. They are of low cost, less toxic to non-target organisms, and easily biodegradable along the food chain. Moreover, the use of indigenous molluscicides rather than imported ones is desirable, especially as strategies for schistosomiasis control programs should be based on long-term operations (Kloos and McCullough, 1982; Duncan, 1987; Ndamba *et al.* 1994; Angaye *et al.*, 2015). Many indigenous plants of the Republic of Sudan have been investigated for molluscicidal activity. *Pulicaria crispa* - locally known as "*attaghar*" in Sudan - is used in folk medicine for treatment of colds, coughs, colic, excessive sweating and as a carminative (Elshiekh and Abdelmageed, 2015).

The present study was undertaken to evaluate the biological activity of different parts of *Pulicaria crispa* (leaves, stems, and roots) on *Biomphalaria pfeifferi* snails in different periods of exposure (24, 48, and 96 hours). The behavior of snails for the first five hours was also observed.

MATERIAL AND METHODS

Animals: The freshwater snail *Biomphalaria pfeifferi* (Krauss, 1848, Gastropoda: Pulmonata) is the most important intermediate host of *Schistosoma mansoni* in Africa in terms of its large geographical range. Snails were collected from Alsileit Agricultural Scheme (Khartoum North) and screened in the laboratory for infection with any type of parasites. Non-infected snails, being kept for two weeks to adapt laboratory conditions, were used in the experiments.

Collection and preparation of plant samples: *Pulicaria crispa* (Forssk.) Oliv, - with synonyms *Pulicaria undulata* (L.) C.A. Mey and *Francoeuria crispa* (Forssk.) Cass - is an annual herb or sometimes a perennial subshrub belonging to the family Asteraceae. The plant is found in in Saudi Arabia, Kuwait, Iran, Iraq, Egypt, Afghanistan, Pakistan, India and parts of north and west tropical Africa (Stavri *et al.* 2008). Identified flowering samples of the herb were collected from the wild in Shambat area, north of Khartoum, Sudan, and brought to the laboratory. The leaves, stems, and flowers were picked, air-dried separately in the shade for at least four days. The plant materials were then ground to powder. 500, 625, 750, 875, 1000, 1125, 1250, 1375, 1500, 1625, 1750, 2000, 2250, and 2500 mg of leaves, stems, and flower powdered materials were accurately weighed and separately put in paper envelopes until bioassay experiments.

Bioassay tests of *P. crispa* materials on adult snails: Only adult snails (6-7 mm diameter) were used in all experiments, in a range of 26-29^{o}C. For evaluation of the toxicity of dried un-extracted plant samples, 500, 625, 750, 875, 1000, 1125, 1250, 1375, 1500, 1625, 1750, 2000, 2250, and 2500 mg of each of leaves, stems, and flowers powdered materials were separately added

Table 1. Effect of different parts of *P. crispa* on *B. pfeifferi* snails exposed for different periods of time.

Exposure period (hours)	Plant part	Regression equation	LC50 (ppm)	LC90 (ppm)	(UL – LL) of activity*	Slope (degree)	r2**
24	Leaves	Y = 11.38X -26.70	616.60	776.25	±2.52	85.0	0.97
	Flowers	Y = 11.35 X -26.70	616.60	812.83	±2.16	84.9	0.98
	Stems	Y = 12.18X -29.21	645.65	812.83	±1.69	85.3	0.99
48	Leaves	Y = 5.41X -9.16	416.87	707.95	±1.34	79.5	0.99
	Flowers	Y = 5.75X -10.26	446.68	758.58	±2.41	80.1	0.96
	Stems	Y = 6.25X -11.84	489.78	794.33	±2.13	80.9	0.97
72	Leaves	Y = 6.01X -9.57	263.03	436.52	±3.55	80.6	0.92
	Flowers	Y = 4.74X -6.68	288.40	537.03	±1.84	78.1	0.97
	Stems	Y = 3.97X -5.20	371.54	776.25	±1.67	75.6	0.97

*upper and lower limits of activity for LC50 and LC90) with 95% confidence limits.** The regression coefficient

to plastic dishes that contained 1500 ml dechlorinated tap water and 25 snails. Therefore, the concentrations used for each sample were 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 800, 900, and 1000 ppm. There were four replicates for each concentration. The exposure period for each experiment was 24 hours followed by other 24 hours recovery period in dechlorinated tap water. The behavior of snails was continuously observed for the first five hours.

Statistical Analysis: The regression equation, lethal concentration that killed 50% and 90% of animal population (LC_{50} and $LC_{90,}$ respectively), fiducial limits (upper and lower limits of activity for LC_{50} and LC_{90}) with 95% confidence limits were calculated by using Probit analysis. The regression coefficient (r^2) to show the degree of homogeneity between the concentration of plant sample and mortality of snails was calculated. Also the slope values (in degrees) to determine the quick response of the snails to the plant parts were calculated.

RESULTS AND DISCUSSION

Table 1 shows the overall results of the experiments that tested the biological activity of different plant parts against snails during the different exposure periods. LC50 and LC90 values revealed that leaves were the most toxic to *B. pfeifferi* snails, then flowers, and finally stems. Also figures 1, 2, and 3 illustrate the regression lines obtained by plotting the "Concentration" and the "Probit" values. Kloos *et al.* (1987) screened fifty local medicinal agricultural in Kenya and found that the higher molluscicidal activity was observed in leaves as

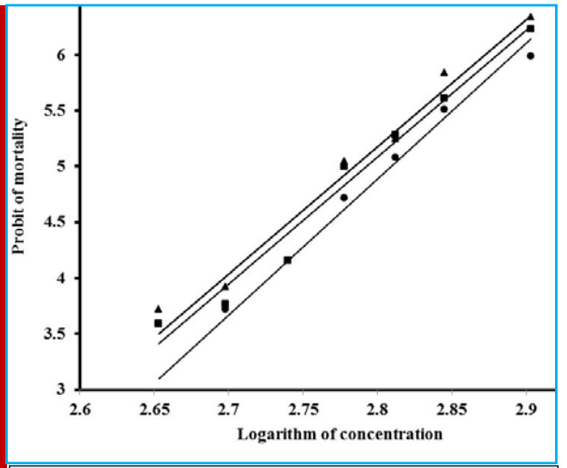

FIGURE 1. Illustration of regression lines resulted from activity of *P. crispa* parts on *B. pfeifferi* snails exposed for 24 hours.

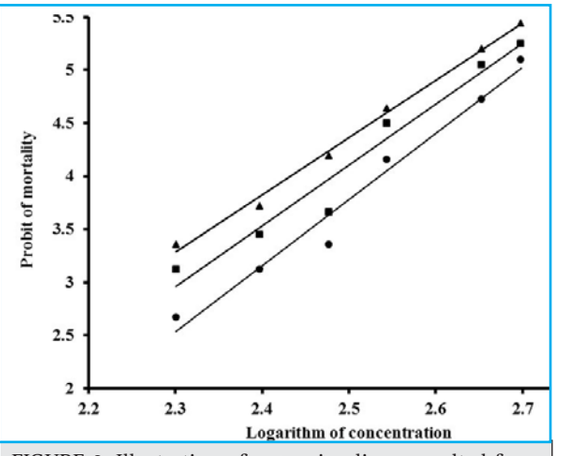

FIGURE 2. Illustration of regression lines resulted from activity of *P. crispa* parts on *B. pfeifferi* snails exposed for 48 hours.

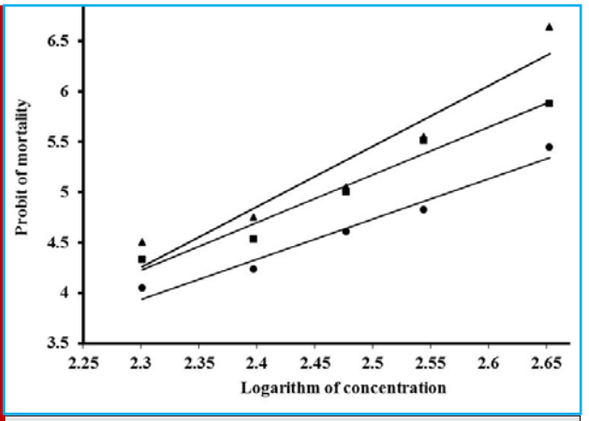

FIGURE 3. Illustration of regression lines resulted from activity of *P. crispa* parts on adult *B. pfeifferi* snails exposed for 72 hours.

period, followed by 48 hours, and finally 24 hours. However, the relationship between the concentration of the plant material and the activity exerted on the snails is not necessarily to be in a constant manner. For example, if the exposure time is doubled, the effective concentration is not necessarily halved (WHO,1965). Unarguably, long exposure is not advised because the longer the exposure period, the greater the chance that snails would be influenced by factors unrelated to the active ingredient(s) responsible for the toxicity (Dam *et al.* 1989). Moreover, our observations showed slight rottenness of the dishes containing plant materials tested for 72 hours.

The mode of action observations during the first five hours showed more sluggish activity of snails immersed in leaves and stems compared to stems, but there were no dead snails reported. Dead snails remained intact inside their shells and appeared to bleed, as shown by

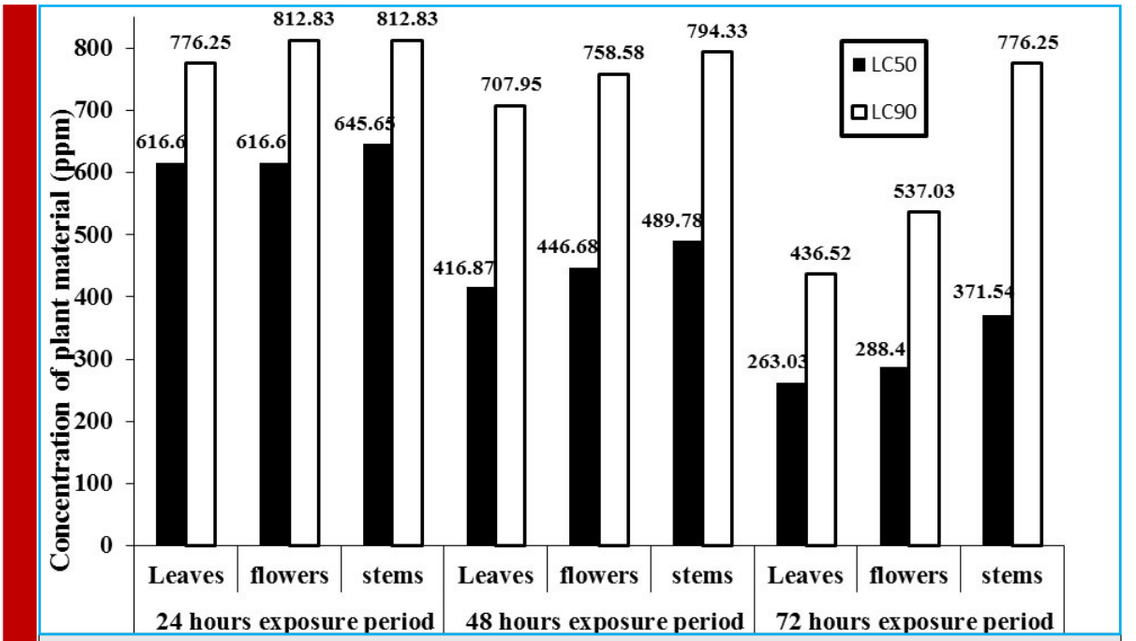

FIGURE 4. Comparison of LC50 and LC90 values of different parts of *P. crispa* on *B. pfeifferi* snails exposed for different periods of time.

opposed to other plant parts. This might be partly due to the fact that the leaves are the main sites of photosynthesis for synthesis of secondary metabolites. Moreover, leaves have a higher surface area volume ratio resulting in more rapid release and higher solubility of any toxicant(s).

The LC_{50} and LC_{90} values - as shown in table 1 and also depicted in figure 4- tend to decrease with the prolongation of the exposure periods in all experiments. This indicates high toxicity with 72 hours exposure

the red color around the shells. It has been known that snails exposed to molluscicides either retract into their shells and expel hemolymph or become swollen and remain extended from the shell aperture, possibly due to disrupted osmoregulation at concentrations around their LC^{50} (Combes and Cheng, 1986).

On conclusion, the herb *Pulicaria crispa* could be considered as one of the promising plant molluscicides in schistosomiasis control programs as long as recently accepted that naturally occurring molluscicides isolated

from various plant sources have shown many desirable advantages over chemical ones.

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Plant regeneration from direct and indirect organogenesis and assessment of genetic fidelity in *Saccharum officinarum* using DNA-based markers

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ABSTRACT

Sugarcane has acquired significant importance in the world economy because of the sugar and ethanol production. Therefore, rapid multiplication of outstanding sugarcane variety is necessary in developing countries. The aim of this investigation was to compare regeneration efficiency of various explants and genetic fidelity of regenerated plantlets certified by using DNA-based molecular markers, that is, random amplified length polymorphism (RAPD) and inter simple sequence repeats (ISSR). The sugarcane plantlets were obtained through direct (axillary buds, apical meristem, and leaf whorl disk) and indirect (callus culture) shoot organogenesis from the variety Co 86032. Among all the explants, highest shoot forming ability was observed in axillary buds showed 97.66±0.66% shoot formation and the highest number of shoots per explants (4.33±0.24) and a total number of regenerated shoots (173.00±8.11) were observed in the leaf whorl disk. Morphological variation was not observed among the regenerated plants from various explants and therefore, genomic DNA was isolated from fresh leaves and genetic fidelity assessment was carried out using RAPD and ISSR. Both the markers produced 1368 and 2271 bands, respectively, including all the tested plants, indicates that plants derived from direct organogenesis did not show any polymorphism. However, a genetic variation has been observed in the plants derived from callus and showed 4.54% polymorphism during analysis. The results suggested that plants regenerated from direct organogenesis are of more true-to-type, whereas genetic variation occurs during indirect organogenesis. Combination of RAPD along with ISSR can be used for detection of genetic variation in the early stage in sugarcane micropropagation. High performance of regeneration and low risk of genetic variation ascertains the efficiency of this operation.

KEY WORDS: FIDELITY, ISSR, MICROPROPAGATION, REGENERATION, RAPD, SUGARCANE

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INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is a member of the Poaceae family and is an imperative cash crop. Sugarcane produces around 70% to 80% of sugar worldwide and 100% in India (Suprasanna et al. 2011; Thorat et al. 2017). In India, sugarcane is cultivated under a wide range of agro-climatic conditions and secured a distinct position after cotton, and as an agro-industrial crop because of the prominent source of efficiently vital product (sugar) as well as by-products (bagasse, molasses, and press mud) playing a major role in the economic progress of small and large-scale industrial sectors. In a tropical climate, the cultivation of sugarcane is more successful in terms of cane yield and sugar recovery throughout the year (Thorat et al. 2015; Singh and Shami, 2017).

Sugarcane is recognized as the most competent crop, which converts solar energy into harvestable chemical energy in the form of sucrose and biomass (Joyce et al. 2010). Sugarcane varieties are highly heterogeneous and usually multiplied by stem cutting method. The stems of sugarcane with two to three nodes are generally used for plantation in many tropical countries (Behara and Sahoo, 2009). Owing to the unavailability of standardized multiplication procedure and contamination by systematic microorganism is the serious challenge to multiply an elite genotype of sugarcane (Lal and Singh, 1994; Thorat et al. 2016).

However, plant tissue culture of sugarcane has received extensive research attention because of its economic consequence (Behara and Sahoo, 2009). During the past four decades, plant tissue culture is used for the large-scale micropropagation in commercial horticulture and agriculture worldwide (Das et al. 1996). Generally, plant tissue culture technique is used for providing the highest number of healthy plantlets within the short duration, which is useful for rapid spreading of newly, released sugarcane varieties in short duration (Fildmann et al. 1994). In sugarcane, various explants like apical meristem (Devarumath et al., 2007), axillary buds (Thorat et al. 2016), leaf whorl disk (Gill et al. 2006; Kaur et al. 2015), callus (Behara and Sahoo, 2009), and cell suspension culture (Thorat et al. 2017) were used for rapid multiplication. During the process, to maintain genetic fidelity of the micro-propagated plantlets with respect to the mother plants are major concerns. Despite the advantages of micropropagation, many genera including sugarcane has been found polymorphic,(Devarumath et al., 2007; Lal et al. 2007, Rizvi et al, 2012 and Thorat et al. 2017).

Since last few years, a range of DNA-based molecular markers have been utilized for fast and recognizable assessment of the genetic similarity of micropropagated plants including sugarcane (Devarumath et al., 2002 and 2007; Lal et al. 2007; Goel et al. 2009; Rizvi et al, 2012; Kshirsagar et al. 2015). The random amplified length polymorphism (RAPD) and inter simple sequence repeats (ISSR) are easy, reliable, quick technique to perform, it did not require past knowledge of the nucleotide sequence of the organism, and requisite less amount of DNA as well as comparatively less expensive and provides effective variation at multiple loci in DNA (Williams et al. 1990; Ceasar et al. 2010 Govindraj et al. 2015).The present work was performed with an objective to establish the rapid and efficient micropropagation method by using various types of sugarcane explants, and study their genetic variation occurred during the process of direct and indirect organogenesis using molecular markers (RAPD and ISSR).

MATERIAL AND METHODS

The sugarcane var. Co 86032 grown in experimental farms of Vasantdada Sugar Institute, Manjari (Bk.), Pune, Maharashtra, India was used in this investigation. The plants were regenerated from direct (axillary buds, apical meristem, leaf whorl disk) and indirect (callus) organogenesis.

DIRECT ORGANOGENESIS

Axillary buds culture

The axillary buds were collected from 6 to 8-month old field-grown sugarcane. The sugarcane were sets cleaned properly by washing under running tap water followed by 0.1% Tween-20 solution for 15 min. After choosing 50 healthy axillary buds, were dissected properly (1–1.5 cm^2) with the help of scalpels and kept in antioxidant solution (0.1% citric acid and 0.1% ascorbic acid) for 15 min followed by surface sterilized with Bavistin™ (0.1%) and streptomycin (0.1%) for 15 min at 25±1°C (Thorat et al. 2016). Sterilized buds were inoculated in primary shoot regeneration medium supplemented with sucrose (30 g/l), casein hydrolysate (0.5 g/l), polyvinyl pyrrolidone-40 (100 mg/l), inositol (20 mg/l), thymine (1 mg/l), and 6-benzylaminopurine (BAP) (3 mg/l) containing 0.5% plant preservative mixture and incubated at 28±1°C with a 16 h photoperiod for1 month (Thorat et al. 2016).

Apical meristem culture

The apical meristems were excised from 4 to 5-month old field-grown sugarcane tops. The cane tops were surface sterilized with the above mentioned procedure. In total, 50 number of apical meristem was obtained by removing the outer layers and the innermost part (2–3

mm) were aseptically transferred to test tubes containing Murashige and Skoog (1962) basal (MS) medium supplemented with kinetin and BAP (Sawant and Tawar, 2011) and allowed to grow for 20–30 days.

Leaf whorl disk culture

Expanded leaves from the harvested shoot tops were removed and washed with 0.1% Teepol™ for 4–5 min followed by a rinse under tap water for 10–12 min. The external whorls of mature leaves were removed till a spindle of 8–10 cm in length and ~1.0 cm in diameter was obtained (Kalunke et al. 2009; Babu and Nerkar, 2012). Then these spindle explants were treated with 70% ethanol for 30 sec followed by 0.1% Bavistin™ and 0.1% streptomycin for 15 min, then surface sterilized with 0.1% $HgCl_2$ for 20 min. These sterilized explants were washed thrice with sterile distilled water to remove the traces of $HgCl_2$. After surface sterilization treatment, leaf whorls were washed thoroughly in sterilized distilled water for 3–4 times and external layers of leaf sheath were pulled out one by one until the cylinder reached approximately 0.5–1 cm in diameter. The 50 cylinders were cut transversely into thin slices (1–1.5 mm thick) and inoculated on MS medium supplemented with 5.0 mg/l naphthaleneacetic acid (NAA) with 0.5 mg/l kinetin (Kaur and Sandhu, 2015). About 30 explants were taken per treatment and number of replications per treatment was three. The cultures were incubated under light (~3000 Lux) at 26±2°C and 16/8 h (light/dark) photoperiod regimes.

INDIRECT ORGANOGENESIS

Callus culture

The thin slices (1–1.5 mm thick) of the leaf whorl after sterilization (above mention), inoculated on callus induction medium containing MS basal medium supplemented with sucrose (20 g/l), coconut water (100 ml/l), polyvinylpyrrolidone-40 (PVP) (500 mg/l), casein hydrolysate (500 mg/l), 2-ethanesulfonic acid (MES buffer) (500 mg/l), thiamine HCl (1 mg/l), inositol (20 mg/l), and proline (500 mg/l) were incubated in dark at 28±2°C for 2weeks. Well proliferated 50 embryogenic calli (0.5 cm^2) were transferred to shoot regeneration medium.

Shoot multiplication

After 1 month-inoculation, regenerated shoots from various explants were isolated without damaging their basal portion. Single shoot or shoots clusters were transferred to culture bottles containing secondary shoot multiplication medium containing liquid MS basal medium supplemented with 30 g/l sucrose, 0.5g/l casein hydrolysate, 100 mg/l PVP, 20 mg/l inositol, 1 mg/l thiamine HCl, 1 mg/l kinetin, 0.5 mg/l NAA, and 2 mg/l 6-benzylaminopurine. The culture bottles were incubated at 26±2°C with a 16/8 h (light/dark) photoperiod regimes for 4 weeks on illuminated shakers.

Rooting of shoots and acclimatization

Elongated shoots (4–5 cm) after 4 weeks from SSRM were transferred to half-strength MS basal medium supplemented with 0.5 mg/l NAA. Root induction was promoted by incubated at room temperature for 16/8 h (light/dark) photoperiod regimes. After 15–20 days, shoot showing prominent roots was considered as rooted plantlets. In total, 10–12-weeks old rooted plants were carefully cleaned with water to remove excess agar, then transferred in plastic bags filled with soil mixture containing 1 part of sand, 1 part of the coco peat, and 1 part of vermicompost (v/v) and irrigated with water for the next 2 weeks under greenhouse condition.

Genetic stability analysis using molecular markers (RAPD and ISSR)

Total genomic DNA from young leaves of the mother plant and in-vitro raised plants was isolated by the method described by Aljanabi et al. (1999). The quality and quantity of genomic DNA were determined by 0.8% agarose gel electrophoresis and by UV-vis spectrophotometry. The final concentration of extracted genomic DNA was made to 50 ng/µl and stored at –20°C till further use. Two sets of primers including arbitrary (RAPD) and semi-arbitrary (ISSR) were used for analysis of genomic DNA ofthe mother plant and in-vitro raised plantlets. In total, 10 primers each of RAPD (OPH series, Operon Technologies, INC. California, USA) (Table 2) and ISSR primers (UBC series, University of British Columbia, Vancouver, Canada) (Table 3) were selected on the basis of preliminary screening and their reproducibility. PCR analysis was done with slight modifications (Williams et al. 1990). For RAPD, DNA amplification was carried out with total reaction mixture volume of 20 µl consisting 50 ng template DNA (1 µl), 10x PCR buffer with $MgCl_2$ (2 µl), 250 µM dNTPs (2 µl), 0.25 µM primer (2 µl), 1U Taq DNA polymerase (0.2 µl), and sterile nuclease-free distilled water (12.8 µl). PCR was performed on Thermal cycler (Applied Biosystem, USA) at initial temperature of 94°C (5 min, 1 cycle), followed by 40 cycles of 1 min at 94°C, 1 min at 37°C, 2 min at 72°C, and final extension cycle of 10 min at 72°C. In the case of ISSR, the reaction mixture was made similar to RAPD. However, PCR was carried out using initial denaturation at 95°C for 5 min followed by 40 cycles 1 min at 94°C, 1 min at 50–54°C (depending upon the primer), 2 min at 72°C, and final extension cycle of 10 min at 72°C. The samples were stored at 4°C until further analysis was carried out. The PCR products were resolved on 1.5 % (w/v) agarose gel with 1x TBE buffer, stained with ethidium bromide and

documented under UV light. The fragment size was estimated using 1 kb DNA ladder.

Statistical Analysis

All experiments were performed twice, with three independent replicates. Genomic DNA was extracted from the same bulk sample to maintain the consistency of results. The data were analyzed using Microsoft Excel 2010 and SPSS (Statistical package for the social sciences; software package version 16). One-way ANOVA (Analysis of variance) was applied to test mean differences of all treatments while the statistically significant difference between mean values was established at p value ≤0.05 while Duncan's New Multiple Range Test was used. The results were expressed as mean ±SE. For RAPD and ISSR analysis, a band with same mobility were counted as an identical band while each amplified product was scored as present (1) or absent (0), bands of low intensity that were difficult to be distinguished as present or absent were not considered.

RESULTS AND DISCUSSION

Somaclonal variations have been frequently reported in tissue culture raised plants. The occurrence of variation during in-vitro propagation is because of the source of explants and the pathway of regeneration (Goto et al. 1998). The present study was conducted to screen in-vitro plant regenerationin sugarcane from direct (axillary buds, apical meristem, leaf whorl disk) and indirect (callus) organogenesis and to confirm genetic stability, in-vitro regenerated plants were evaluated through RAPD and ISSR markers. Micropropagation protocol for sugarcane was standardized in an earlier report through apical meristem (Sawant and Tawar, 2011), axillary buds (Thorat et al. 2016), direct shoot regeneration from leaf whorl disk (Kaur and Sandhu, 2015) and indirect organogenesis from embryogenic callus (Gill et al. 2004). Shoot formation was observed from all types of explants in sugarcane var. Co86032 and the regenerated shoots were multiplied on SSRM medium. Numbers of regenerated shoots were varied by type of explant and method of regeneration and depicted in Fig. 1 and Table 1.

Observations were taken after 30 days of initial culture. Different explants showed healthy regenerated shoots. Among all types of explants, a number of explants showed shootsformation were observed highest from axillary buds culture (48.33±0.33) followed by the apical meristem (46.67±0.88), leaf whorl disk (40.00±1.00), and lowest in callus (37.33±1.76). The variation of shoot proliferation in different explant is might be because of

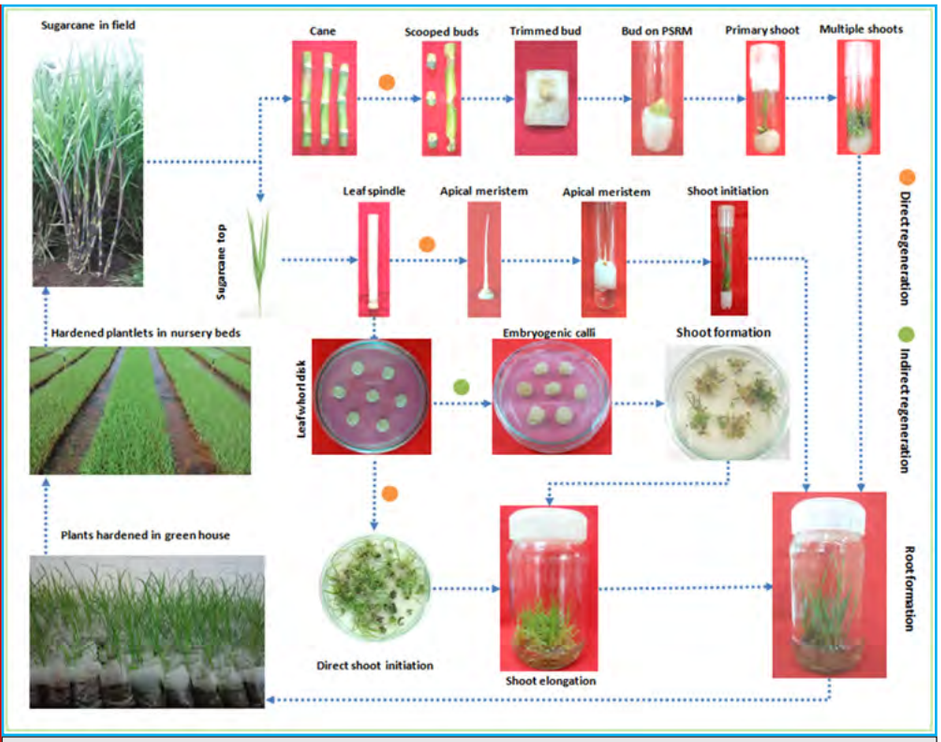

FIGURE 1. Diagrammatic/schematic representation of micropropagation protocol for sugarcane (*Saccharum officinarum* L.) using direct and indirect organogenesis.

Table 1. Sugarcane (*Saccharum officinarum* L.) shoots formation from various sections.

Sr. No.	Explant	Mode of organogenesis	No. of explant inoculated	No. of explant regenerated	Total No. of regenerated Shoots (30 days)	No. of regenerated shoots/ explants (30 days)	Total No. of regenerated shoots (60 day)	Shoot length (mm)	Explant showed roots (%)	Root length (mm)	No. of Roots/ shoots
1	AB	Direct	50	48.33±0.33a	60.0±2.08c	1.24±0.03c	240.00±8.32c	101.16±10.66	91.99±1.11	25.33±1.66	5.63±0.42
2	AM	Direct	50	46.67±0.88b	98.66±4.80b	2.11±0.06b	394.00±19.22b	100.59±12.14	93.36±2.10	29.33±1.20	5.96±0.20
3	LD	Direct	50	40.00±1.00c	173.00±8.11a	4.33±0.24a	693.33±32.44a	99.97±9.86	90.43±1.52	26.33±1.85	5.13±0.34
4	CC	Indirect	50	37.33±1.76d	51.33±6.17c	1.40±0.23c	205.33±24.69c	98.81±8.52	89.74±1.47	25.66±1.42	4.95±0.11

Note: AB- Axillary bud, AM- Apical Meristem, LD- Leaf whorl disks, and CC- Callus from leaf whorl disks.

Table 2. Molecular polymorphism analyzed by RAPD markers.

Sr. No	Primer code	Primer sequence (5' to 3')	Direct organogenesis									Indirect organogenesis			Size range (bp)
			Axillary buds			Apical meristem			Leaf whorl disk			Callus culture			
			*n*SB	*n*MB	*n*PB	*n*SB	*n*MB	*n*PB	*n*SB	*n*MB	*n*PB	*n*SB	*n*MB	*n*PB	
1	OPH-01	GGTCGGAGAA	4	4	0	4	4	0	4	4	0	4	4	0	250-3000
2	OPH-02	TCGGACGTGA	4	4	0	4	4	0	4	4	0	4	4	0	250-3000
3	OPH-04	GGAAGTCGCC	5	5	0	5	5	0	5	5	0	5	5	0	250-2000
4	OPW-06	ACGCATCGCA	4	4	0	4	4	0	4	4	0	4	4	0	250-1000
5	OPH-09	TGTAGCTGGG	1	1	0	1	1	0	1	1	0	1	1	0	250-1000
6	OPH-11	CTTCCGCAGT	5	5	0	5	5	0	5	5	0	5	5	0	250-1000
7	OPH-13	GACGCCACAC	6	6	0	6	6	0	6	6	0	6	6	0	250-2000
8	OPH-16	TCTCAGCTGG	1	1	0	1	1	0	1	1	0	1	1	0	250-1000
9	OPH-17	CACTCTCCTC	2	2	0	2	2	0	2	2	0	2	2	0	500-1000
10	OPH-18	GAATCGGCCA	6	6	0	6	6	0	6	6	0	6	6	0	250-500
Total			38	38	0	38	38	0	38	38	0	38	38	0	
Percentage (%)				100	0		100	0		100	0		100	0	

*n*SB- total number of scorable bands, *n*MB- number of monomorphic bands, *n*PB- number of polymorphic bands.

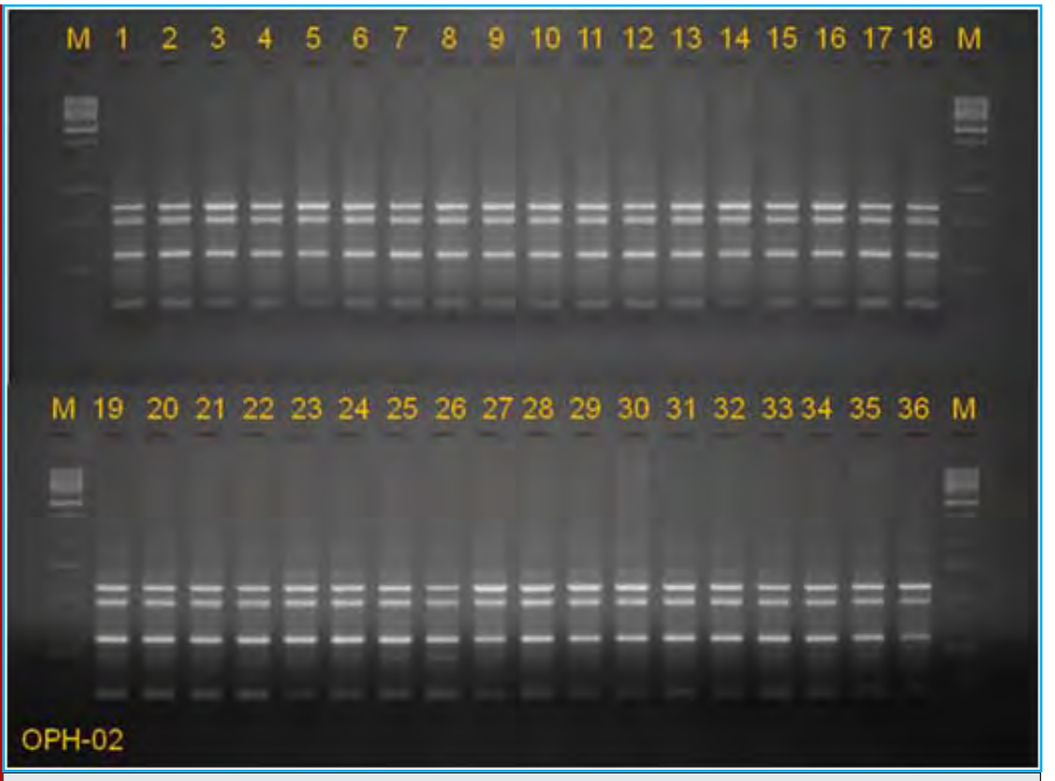

FIGURE 2. RAPD profile of regenerated plantlets from: axillary buds (1-9), callus (10-18), apical meristem (19-27) and leaf whorls (28-36), 1 kb DNA marker (M).

incompatibility of tissue with the nutrients in medium, tissue damage during pretreatment and surface sterilization and moreover inabilities of cells to further re-differentiate into growing tissue (Thorat et al. 2016). However, the number of shoots regenerated from leaf whorl disk was highest (173.00±8.11) followed by the apical meristem (98.66±4.80), axillary buds (60.00±2.08), and lowest in callus culture (51.33±6.17). The possibility of variation was because of non-response of explant like browning and development of non-regenerable callus. The highest number of regenerated shoots per explants were observed in leaf whorl disk (4.33±0.24) followed by the apical meristem (2.11±0.06), callus from leaf whorl disk (1.40±0.25), and the lowest was in axillary buds (1.24±0.03). Similar results were reported in sugarcane variety CoPb 91 and found four shoots per spindle, after 28 days of initial culture of the leaf whorl disk (Kaur and Sandhu, 2015). In apical meristem culture of sugarcane var. HSF-240, CP-77-400, and CPF-237 showed 5.83 shoots per explants after 3 weeks is almost thrice compare to current results. The highest numbers of healthy shoots in terms of shoot length were found in the range from 101.16±10.66 mm (axillary buds) to 98.81±8.52 mm (callus culture).

Similar results in terms of shoot length (105 mm) were observed by Khan et al. (2008) in the various sugarcane cultivars. After 30 days of initial culture, all the regenerated shoots were transferred to SSRM for further multiplication and shoot elongation. It was observed rapid multiplication of shorts in the second subculture and found approximately four times shoot multiplication after 60 days. The highest number of multiple shoots was observed in leaf whorl disk (693.33±32.44) followed by the apical meristem (394.00±19.22), axillary buds (240.00±8.32), and callus (205.33±24.69). Kaur and Sandhu (2015) found that 448 shoots were regenerated from leaf whorl disk after second subculture (2 months) in sugarcane cultivar CoPb 91. Over a period of 60 days of initial culture, numbers of plants regenerated from direct regeneration were more comparing with indirect regeneration.

All the regenerated shoots after 60 days of culture were transferred to the rooting medium for developing roots. Root formation was observed from almost all the shoots after 2weeks of culture on rooting medium. In the present investigation, no significant differences were found with respect to the rooting percentage, root length, and number of roots per shoots (Table 1). The

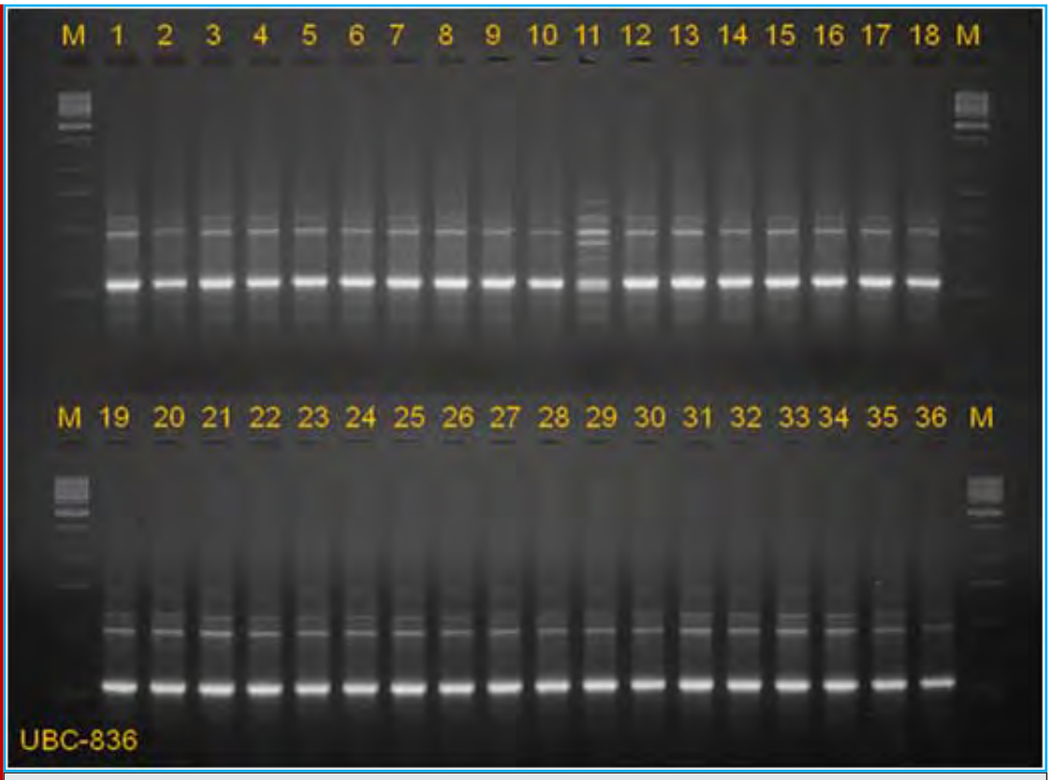

FIGURE 3. ISSR profile of regenerated plantlets from: axillary buds (1-9), callus (10-18), apical meristem (19-27) and leaf whorls (28-36), 1 kb DNA marker (M)

Table 3. Molecular polymorphism analyzed by ISSR markers.

Sr. No	Primer code	Primer sequence (5' to 3')	Direct organogenesis									Indirect organogenesis			Size range (bp)
			Axillary buds			Apical meristem			Leaf whorl disk			Callus culture			
			*n*SB	*n*MB	*n*PB	*n*SB	*n*MB	*n*PB	*n*SB	*n*MB	*n*PB	*n*SB	*n*MB	*n*PB	
1	UBC-817	(CA)8A	7	7	0	7	7	0	7	7	0	7	7	0	500-1500
2	UBC-835	(AG)8YC	4	4	0	4	4	0	4	4	0	4	4	0	250-1000
3	UBC-836	(AG)8YA	4	4	0	4	4	0	4	4	0	7	4	3	500-1000
4	UBC-844	(CT)8RC	7	7	0	7	7	0	7	7	0	7	7	0	500-2000
5	UBC-849	(GT)8YA	6	6	0	6	6	0	6	6	0	6	6	0	500-1000
6	UBC-850	(GT)8YC	5	5	0	5	5	0	5	5	0	5	5	0	500-1000
7	UBC-855	(AC)8YT	9	9	0	9	9	0	9	9	0	9	9	0	250-3000
8	UBC-857	(AC)8YG	6	6	0	6	6	0	6	6	0	6	6	0	250-1000
9	UBC-864	(ATG)6	7	7	0	7	7	0	7	7	0	7	7	0	250-2500
10	UBC-868	(GAA)6	8	8	0	8	8	0	8	8	0	8	8	0	750-3000
Total			63	63	0	63	63	0	63	63	0	66	63	3	
Percentage (%)				100	0		100	0		100	0		95.45	4.54	

*n*SB- total number of scorable bands, *n*MB- number of monomorphic bands, *n*PB- number of polymorphic bands.

highest numbers of profuse roots in terms of root length were found in the range between 29.33 and 25.33 mm and number of roots per shoot was ranging from 5.96 to 4.95 (Table 1). Kaur et al. (Kaur and Sandhu, 2015) showing 100% root induction after 14 days in sugarcane cultivar CoJ 83. Khan et al. (2009) reported that shoots on rooting medium showing 38 mm in length after 12 days of incubation. The rooting responses in all the cultures are equal and as such no definite differentiations were observed. All the regenerated plants were acclimatized in greenhouse conditions. Around 95% plants were survived.

The present study was performed to screen the plantlets regenerated from direct shoot organogenesis (axillary buds, apical meristem, and leaf whorl disk) and indirect shoot organogenesis (callus culture) of sugarcane var. Co 86032. PCR-based markers like RAPD and ISSR were employed in genetic fidelity assay. The plants analyzed, representing in-vitro rooted plantlets after 1st multiplication passages. The leaves were collected from these samples randomly. A total of 15 RAPD and 15 ISSR markers were used for preliminary screening with the mother plant of sugarcane. Based on the reproducible band, only 10 of each were selected.

Of the 15 RAPD primer used for the preliminary screening, only 10 primers formed reproducible and scorable banding pattern. These 10 primers produce 38 distinct bands ranging from 1 (OPH-09) to 6 (OPH-13 and 18) with an average of 3.8 bands per primer and produced a unique set of amplification product ranging from band size of 250 bp to 3000 bp. A total of 1368 bands (No. of plants analyzed from various explants × No. of band classes with all RAPD primers) was generated in all plantlets from different sections. Primer OPH-09 and OPH-16 amplified the lowest number of one band from each primer. However, OPH-13 and OPH-18 amplified the maximum number of 6 bands each. Overall no polymorphism was detected among the plants derived from direct and indirect organogenesis (Table 2). The RAPD profiles of the regenerates are depicted in Fig. 2. Similar results were reported in micro-propagated shoots of *Curcuma longa* (Salvi et al. 2001), sugarcane (Khan et al. 2009), *Gerbera jasmesonii* (Bhatia et al. 2011), *Momordica dioica* (Rai et al. 2012), *Terminallia bellerica* (Dangi et al. 2014), and *Dendrocalamus strictus* (Goyal et al. 2015). Zucchi et al. (Zucchi et al. 2002) reported that polymorphism occurred in sugarcane shoots regenerated from meristem cultures because of the number of subcultures and RAPD analysis was used for detection of polymorphism.

Out of 15 ISSR primers used in the preliminary screening, only 10 primers produced clear and reproducible bands. The 10 ISSR primers produced 66 distinct and scorable bands in the size range of 250–3000 bp. The number of scorable bands from for each primed varied from 4 (UBC-835, 836) to 9 (UBC-855), with an average 6.6 bands per primer (Table 3). A total of 2271 bands were generated in all plantlets from different sections. The polymorphism was not occurred in regenerated shoots from axillary buds, apical meristem, and leaf whorl disk during the analysis. Erig and Schuch (2003) and Hsie et al. (2015) observed similar results in sugarcane and showed 100% true-to-type plantlets. The present results enlighten the fact that plants developed from direct organogenesis are highly stable than those obtained from indirect oraganogenesis (callus) (Kamenickà and Rypák, 1989). However, plant raised from indirect organogenesis (through callus phase) has observed that only one primer (UBC-836) showed three polymorphic and four monomorphic distinct bands (Figure 3). In ISSR primer analysis total 95.45% monomorphic and remaining 4.55% polymorphic bands were observed (Table 3). Similar results of polymorphism also observed during micropropagation in *Swertia chirayita* (Joshi and Dhawan, 2007) and in *Gerbera jamesonii* (Bhatia et al. 2011).

The possible reasons for polymorphism observed during analysis that occurred in in-vitro raised plantlets regenerated from mother plants using indirect organogenesis might be because of source of application of growth regulators during callus induction, shoot regeneration, duration of cell, and tissue multiplication, accumulating mutations during the process of indirect organogenesis, etc. (Goel et al. 2009; Rizvi et al. 2012; Chavan et al. 2014; Kshirsagar et al. 2015; Thorat et al. 2017). Similarly, ISSR primers were competent to detect polymorphism in *Camellia chinensis* (Devarumath et al. 2002), *Stevia* (Martin et al. 2004), *Gerbera jamesonii* (Bhatia et al. 2011), *Tylophora indica* (Sharma et al. 2014), *Dendrocalamus strictus* (Goyal et al. 2016), *Morus alba* (Saha et al. 2016) and *Saccharum officinarum* (Thorat et al. 2017).

The regenerated plantlets did not show any measurable dissimilarity in morphology when compared to the respected mother plant. However, sometimes a variation occurs during micropropagation and cannot be observed because the structural difference in the gene product does not always change its biological activity to extend for phenotypic expression. The presence or absence of genetic variation during in-vitro regeneration depends upon the source of the explants and the method of the regeneration (Goto et al. 1998) and finest levels of plant growth substances have also related with somaclonal variation (Martin et al. 2004). RAPD and ISSR are the simple, cost-effective PCR-based techniques used in this study to check the genetic similarity in in-vitro regenerated plants. This marker amplifies the different loci of the genome which allows better chances for identi-

fication of variation at the genetic level (Martin et al. 2004). Thus, confirmation of the similarity at the genetic level is needful by DNA analysis techniques. Therefore, in this study two DNA-based molecular markers (RAPD and ISSR) were employed for genetic fidelity analysis of both direct and indirect organogenesis derived plantlets of Co86032.

CONCLUSION

In the present investigation,the molecular study revealed that the micropropagated plants from direct organogenesis were genetically similar with respect to the mother plant and no differentiation was induced during micropropagation and plant regenerated from indirect organogenesis, that is, callus culture found optimum levels leads to somaclonal or epigenetic variation. A plant regenerated from adventitious buds showed the lower tendency for genetic differentiation (Joshi and Dhawan, 2007).Therefore, the routine checkup is necessary to identify the genetic variation of the micropropagated plants and helping to produce true-to-type progeny. Although, RAPD and ISSR has been proved as the most suitable techniques to detect genetic variation provoked during micropropagation (Devarumath et al. 2002; Shu et al, 2003).

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Efficacy of closed loop feedback system with augmented virtual reality visual cues training on gait and functional performance in stroke patients

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ABSTRACT

The traditional gait training techniques, which are not based on principles of motor control lack requisites in terms of training intensity, duration and enough practice to have any meaningful carryover effect. Therefore, the aim of this study is to investigate the efficacy closed loop visual cues incorporated augmented reality environment on functional gait and community ambulation in stroke patients. A randomized control trial with control group was designed and 14 subjected were recruited in each group. Four weeks of augmented reality based closed loop visual cue training was given to experimental group. Compared to control group, the Walking speed, cadence, stride length of paretic and non-paretic limb, symmetrical walking and SIS-SF score improved 25.1%, -5.5%, 29.3%, 8.3%, 5.3% and 29.1% respectively. The finding of our study supports the beneficial effect of augmented reality based closed loop visual cue training for improving the gait and functional ambulation in stroke patients.

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INTRODUCTION

Stroke is one of the most devastating condition leading to life long morbidly and mortality. The stroke survivors suffer from various sensorimotor impairment of gait and posture leading to moderate to severe disability (Duncan et al 2002). Patients with stroke presents with multiple postural deficits including loss of anticipatory postural reaction, postural sway, uneven weight bearing and inability to maintain upright posture. These deficits increase the risk of falling and ultimately affect the ambulation and activities of daily living (Dickstein et al 2000). Depending upon the severity of stroke most patient recover gait function, though a very small percentage of stroke survivors become community ambulatory (Mackintosh et al 2005). Recent studies proposed that the post stroke impairment of mobility functions, such as, reduced walking speed, asymmetrical weight bearing, unequal step and stride length etc. may be due the inability of the patients to regulate the anticipatory postural reaction (Hill et al 1997). Over and above, the impaired coordination directly affects all the aspects of ambulation including; turning, obstacle avoidance, relative foot placement and velocity regulation, required for independent community ambulation (Roerdink et al 2007). Synthesis of recent literature supports an intensive task specific gait training targeting symmetrical gait pattern may improve ambulatory function (Hollands et al 2012)

The existing evidence suggest that the visual inputs are most import external sensory cues regulating walking and due the impaired sensory inputs the stroke survivors excessively rely on vision to maintain dynamic stability (Kim et al 2012). Gait training incorporating external sensory feedback, which has been found to be very effective in Parkinson's disease (Fuzail et al 2007) have recently begun to be used to investigate functional walking tasks in stroke populations (Bonam et al 2004). In a natural closed loop feedback control system, the physical motion of the body generates the visual cue in response to ambulation and in the absence of movement these cues are not generated (Hollands et al 2010).

The traditional gait training techniques, which are not based on principles of motor control lack requisites in terms of training intensity, duration and enough practice to have any meaningful carryover effect (Rizzo et al 1997). Novel interactive Virtual reality (VR) technologies, creating an immersive environment for stroke patients simulating real-world experience, can help address the limitations posed by traditional approaches (Cikajlo et al 2009). Augmented reality (AR) can improve the implicit knowledge of movement by immersing the desired training regimen in a real-world environment (Azuma et al 2001). A recent systemic review had showed strong scientific evidence supporting the beneficial effects of virtual reality on upper limb motor recovery in stroke patients (Viñas Diz 2016). A recent home based virtual reality training had shown beneficial functional training effects; suggesting that it may be useful as a neurorehabilitation tool (Villiger et al 2017).

To the best of our knowledge, till date, there is no randomized controlled trial demonstrating the efficacy of closed loop visual cues incorporated augmented reality environment on functional gait and community ambulation in stroke patients. Therefore, the aim of this study is to investigate the efficacy augmented reality based closed loop visual cue training on gait and functional ambulation in sub-acute stroke patients.

MATERIAL AND METHODS

Design: A randomized control trial comparing the effect of proposed intervention with control group was designed. Based on the 80% power and an alpha value of .05 the sample size calculated to be 14 participants in each group. The subjects were randomly selected based on lottery method and allocated to either closed loop visual cue augmented reality group or to control group receiving traditional gait training. The subjects were selected based on the following inclusion criteria; (a) patients having single episode of stroke (b) onset of stroke must have occurred before months, (c) presence gait deficit due to hemiplegia, (d) patient must be able to walk independently, and (e) must be medically stable without any visual or cognitive defect. Patient having other comorbidity affecting gait function were excluded from the study.

All the subjects recruited in the study underwent a structured one-hour physical therapy session 6 day a week for four consecutive weeks. In addition to this the subjects in the experimental group subjects participated in an additional closed loop visual cues training through a head mounted device (HMD) with partial bodyweight supported treadmill training (Walker et al 2010). The HMD generated visual cues matched to walking symmetry bringing the sensory feedback signals closer to the eyes, making the sensory effect more pronounced, easier to follow and to learn. The closed loop visual cue training was provided every alternate day for 4 weeks.

Outcome measurements

The following outcome measurements were taken by a therapist at baseline and again after 4 weeks of training. Gait function were measured using GAITRite (GAITRite; CIR system Inc., Havertown, PA, USA). The standard GAITRite walkway contained six sensor pads encapsulated in a rolled-up carpet with an active area of 3.66

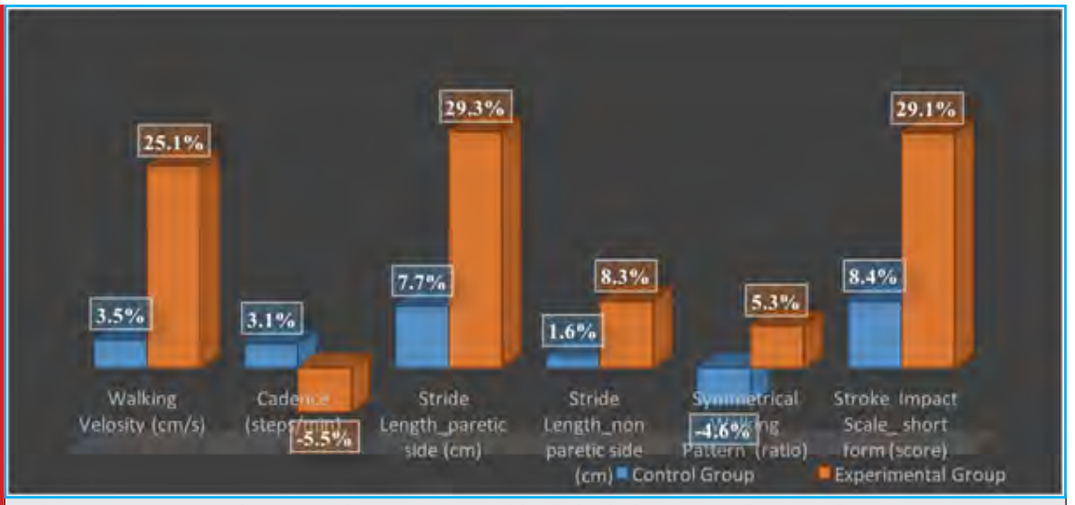

FIGURE 1. Percentage changes in the outcome variables in both the groups after 4 weeks.

m (length) 0.61 m (width). GAITRite will be used for measurement of the spatiotemporal parameters, including gait velocity, cadence, step length, and stride length (Mc Donough et al 2001). (Uden et al 2004) investigated the reliability of measurements performed using the GAITRite system as a video-based analysis system and found excellent reliability (Intraclass Correlation Coefficient (ICC) Z0.94).

A short for stroke impact scale (SF-SIS) was used to measure the perceived community participation content, convergent, and discriminant validity (Rachael et al 2016).

RESULTS

Total 28 patients were enrolled in the study with a mean age of 44.7+12.3 years and the mean stroke onset was found to be 20.5+9.2 months. Fourteen subjects were randomly allocated in control and experimental group. Two patients dropped out of the control group and 3 subjects dropped out from the experimental group due to various reason.

An independent t-test was performed to check the uniformity of data which showed that there is no difference in all the selected variable. The results are provided in below mentioned table 1.

It was found that, in the group getting general physical therapy program, there was an increase of 4% in walking velocity, 3% increase in cadence, 8% improvement in walking symmetry, 2% increase in stride length of the paretic limb, 5% decrease in stride length of the non paretic limb and 8% improvement in the SIS_SF scores. A paired t-test was conducted, with an alpha level of .05 between the baseline and post test scores,

Table 1. Baseline Characteristic

Variables	CG		EG		T	p
	Mean	SD	Mean	SD		
Age (years)	46.5	13.4	42.7	11.3	.727	0.475
Stroke Onset (months)	20.5	11.5	20.6	6.5	-.012	0.991
Walking Velocity (cm/s)	35.3	9.8	38.5	11.6	-0.717	0.481
Cadence (steps/min)	71	7.0	69.8	8.8	0.357	0.725
Stride length-paretic side (cm)	0.63	0.10	0.6	0.1	0.222	0.827
Stride length-non paretic side (cm)	58.5	3.2	58.8	3.0	-0.247	0.808
Symmetrical Walking Pattern (ratio)	59.8	2.4	60.5	3.2	-0.529	0.603
Stroke Impact Scale_ short form (score)	20	4.3	22	3.4	-1.127	0.272

CG: Control Group, EG: Experimental Group, SD: Standard deviation

Table 2. Result of pairwise comparison of baseline and post test scores of outcome variables in the control group.

	CG (M±SD)	EG (M±SD)	T	p
Walking Velocity (cm/s)	35.3±9.8	36.5±13.5	.438	.670
Cadence (steps/min)	71±7	73.2±8.5	1.116	.288
Stride length-paretic side (cm)	58.4±3.7	59.3±5.9	.557	.558
Stride length-non paretic side (cm)	59.8±2.4	57.1±5.5	1.817	.096
Symmetrical Walking Pattern (ratio)	0.63±0.1	0.67±0.13	1.339	.208
Stroke Impact Scale_ short form (score)	20±4.3	22±5.8	1.301	.220

CG: Control Group, EG: Experimental Group, M±SD: Mean ± Standard deviation

Table 3. Result of pairwise comparison of baseline and post test scores of outcome variables in the experimental group.

	CG (M±SD)	EG (M±SD)	T	p
Walking Velocity (cm/s)	38.5±11.6	48.1±11.1	4.545	0.001
Cadence (steps/min)	69.8±8.8	66±6.9	2.329	0.042
Stride length-paretic side (cm)	58.8±2.9	63.7±4.1	3.331	0.008
Stride length-non paretic side (cm)	60.5±3.2	63.6±5.5	1.665	0.127
Symmetrical Walking Pattern (ratio)	0.62±0.14	0.8±0.07	4.225	0.002
Stroke Impact Scale_ short form (score)	22±3.4	28±4.6	3.545	0.005

CG: Control Group, EG: Experimental Group, M±SD: Mean ± Standard deviation

which showed that none of the improvement is statistically significant (table 2)

After comparing the baseline and post test scores in the group getting closed loop visual cues training, it was found that there was an increase 25% in the waking speed, which was found to be statistically significant (t=4.545, p=.001). The cadence showed a decreased of 5% which was statistically significant (t=2.329, p=.042). The stride length of the paretic limb increased by 8% which was found to be statistically significant (t=3.331, p=.008). Even though, there was an increase of 5% in stride length of the non paretic limb, it was found to be statistically not significant (t=1.665, p=.127).

Compared to control group, the Walking speed, cadence, stride length of paretic and non paretic limb, symmetrical walking and SIS-SF score improved 25.1%, -5.5%, 29.3%, 8.3%, 5.3% and 29.1% respectively. An independent t-test was utilized to find the effectiveness of both the treatment regimes. The result presented in table 4 suggests that, all the outcome variables showed a statistically significant improvement in the experimental group getting closed loop visual cues.

Table 4. Result of comparison between the control and experimental group on all outcome variables.

Variables	CG		EG		T	p
	Mean	SD	Mean	SD		
Walking Velocity (cm/s)	36.5	13.5	48.1	11.1	-2.236	0.036
Cadence (steps/min)	73.2	8.4	66.0	6.9	2.213	0.038
Stride length-paretic side (cm)	0.68	0.13	0.8	0.1	-2.822	0.01
Stride length-non paretic side (cm)	58.3	4.6	63.7	4.1	-3.012	0.007
Symmetrical Walking Pattern (ratio)	57.1	5.5	63.6	5.5	-2.858	0.009
Stroke Impact Scale_ short form (score)	22	5.8	28	4.6	-2.969	0.007

CG: Control Group, EG: Experimental Group, SD: Standard deviation

DISCUSSION

The aim of this study was to find the effectiveness a novel method utilizing the augmented reality based closed loop visual cue training to improve the gait function for better community participation in stroke patients. The result of this study has showed an improvement of all gait related variable in the experimental group except stride length of the non paretic limb, which may be due to the relative improvement of the paretic limb's stride length. The result of our study is similar to the study done by (Lee et al 2014). where they have used a virtual reality based postural control for improving the gait function in the stroke patients. Their study showed a significant difference in all gait variables except cadence but in our study we found that the cadence was significantly decreased by 5%. This decrease may be in response to the significant increase in the paretic limb's stride length, which would have caused longer but less steps/minute. We also found an insignificant increase in the non paretic limb's stride length, which is in contrast with the study by (Lee et al 2014). This may be due to the improved motor control of the paretic side, causing leveling of stride length on both the side.

A recent study by XI et al (2017) suggests cortical plasticity by increased activation of cortical regions in stroke survivors as a probable mechanism for better recovery of mobility function associated with virtual reality based training. Another important finding of our study was 29% significant improvement in symmetrical walking pattern in the experimental group subjects. This finding is similar to the result of the study conducted by (Sami et al 2015) here the symmetrical walking pattern significantly improved due to increased muscle strength and coordination.

As stroke patients are unable to cope with the challenges of varying environmental demands required for community walking due to their impairments. As stroke patients live a relatively sedentary lifestyle any interventions which consistently augments activity level and induces demand on lower limb's motor control may improve the walking pattern (Hendricksan et al 2014). Therefore, the improvement seen in our study may be attributed to the determined efforts of the patients in response to the closed loop visual cues for effectively controlling their lower limbs.

The most noteworthy finding of our study was a substantial improvement in perceived community participation among the subjects closed loop visual cue training group. We saw a 29% increase in perceived community participation which is in line with the findings of Sami et al 2015. (Warren et al 2016) in their study established a direct relationship between decreased community participation and slower walking speed, which may explain the magnified perception of community participation due to increased walking speed.

CONCLUSION

The finding of our study supports the beneficial effect of augmented reality based closed loop visual cue training for improving the gait and functional ambulation in stroke patients.

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Effect of monosodium glutamate on striato-hippocampal acetylcholinesterase level in the brain of male Wistar albino rats and its implications on learning and memory during aging

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ABSTRACT

Glutamate is one of the most abundant excitatory neurotransmitters of brain, plays an important role in learning and memory. Monosodium glutamate (MSG) is a sodium salt of L-glutamate, a well-known flavor enhancer. Because of its capacity to increase the palatability, nowadays it is most widely used in all households and fast foods. The present study investigates the effect of MSG on striato-hippocampal acetylcholinesterase (AChE) level in the brain of male *wistar* albino rats and its implications on learning and memory during aging. The experimental groups consisted of 3 – 4 weeks old *Wistar* albino rats and were divided into group I and group II. These groups were again subdivided into control group treated with laboratory diet, 100 mg MSG, 400 mg MSG, 2 g MSG and 4 g MSG group treated with various doses of MSG/ kg body weight for sixty days. Immediately after 60 days of MSG treatment, the group I was used for the analysis of body weight, T-maze test, novel object recognition test (NOR) and AChE level while group II was kept free of MSG for further sixty days and later used for the above experiments. The results showed that group I animals neither have a dose dependent increase in body weight, a decline in the T-maze response, reduced discrimination index in NOR test and an increase in AChE level. However, the changes were significant at 4 g MSG treated group. The group II animals did not showed any significant differences when compared to control group. Thus the study revealed that a long term administration of MSG has a capacity to cause cognitive impairment but is not perpetual, during the life time, due to normal diet and natural healing mechanisms in the body ameliorates the toxic effects of MSG and showed gradual increase in the cognitive parameters, however it take a long time.

KEY WORDS: MSG, T-MAZE, NOVEL OBJECT RECOGNITION TEST, ACETYLCHOLINE ESTERASE

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INTRODUCTION

Monosodium glutamate is a sodium salt of L-glutamate, a well known flavor enhancer and a source for the unique flavor called "umami". Free glutamic acid produces Umami or pleasant savoury taste, one of the five primary tastes. The presence of MSG in food increases the palatability or alters and magnifies the desirable taste, thus it is an essential part of human diet and commonly found in most of the Asian Western diets. Body can never discriminate the glutamate coming from the monosodium glutamate and natural foods. The glutamate released from food or from the MSG is absorbed into the enterocytes from the lumen. Glutamate is an important oxidative substance for intestinal mucosa and additionally is a precursor for arginine, proline and glutathione. Glutamate is one of the most abundant excitatory neurotransmitter in brain acting through NMDA (N-methyl-D-aspartate) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors and play a pivot role in learning and memory (Riedel et al., 2003; Jinap & Hajeb, 2010; Moneim et al., 2018).

The Food and Drug Administration (FDA) reported that an unknown percentage of the population might react to MSG and develop symptoms that constitutes a syndrome including burning sensation at the back of the neck, forearms, chest, headache, chest pain, facial pressure/tightness, nausea, palpitation, numbness at the back of the neck, radiating to the arms and back, tingling, warmth, weakness in face, temples, upper back, neck and arms, drowsiness and weakness collectively called MSG symptom complex (FDA, 1995). Animal studies also show that increased administration of MSG increased blood glutamate level (Bogdanov et al., 1996). Neonatal administration leads to immature blood brain barrier (Boonnate et al., 2015), degenerative changes in hypothalamic arcuate nucleus (Holzwarth-Mcbride et al., 1976), reduced serotonin and cognitive functioning (Moneim et al., 2018), pyknotic Purkinje and granule cells with inflamed cells in the cerebellum (Hashem et al., 2012). Another study showed that 4 g MSG treated group had an increase in the body weight, but mild doses did not show any increase in the body weight (Sari et al., 2018).

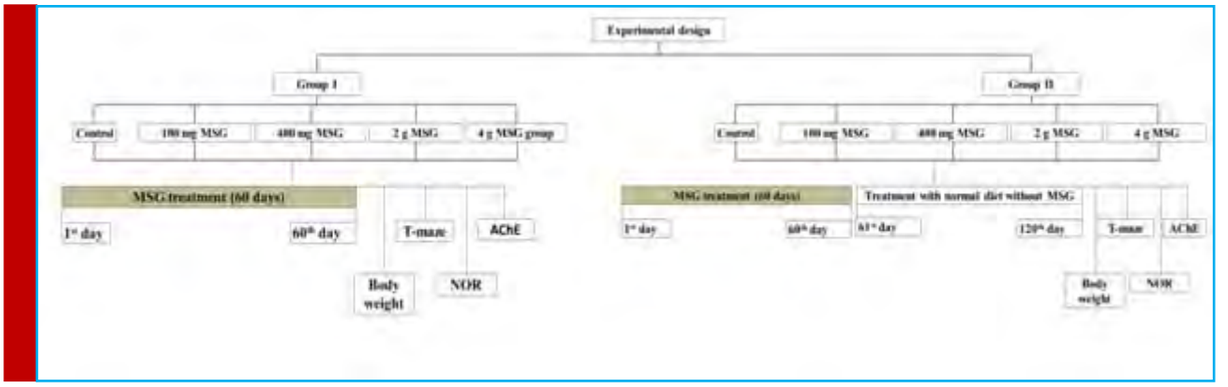
The present study investigates the effect of monosodium glutamate on striato-hippocampal acetylcholinesterase level in the brain of male *wistar albino* rats and its implications on learning and memory during aging.

MATERIALS AND METHODS

Male Wistar albino rats, about 30 -50 g weight and approximately 3 – 4 week rats were used for the study. The rats were maintained under laboratory conditions in the animal house of the Department of Life Sciences (Reg#426/2/CPCSEA). All experiments were carried out according to the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India and approved by the Institutional Animal Ethics Committee (IAEC) of the University of Calicut, Kerala.

The rats were divided into two groups namely group I and group II, which is again subdivided in to Control: treated with normal laboratory diet, 100 mg MSG group: treated with 100 mg MSG/ kg body weight, 400 mg MSG group: treated with 400 mg MSG/ kg body weight, 2 g MSG group: treated with 2 g MSG/ kg body weight and 4 g MSG group: treated with 4 g MSG/ kg body weight. MSG was dissolved in water and given by oral gavage for 60 days. The weights of the rats were observed during the treatment period. Immediately after sixty day of MSG administration group I rats were used for T-maze test, novel object recognition test (NOR) test and the estimation acetylcholinesterase (AChE). Group II rats were allowed to have normal diet without MSG for further 60 more days and later was used for the above experiments. The experimental design is summarised below:

T-maze test: The T-maze test was used for assessing spatial working memory in rodents, especially for



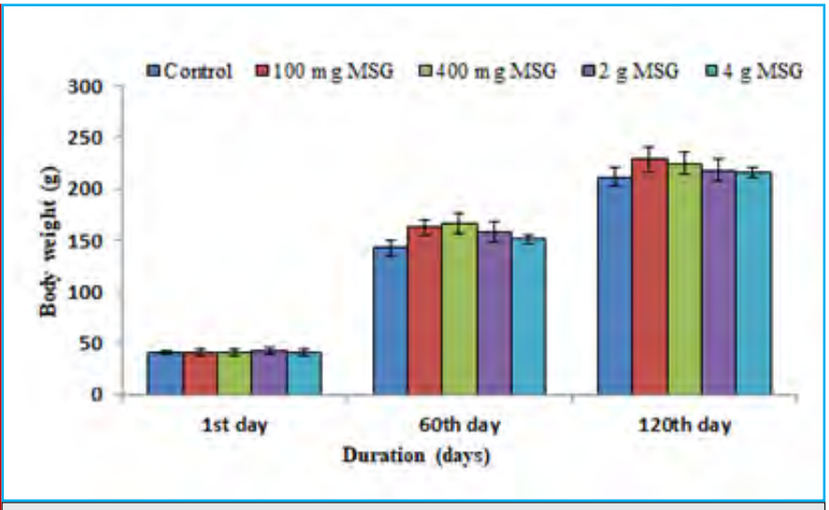


FIGURE 1. Shows the comparison of body weight of the control rats and MSG treated groups. Values are Mean + SD, n = 6 animals per group.

delayed alternation tasks. T-maze apparatus consists of a T shaped hollow wooden box with 60 X 10 cm long tail, 50 X 10 cm short arms and 40 cm high walls. The experimental rat was exposed to the T-maze 2 days before the test for habituation. On the experiment day, after acclimatization, rats were place at the starting end of the long tail of the T-maze and allowed to explore the apparatus. In one of the short arm of the T-maze, animal feed was placed in such a way that the rat is not able to see it, as soon as it reaches the end of the long arm. When the animal reaches the food, that move was taken as a positive response. When it moves to the other end, that move was taken as a negative response. Ten trials were given for a set with a gap of 5 minutes per set. The trials were continued till a set gets at least 90 % positive response. Then the total number of positive responses was added in all the sets and divided by the total number of trials given in all the sets to get the percentage of positive response (Deacon, 2013).

Novel object recognition test (NOR): NOR test is a non-force driving and spontaneous test for recognition memory, introduced by Ennanceur and Delacour in 1988. The apparatus consists of a wooden box of size 60 X 50 X 45 cm with different shaped objects. NOR test has 3 phases including a habituation phase, a training phase and a test phase in each rat. During the habituation phase, the rat was placed in the NOR apparatus and allowed to adapt for 30 min without any objects. The next day, during the training phase, two identical objects were presented to the rat and allowed to explore the objects for 10 minutes. Twenty-four hours after the training phase, during the test phase, one of the old objects was replaced with a novel object and presented to each rat and allowed to explore the objects for 6 minutes. The object exploration time was recorded and find out discrimination between two objects (familiar and novel) by using a discrimination index (DI). DI = novel object exploration time/ total exploration time of both objects) – (familial object exploration time/ total exploration time of both objects) × 100 (Ennaceur & Delacour, 1988; Win-Shwe & Fujimaki, 2012).

Estimation of Acetylcholinesterase (AChE): AChE activity was determined by the method of Ellman *et al.,* 1961. The enzyme acetylcholinesterase catalyses the hydrolysis of acetylcholine into thiocholine and acetic acid. Thiocholine reacts with DTNB (5, 5'-dithiobis (2-nitrobenzoic acid) to form yellow coloured anions of 5-thio-2- nitrobenzoic acids, which is measured spectrophotometrically at 412 nm. The enzyme activity was expressed as nano moles of conjugate formed/ Min/ mg of protein with the molar extinction coefficient 2.52 X 10^4 M^{-1} cm^{-1} (Srikumar et al., 2004).

Statistical analysis: Statistical analysis of the data was carried out by applying the analysis of variance (ANOVA), followed by Tukey's test with the help of SPSS software, version 21. The values were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Monosodium glutamate, the most widely and extensively used food additive and the source of fifth taste Umami. Because of its palatability and taste enhancing capacity, kids are more addictive to foods such as chips, soups, meats, canned foods, which are marinated by MSG. In the present study, Figure 1 shows the body weight of the group I and group II rats treated with different doses

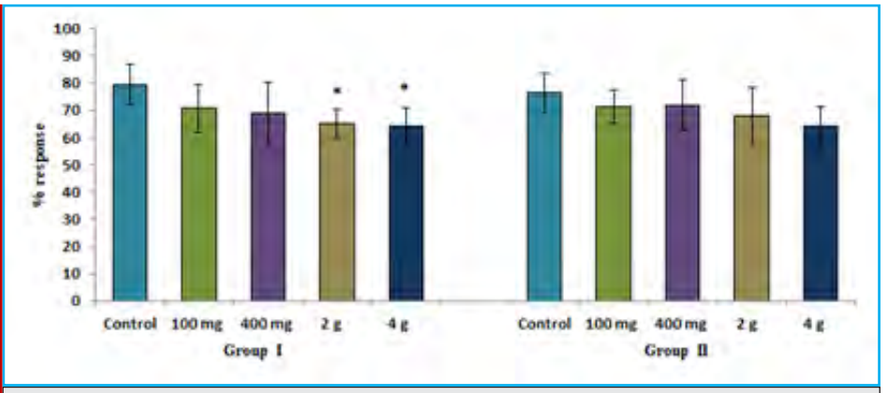


FIGURE 2. Shows the comparison of the percentage of response in T-maze of the control rats and MSG treated groups on 60th and 120th days after the sixty days of MSG treatment. Values are Mean + SD, n = 6 animals per group. *p ≤ 0.05 when compared with the control group.

of MSG/ kg body weight. It showed that both group I and group II have a non-significant increase in the body weight.

Figure 2 shows the percentage response in the T-maze of the group I and group II rats treated with different doses of MSG/ kg body weight. Immediately after the MSG treatment, the group I rats displayed a dose dependent decline in the percentage response in T-maze while 2 g and 4 g MSG treated groups showed a significant decline when compared to the control group. The group II rats, which had no MSG exposure during the last 60 days also showed a decline in the T-maze response while it has no significant difference when compared to the control group.

Figure 3 shows the DI during thirty minute exploration of NOR apparatus of group I and group II rats treated with different doses of MSG/ kg body weight. In group I rats, the subgroups treated with various doses of MSG showed a dose dependent decline in the DI of NOR test while the high dose, 4 g MSG treated subgroup showed a significant ($p \leq 0.05$) decline when compared to the control group. The group II rats did not show any significant difference when compared with the control group even though there is a visible decline.

Figure 4: shows the enzyme AChE level in the striatum and hippocampal region of the brain in Group I and group II rats treated with different doses of MSG/ kg body weight. The group I rats showed a non-significant elevation in the enzyme AChE level in the striatum and hippocampal region of the brain. However, the subgroup treated with 4 g MSG/ kg body weight showed a significant increase ($p \leq 0.05$) in the enzyme AChE in hippocampal region of the brain when compared to the

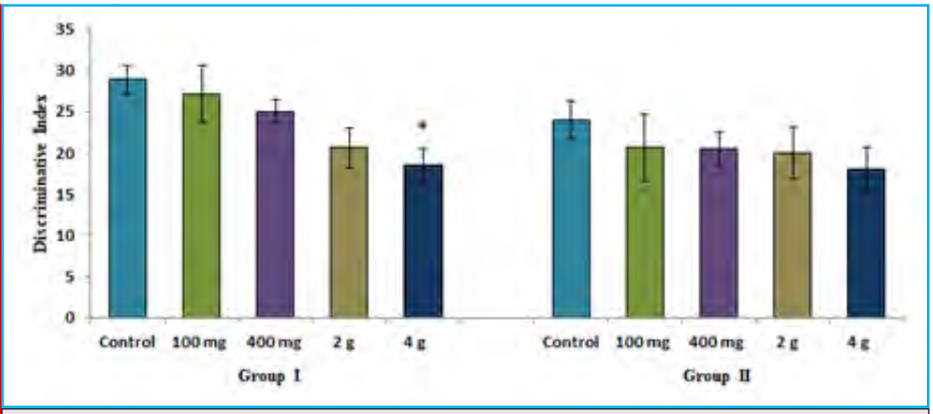


FIGURE 3. Shows the DI during thirty minute exploration of NOR apparatus of group I and group II rats treated with different doses of MSG/ kg body weight. Values are Mean + SD, n = 6 animals per group. *p ≤ 0.05 when compared with the control group.

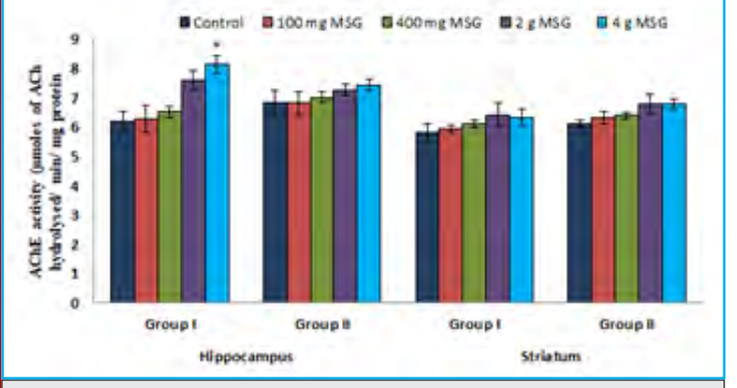


FIGURE 4. Shows the enzyme AChE level in the striatum and hippocampal region of the brain in Group I and group II rats treated with different doses of MSG/ kg body weight. Values are Mean + SD, n = 6 animals per group. *$p \leq 0.05$ when compared with the control group.

control group. The group II rats did not show any significant difference in the AChE level in straitum and hippocampal region of the brain when compared to the control group.

These results are also validated by earlier studies, which shows that MSG has the capacity to induce obesity associated with an increase in Lee index, fat accumulation, dyslipidemia and insulin resistance (Lobato et al., 2011; Alarcon-Aguilar et al., 2007). MSG increases insulin resistance and thereby increases blood insulin level, blood glucose plasma levels and also an increase in the plasma total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and free fatty acid levels. It also reduces the cortisol induced lipolysis (Zhang et al., 2010).

Monosodium glutamate induced obese rats displayed adipose tissue hypertrophy, elevated levels of insulin, leptin and slightly elevated serum glucose. The condition which causes increased blood sugar level and insulin level is called as insulin resistance, may be it is because of the activation of phosphotyrosine protein dephosphorylation and reduced level of protein tyrosine phosphorylation, which reduces the development of Gαi2 G-protein, i.e., essential for the activation of insulin signaling to GLUT4 and transportation of glucose (Baculikova et al., 2008). A clinical study in humans from Thailand revealed that MSG consumption is associated with the risk of having metabolic syndrome and being overweight (Pepino et al., 2010). Another study evidenced that an intravenous administration of MSG produces obesity, increased blood glucose, insulin, total cholesterol, and triglyceride levels (Nagata et al., 2006), but it did not show any polyphagia which may be due to the excessive stimulation of the vagus nerve (Balbo et a., 2000). Neonatal administration of MSG produces hypothalamus induced obesity (Braga et al., 2001). Another study showed that 4 g MSG treated group has an increase in the body weight, but mild doses did not show any increase in the body weight (Sari et al., 2018).

The percentage response in the T-maze and DI of NOR obtained from the group I explains the poor cognitive functioning. The increased level of AChE may break the neurotransmitter acetylcholine, which act through NMDA and AMPA receptors to assist in cognition. But after administration of MSG, in group II the toxic effects are gradually reducing and it is slightly fast in low dose treated subgroups and very slow in high dose treated subgroups. A study showed that the neonatal treatment of MSG induces obesity, elevated insulin secretion and acetylcholinesterase level. MSG also increases the activation of parasympathetic tone, which cause hyper secretion of insulin from the pancreas (Balbo et al., 2000). Measurement of neurotransmitters and neurotransmitter related enzymes in hypothalamic nuclei from MSG-treated rats revealed normal levels of norepinephrine, serotonin and glutamic acid decarboxylase, but reduced levels of choline acetyltransferase, dopamine and an elevated level of acetylcholinesterase in hypothamic nuclei. It is confirmed that MSG-induced endocrine deficiency syndrome results in the destruction of cholinergic and dopaminergic tuberoinfundibular systems in the hypothalamus (Nemeroff et al., 1978). Another study showed that subchronic MSG dose was anxiogenic and showed a slightly retardant effect on spatial working memory (Onaolapo et al., 2012). Postnatal treatment of MSG in mice showed that there is a decrease in choline acetyl-

transferase and a huge increase in AChE activity (Ross et al., 1975). Hassan et al., (2014) showed that MSG consumption may have immunotoxic effects on the thymus and spleen of adult rats, which is reversible though the normal structure of the spleen would need time to be regained (Hassan et al., 2014).

CONCLUSION

Monosodium glutamate is a salt of L-glutamate, which is an abundant neurotransmitter and essential for cognitive functioning but high doses is shown to have neurotoxic. Low dose of MSG treatment doesn't cause any much decline in cognition but it increases body weight. However, high dose of MSG administration increases the body weight and significantly reduce the cognitive functioning due to the decline in acetylcholine and elevation of acetylcholine esterase. But during the life time, due to normal diet and natural healing mechanisms in the body ameliorates the toxic effects of MSG and showed gradual increase in the cognitive parameters. More detailed studies are also needed to reveal the exact mechanism behind the fluctuation of cognition.

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CONFLICTS OF INTEREST

Nil

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Effect of aluminium chloride on bond strength of adhesive resins

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ABSTRACT

Bond strength of the composite restorations is one of the important factors to achieve success in restoring teeth. This in vitro study evaluated the effect of a hemostatic agent on shear bond strength of a two-step self-etch and a Universal bonding agents. Forty extracted human premolars without any caries, cracks and restorations were selected. After disinfection process, the mesial or distal dentin surfaces were exposed and polished for the same time to gain similar smear layers. They randomly assigned to four groups as follows: (n=10) A: ViscoStat Clear + All-Bond SE / B: ViscoStat Clear + All-Bond Universal / C: All-Bond SE / D: All-Bond Universal. ViscoStat Clear was applied according to manufacturer's orders. Then it was rinsed with constant pressure and the rinsing time was as long as the application time. After 1000 rounds of thermocycling, SBS test was performed. Data were analyzed with 1-way ANOVA.Not significant statistical differences were obtained between control and test groups. Based on the results of this study, there is no statistical differences between control groups and ViscoStat Clear-contaminated groups when using a self-etch or universal bonding agent.

KEY WORDS: SELF-ETCHING ADHESIVE, SHEAR BOND STRENGTH, UNIVERSAL BOND, VISCOSTAT CLEAR

INTRODUCTION

Bond strength of composite restorations is one of the important factors to achieve success in restoring the teeth. Low bond strength can lead to debonding the restorations. In bonding application process, isolation is an important factor. If the surface is contaminated with blood or saliva, bond strength will be reduced. In some cases, blood and saliva control is very difficult especially when the margins of the restorations are under the gum and the operator isn't able to put a rubber dam, (Tuncer et al., 2014). Therefore in these cases, the use of hemostatic agents with the retraction cords is recommended. Sometimes the restoration's margin is not under the

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gum but because of a chronic gingivitis, blood control is difficult. (Tuncer et al., 2014) Another way to control bleeding is the use of lasers and electrosurgery, (Tuncer et al., 2014) but they are not routinely used. The result of application a hemostatic agent might be a reduction in bond strength. To solve this problem, in some studies the rinsing time was increased and some kinds of cleansing methods were selected (Ajami et al., 2013, Chaiyabutr and Kois, 2011), primer application time was increased (Kuphasuk et al., 2007) 6, and also the hemostatic agents and the bonding agents that are more compatible with each other were chosen, (Mohammadi et al., 2012, Fathpour and Khoroushi, 2013.

Some studies showed a SBS reduction when hemostatic agents were used. (Mohammadi et al., 2012, Tuncer et al., 2014, Bernades Kde et al., 2014, Sharafeddin and Farhadpour, 2015) And some of them didn't show any significant differences between test groups and control ones.(Kuphasuk et al., 2007, Fathpour and Khoroushi, 2013, Kimmes et al., 2006) Universal bonding agents or multi-mode one bottle adhesive resins can be used in all of the etch-&t-rinse, self-etch and selective etch techniques. The technician should select one of the methods according to the cavity and patient's condition.(Hanabusa et al., 2012) In the past studies, there were controversial results about using self-etch adhesive resins with different kinds of hemostatic agents. (Kuphasuk et al., 2007, Arslan et al., 2013).

Nowadays the use of self-etch adhesive resins has increased because there is less technical sensitivity and also procedure time has decreased in this technique, (Arslan et al., 2013, Kuphasuk et al., 2007). Also because of the same penetration depth of resins as the depth of etching, the post-operative sensitivity of patients has decreased, (Mena-Serrano et al., 2013). In this study, the effect of aluminum chloride, as a hemostatic agent, on shear bond strength of the self-etch and the universal bonding agents was evaluated.

MATERIAL AND METHODS

This study is an experimental, in vitro and a double-blind one. Forty extracted human premolars without any caries, cracks, and restorations that had approximately the same size were selected. The sample size of each group was obtained via the data from the similar study with sample size formula. (Sharafeddin and Farhadpour, 2015) Samples were kept in distilled water at room temperature. All teeth were cleaned with a rubber cap and the pumice powder. They were stored in 0.2% Thymol solution for a week then kept in distilled water until the test time. The samples were mounted in self-curing acrylic-resin molds. They were numbered from one to 40 and randomly assigned to four groups. These numbers were kept by someone who did not play a role in the bond strength test. The mesial or distal surface of each tooth was cut by a long, flame bur (Meisinger, Neuss, Germany) with the red round bar until the dentin was exposed. Then the exposed dentin was polished with a 600 grit paper disk for 15 seconds to gain approximately similar smear layer.

In group A, dentine was contaminated with aluminum chloride (ViscoStat Clear, Aluminum chloride 25%, Ultradent, South Jordan, Utah, USA) according to the manufacturer's instruction.(application time: 5 minutes) It was rinsed with constant pressure and the rinsing time was as long as the application time. Then All-Bond SE (6^{th} Generation, self-etching bonding agent, Bisco, Schaumburg, Illinois, USA) was applied in accordance with manufacturer's instruction. Two layers of bonding agent were applied and gently air dried after scrubbing each layer. Then light cured for 20 seconds using a light cure device with an output intensity of 900 mw/cm^2 (Demetron LC, Kerr, Orange, California, USA). Forty plastic cylinders were made with internal diameter of two mm and four mm height. Ten cylinders were prepared with the following procedure: The composite resin (All-Purpose Body, A_3 shade, Bisco, Schaumburg, Illinois, USA) was placed inside the cylinder and fixed with finger on the dentin of the sample. Excess of the composite was removed carefully with a scalpel blade. Then incrementally cured for 40 seconds. The plastic cylinder was cut out.

In group B, dentine was contaminated with aluminum chloride (ViscoStat Clear, Aluminum chloride 25%, Ultradent, South Jordan, Utah, USA) according to the manufacturer's instruction. And rinsed with constant pressure. The rinsing time was as long as the application time. Then All-Bond Universal (Universal, Bisco, Schaumburg, Illinois,USA) was applied in accordance with manufacturer's instruction. Two layers of bonding agent were applied and gently air dried after scrubbing each layer. Then light cured for 20 seconds using a light cure device with an output intensity of 900 mw/cm^2 (Demetron LC, Kerr, Orange, CA, USA). Composite resin (All-Purpose Body, A_3 shade, Bisco, Schaumburg, Illinois, USA) was placed and cured on this surface like group A.

In group C (control group), All-Bond SE was used without ViscoStat Clear, and the composite cylinder was applied as the same way in group A.

In group D (control group), All-Bond Universal was used without ViscoStat Clear, and composite resin was placed and cured the same way.

Intensity of the light-curing device was measured (Optilux 501, Demetron, Kerr, Orange, CA, USA) between the procedures.

The specimens were immersed in distilled water after preparation process at room temperature for 24 hours.

Before the shear bond test, specimens were subjected to 1000-round thermocycling (MP Based, KARA 1000,Tehran, Iran) procedure at 5-55 °C with a dwell time of 30 seconds and a transfer time of 30 seconds.

In group B and D (universal groups), the self-etch technique was selected to use the universal bonding agent.

Shear bond strength test was performed with a universal testing machine (Zwick/Roell, Z020, Ulm ,Germany) at a crosshead speed of 0.5 mm/min. Data were analyzed by 1-way ANOVA.

Ethical clearance registration number at Islamic Azad University of Medical Sciences-Dental Branch, Tehran, Iran is "IR.IAU.DENTAL.REC.1396,24". And the ethical ID at IRCT (Iranian Registry of Clinical Trials) is "IRCT2017101136711N1".

This study has been conducted in full accordance with Iranian Registry of Clinical Trials. In our country, ethical issues are reviewed by this center. And the study has been independently reviewed and approved by this ethical board. (It is a publicly accessible database)

RESULTS AND DISCUSSION

In this study shear bond strength of the composite resin cured to the dentin of the 40 extracted human premolars that were contaminated or uncontaminated with aluminum chloride, were evaluated. Mean shear bond strength values ± standard deviations of the groups are in Table 1. One-way ANOVA revealed not significant differences in shear bond strength values among self-etch groups and universal groups. Group C (All-Bond SE without the hemostatic agent) indicated the most SBS value (58.44 ± 24.07) and group B (All-Bond Universal + hemostatic agent) showed the least SBS value. (41.73 ± 25.78) Also, there were no statistically significant differences among four groups. ($P<0.2$) The shear bond strength of the both types of bonding agents has not been statistically affected by using the hemostasis. ($P<0.4$). Figure 1 is indicating the comparison of the mean shear bond strength values.

The results of this study showed that the use of aluminum chloride (ViscoStat Clear), as a hemostatic agent, has no significant effect on the shear bond strength of composite resin that is bonded with a self-etch (All-Bond SE) or a Universal bonding agent on the dentin. After the completion of the test, the data of the current study were placed in the sample size formula and the test power was 80%, so the sample size was sufficient. Aluminum chloride is a useful substance to control bleeding. It has the least side effects and nowadays it is used routinely in clinical procedures.(Kuphasuk et al., 2007) Due to the fact that it doesn't contain iron, like ferric sulfate, it doesn't cause discoloration.(Tarighi and Khoroushi, 2014) Therefore this substance was selected in this study. Most caries end up in dentin, also many of the restorations that we have to use a homeostasis with retraction cords are class v cavities. In many of these cases, decays have reached the root surface and there is no enamel. As a result, dentin was selected for testing in this study.

Table 1. Shear bond strength (MPa) of the specimens (n=10)

Group	Mean+S.D	C.V
A (ViscoStat clear+All-bond SE)	57.13 ± 38.63	68
B (ViscoStat clear+All-bond universal)	41.73 ± 25.78	62
C (All-bond SE)	58.44 ± 24.07	41
D (All-bond universal)	47.20 ± 25.53	54

In the past studies, a 0.5% chloramine solution was used to disinfect the teeth.(Ajami et al., 2013, Tuncer et al., 2014, Arslan et al., 2012) In this study, because of the scarcity of chloramine, the alternative substance,Thymol, has been used.(Khoroushi et al., 2016, Trakyali and Oztoprak, 2010)

One of the important issues in this process that affects the result, is the removal of the extra composite from the surrounding composite cylinder, which is carefully done in this study. Also, the depth of the dentin is important. As we move towards the pulp, the diameter of the dentinal tubules increases and the resin tags will then be different, (Lopes et al., 2009). The thickness of the smear layer, especially when using self-etching adhesives, is very important. Because in this method, the individual teeth are not etched, the smear layer is not removed and it is only changed, (Arslan et al., 2012). Since the achievement of the same dentin depth is difficult, in this study, an attempt has been made to test the superficial dentin. Also, to reach the same smear layer, cutting and polishing steps were identical. Certainly, the accuracy of this study is less than clinical studies, and this is a disadvantage. In an in vitro study, all the conditions in the clinic can't be achieved. But for the shear bond strength test and using the universal testing machine, the study must be in vitro and clinical studies are not possible.

According to the previous studies, hemostatic agents have acidic PH in the range of 0.7 to 3. They can remove the smear layer and cause demineralization of the enamel and the dentin, (Fathpour and Khoroushi, 2013). One study showed that using aluminum chloride doesn't have any effects on etch-&t-rinse bonding agent's strength. In the study mentioned above, ViscoStat and ViscoStat Plus were used as the hemostatic agents. (Kimmes et al., 2006) The use of this hemostasis

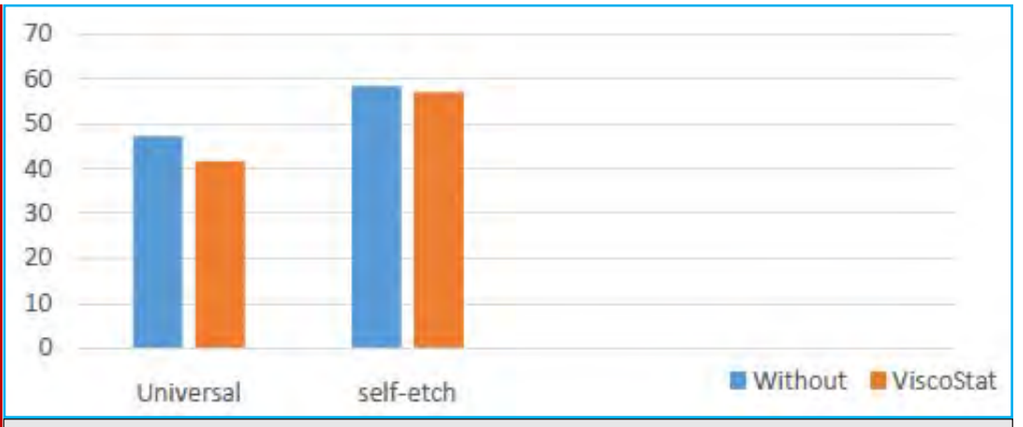

FIGURE 1. Mean shear Bond Strengths (MPa)

without washing, has a negative effect on bond strength, but if it is removed with plenty of water, it doesn't have any effects on the bond strength, (Kimmes et al., 2006). At least, by washing the hemostasis, the strength will be higher than the situation that the hemostasis is not washed. (O'Keefe et al., 2005). In the etch-&t-rinse technique, due to the use of phosphoric acid, aluminum does not remain on the surface, (Harnirattisai et al., 2009). Some papers reported a decrease in bond strength even after washing the surface with plenty of water. They had examined the specimens under SEM and reported that the reason for this reduction was the presence of aluminum on the dentine surface, and also the poor acidity of self-etching bonding agents, (Kuphasuk et al., 2007, Mohammadi et al., 2012, Ebrahimi et al., 2013). Therefore, according to these results of the past studies, it is concluded that washing of the surface with plenty of water is very important, especially when using self-etching technique.

Some articles showed that the duration of the application time of the hemostasis and its concentration are important. The longer time it remains on the surface, the more smear layer is removed and more dentinal tubules are open. (Lahoti, 2016) But if it remains on the surface too much, it could cause over-etching. The studies that showed a reduction in the strength of a self-etch bonding agent after using aluminum chloride 25%, claimed that if the aluminum chloride remains less than 5 minutes on the dentin, it can not remove the smear layer and open the dentinal tubules. On the other hand, because of the poor acidity of self-etching bonding agents, the ability of etching and the penetration of the resin is poor. And bond strength decreases, (Kuphasuk et al., 2007). Based on the results of the previous studies, the property of demineralization of aluminum chloride increases the effect of etching of self-etching bonding agents and prevent the bond strength reduction due to the presence of aluminum, (Kuphasuk et al., 2007, Harnirattisai et al., 2009).

Some studies reported that there are no significant differences between the control groups and ViscoStat Clear-contaminated groups. Also according to an article, some chemical reactions can prevent the bond strength reduction (Khoroushi et al., 2016). After using ViscoStat Clear , the calcium in the hydroxy apatite is replaced by aluminum in $AlCl_3$. This reaction generates $Al(OH)_2H_2PO_4$ which is insoluble. (Ajami et al., 2013, Fathpour and Khoroushi, 2013) As a result, 10-methacryloyloxydecyl dihydrogen phosphate, that is a part of the self-etching adhesives, brings a chemical bond with the remaining minerals in the dentin. Insoluble salts are formed after the use of $AlCl_3$ on the dentin surface. It is probable that due to the formation of a number of chemical bonds, there is no statistically significant difference between ViscoStat Clear- contaminated groups and the control groups, (Khoroushi et al., 2016).

According to one of the studies about self-etching adhesives, the PH of different kinds of these bonding agents is important. It can cause different results, (Tuncer et al., 2014). Both of the adhesive resins that were used in this study were the self-etch adhesive systems. (All-Bond Universal and All-Bond SE) The PH of All-Bond SE is 2.2 and the PH of All-Bond Universal is 3.2 that means All-Bond SE is more acidic. By given the mean of the data of this study, it seems that perhaps the use of this substance in the clinic may be somewhat reduced SBS of the composite resin. And it seems that the Universal Bonding adhesive was more affected by aluminum chloride. Perhaps the reason why the Universal Bonding adhesive was more affected by aluminum chloride is its higher PH and less ability to demineralize the dentine. But since this is an experimental study, it can not clinically prove this issue. According to these controversies mentioned above, more studies are needed.

CONCLUSION

Based on the results of this study, there is no statistical differences between control groups and ViscoStat Clear-contaminated groups.

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Intestinal microbiome and related diseases: A recent updated review

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ABSTRACT

The set of bacteria, viruses and fungi that live on human bodies, is called in all microbiota and its genes are called microbial. Microbial, because of its products, its response to the environment and its reciprocal response to other systems is considered as one of the organs of the body. Sometimes, it is also called the second genome of human beings and the microbial genes that form microbial are more than 1% of human beings' genes and in bowel there are more than three million bacterial genes. One of the most important parts of body that its microbial has attracted the attention of scientists very much is the microbial of alimentary canal. A set of unicellular organisms in our bowel, called intestinal microbial appears as the important index of health. Errors and mistakes in a suitable microbial colonization in the early life causes food sensitivity, allergic responses, type 1 diabetes and other disorders of autoimmune. The important role of the intestinal microbiota in weight adjustment is through harvest and appetite control. Intestinal microbiota has an important role in the pathology of various inflammatory bowel diseases and also developing colorectal, gastric and prostate cancers and cardio metabolic disorders.

KEY WORDS: GUT MICROBIOME, INSULIN RESISTANCE, PROBIOTICS, BIFIDOBACTERIUM

INTRODUCTION

The set of bacteria, viruses and fungi that live on human bodies, is called in all microbiota and its genes are called microbial (Microbial, because of its products, its response to the environment and its reciprocal response to other systems is considered as one of the organs of the body. Sometimes, it is also called the second genome of human beings and the microbial genes that form microbial are more than 1% of human beings' genes and in bowel there are more than three million bacterial genes, (Petersen *et al.* 2017, Greenhalgh *et al.* 2016 Miliani *et al.* 2018).

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One of the most important parts of body that its microbial has attracted the attention of scientists very much is the microbial of alimentary canal. Human being's alimentary canal is an organized microbial composition that carries more than ten times of human being's cells (Vinke *et al.* 2017). Therefore, intestinal microbiome is considered as the mammalian super organism, (Arboleya *et al.* 2016).Genetic, the birth method, nutrition patterns of infants and taking antibiotics, the living health conditions and dietary habits for a long time have all important rolls in the formation of the intestinal microbes, (Caesar *et al.* 2016). However, it seems that alimentary canal of every mature person might have a unique and slightly stable microbiota. The early researches about the analysis of ribosomal DNA 16S, are used to determine and count the distal and fecal intestinal microbiota. More than 90% of the bacterial phenotypes that exist in the intestinal microbiota in healthy human beings, are three bacterial classes that include *Bacterioides*, *Furmicut* and *Actinobacteria* (Haghes *et al.* 2017).

In the early years of 1900s, two Nobel Prizes in physiology and medical science were offered to the scientists that determined the relation between microbes and human health. The first Nobel Prize was offered to Robert Koch that linked microbes to infectious diseases, while the second prize was offered to Ilya Mechnikov who, for the first time, used the alive micro-organisms to protect human health. Now a complicated set of relations between microbiota and human beings has been determined (Vinke *et al.* 2017).

The microbial has an important role in the formation of human being's characteristics. For example, one of the roles of intestinal microbial flora is its effect on the obesity. The fundamental metabolic mechanisms of this resistance include the absorption of glucose and the production of a short chain of fatty acids from duct and the decrease of lipogenesis related to the liver, the decrease of fatty acid oxidation, the decrease of accumulation of triglycerides in adipocytes (Sicard *et al.* 2017) and also the intestinal microbiota has an important role in food digestion and in the host immunologic response (Dominianni *et al.* 2016).

DISEASES AND INTESTINAL MICROBES

Necrotizing Enter colitis: The growth of intestinal microbial in the early life and its importance in infants who are at risk for developing necrotizingenter colitis have been determined. The intestinal microbial of the infants with NEC was determined by the difference in the microbial composition such as the increase in frequency of *Gamma-proteobacteria* that is a common feature of intestinal microbial in the condition of disease (Timothy et al. 2016).

The difference in the microbial composition in fecal microbiota is the first step to develop NEC. Probiotics or the method of nutrition can change the microbial composition into an intestinal microbial, resistant to the disease. In order to decrease the outbreak of NEC in premature infants, a diet that consists of breast milk has been recommended and the milk supplement given by mother has been considered as a way to prevent NEC. In addition to milk supplement, several probiotics have been successful in preventing the severe NEC and decreasing the mortality of premature infants for any reason, including infants with low weights at birth. The recent investigations about the infants with NEC, have shown the increase in frequency of *Proteobacteria* such as *Citrobacter* in fecal microbiota. Besides, Versalovic and *et al* (2017), have recently reported a more ratio of *Gamma-proteobacteria* in fecal microbiota before the NEC diagnosis in infants. Healthy diets for NEC include taking non-edible (injectable) antibiotics for a long time that can decrease the intestinal microbial diversity and prevent colonization by a variety of microbes (Versalovic *et al.* 2017).

The Infection of Clostridium Diphyles and Acute Gastroenteritis: Ecological and microbial disorders may be cured by the use of anti-microbial medicines and chemotherapy and can be improved by probiotics. In the last decade, outbreak of the disease along with Clostridium diphylesin infants has increased. Gorbach and his colleagues have shown the successful treatment of this disease by the use of Lactobacillus rhamnosus strain taken from human beings. This strain is usually known under the title of LGG probiotic. Several investigations about infants have shown that probiotics may be effective for suppressing diarrhea along with antibiotic; and probiotics may increase the maintenance of microbial diversity as a mechanism for soothing the disease phenotype (Johnson et al. 2016).

Irritable Bowel Syndrome: The difference in composition or intestinal dissbiosis microbial in irritable bowel syndrome was reported in 2011, and the effects of the disease based on the differences in bacterial composition were discovered. The increase of *Gamma-proteobacteria* in adult children with IBS and its relation with a group containing intestinal pathogens was accompanied by the increase of pains; and it explained the decrease of *Bifidobacterium* classes and *Verrucomicrobium* in the infants with IBS diarrhea. The consumption of *Bifidobacterium* in adults with IBS decreased the symptoms and determined the possible advantage of probiotic treatments in IBS with probiotic strains which have been selected carefully. The significance of intestinal *Bifidobacterium*

may explain the relative success of compound solution of *Bifidobacterium – lactobacillus* (Johnson *et al.* 2016).

Inflammatory Bowel Disease: The difference in the intestinal microbial composition in different investigations on patients with colon disease and wounded colitis has been reported (Johnson *et al.* 2016). This imbalance and disorder in the host microbiota, called dissbiosis, can form the inflammatory response by the host. Such differences include the decrease of bacterial fila ratios of *Bacteroides* and *Freiquito* and the development of *Protobacterium filum* in patients with inflammatory bowel disease. The relative increase of *Proteobacteria* and *Gamma-proteobacteria* in recent investigations emphasizes on the possible significance of gram-negative bacteria in adults and IBD of infant. The particular components of *Gamma-proteobacteria* help diagnose the infants with IBD; a special example is the increase of *E.coli* and *Shigella* classes in the infants with ulcerative colitis, (Johnson *et al.* 2016).

Celiac Disease: Although the microbial composition of the small intestine in patients with celiac disease is not different, differences in intestinal microbial in stool samples, collected from the patients with celiac disease, have been determined. The equivalent changes have been reported in stool microbial and stool and urine metabolism in the infants with celiac disease, in comparison with healthy controls. In the PROFICEL investigation, it was determined that breast feeding with its possible "feeder" effects on the useful microbes has an influence on the relative talent to develop celiac disease. In a report, the relative timing of the arrival of gluten in early childhood had an influence on the relative talent to develop the disease and disease phenotypes were improved by the changes in intestinal microbial and metabolism (Timothy et al. 2016).

An interesting aspect of celiac disease was the presence of the bacterial metabolizing gluten, such as rothia in edible microbial. Gluten metabolizing microbes in oral or intestinal microbial may decrease the relative talent of people with genetic background to the celiac disease. The probiotic solutions in the future may include the following issues: paying attention to the probiotics with gluten metabolism genes and the probiotic ability to increase the function of gluten metabolizing bacteria in microbial (Johnson *et al.* 2016).

The Effect of Microbial on Depression: Just in a few numbers of investigations, the anxiety effects of probiotics in clinical trials have been investigated. In a research, the positive effects of lactobacillus probiotics helveticus R0052 and lactobacillus *B.longum* R0175 in an animal model were approved in adult volunteers in a random two-way blind trial. The active treatment decreased psychological distress and subsequently, decreased the urinary cortisol (Johnson *et al.* 2016).

The Role of Microbiota in the Autistic Spectrum Disorders (ASD): ASD is a group of evolutionary neurological disorders that includes autism, childhood disruptive disorder and inclusive growth disorder. ASDs along with disabilities, include social retirement, speech disorder and repetitive behavior. Gastrointestinal (GI) disorders in infants with autism are very common and it has been shown that the number of GI symptoms is related to autism very much. Particularly, *Bacteroides* in patients with autism increased significantly, while in people watching the pyromicots were more. On the contrary, in another investigation, patients with autism and (ASD + GI) GI problems were compared with patients with only GI problems and it was shown that *Bacteroides* decreased and pyromicots increased and there were *Betaproteobacteria* (Sutarla) in patients with ASD + GI (Greenhalgh *et al.* 2016).

Etiology of ASD is very complicated and multifaceted and includes gene, environment and diet; and besides, it has been shown that intestinal microbes link to the brain through the intestine-brain axis in order to improve the growth of brain and the function and behavior with the brain.The recent evidence shows that intestinal microbes influence the development of central neurological system and the response to the stress (Bailey *et al.* 2015).

The Intestine-brain Axis in Health and Disease: The term, "intestine-brain" or "brain-intestine" axis is used increasingly to define a neurohumoral two-way communication system. It consists of neural paths and humoral paths that include cytokine, hormones and neuropeptides as signal molecules. The axis brain-intestine and intestine-brain microbes includes central neurological system, the nerves and glands and neurological immune system, sympathetic and parasympathetic nerves of automatic neurological system, the intestinal neurological system and intestinal microbes. By the use of this two-way communication network, the received signals from the brain can influence the secretion of the alimentary canal and mutually, visceral messages from the alimentary canal can influence the function of brain. Intestinal microbes through the intestine-brain axis are related to the function of brain and also neurological diseases, (Dianan and Cryan, 2017)

Tiny Bowel Habitats and (ASD): Bolte *et al* (2016) have published the relation of ASD and low degree intestinal infection with *Clostridium tetani.* This bacterium belongs to the piermicots branch. They are mandatory anaerobic rod-shape. Some of the most important

biological pathogens that belong to this kind of bacterium are *Clostridium botulinum*, *Clostridium diphyles* and *Clostridium tetani*. These species are known for the production of neurotoxin and the poison produced by *Clostridium tetani* in the intestines of laboratory animals can be transferred to the central neurological system through vagus nerve and leads to disorder in neurons. It has been shown that this kind of inhibition may lead to the thousands of behavioral defects observed in the infants with ASD. They made investigations on the infants with autism and in the vancomycin treated infants they showed the improvement of digestive problems such as stomachache, constipation or diarrhea. Besides, the behavioral developments were observed by the considerable decrease in the aggression, the increase of eye contact and remarkable improvement in speech. However, these changes depended on vancomycin treatment and all the symptoms relapsed after they stopped taking antibiotics, (Bolte *et al*. 2016).

The research done by Sandler *et al* (2015), has provided one of the first obvious scientific documentations on the link between the intestine-brain axis and ASD. Fine gold and *et al* (2016), investigated the feces of infants with ASD and compared it with the control group that confirmed the above-mentioned results. Parracho and *et al* (2014), stated that the comparison of the feces of infants with ASD with the healthy brothers and sisters and the unrelated healthy control group showed that the infants with ASD had higher levels of some species of clostridium. Besides, control groups with normal growth and infants with ASD and sibling with normal function showed that infants with ASD had higher levels of *Bacteroides* and lower levels of piermicots than the control group, (Timothy et al. 2016).

Besides, the infants with ASD showed a lower level of some species of *Bifidobacterium* while *Desulfovibrio* was higher. The research done by Vang and *et al* (2014), showed a lower level of *Bifidobacterium* in the infants with ASD as well. In fact, the research done by Finegold and *et al* (2016), showed that there was *Desulfovibrio* in half of the people with autism and some siblings. More interestingly, none of the control people had *Desulfovibrio*. It was known that the infants with ASD don't have enough sulphur. Aldred and *et al* (2014), showed that the people with autism, in comparison to the healthy people, have lower levels of plasma sulfate but a considerable higher levels of urinary sulfate (Haghes *et al*. 2017).

The presence of revitalizing sulfate bacteria such as *Desulfovibrio* in the infants with ASD can be one of the observed disorders in the sulphur metabolism. Besides, the intensity of ASD behavior has a positive relationship with the increase of *Desulfovibrio* species and was also known that there were *Hemophilus parainfluenza* bacteria in the patients with IBS with more frequency. The recent investigations on microbiota in adults with different sub-types of IBS also confirmed the bacterial dissbiosis specific to the intestineand in the patients with IBS-D, *Bifidobacteria* decreased and *Gamma-proteobacteria* increased (Greenhalgh *et al*. 2016). Metabolomics in ASD investigation showed that the infants with autism had abnormal levels of urinary hypores, hypo-hydroxy-4 and N-methyl-2-pyridon-5-carboxamide (2PY) than the control group.

The blood plasma of the patients with autism showed the increase in most of saturated fatty acids, except propionic acid and the decrease in most of unsaturated fatty acids that can be related to the several processes including oxidative stress to disorders in mitochondria and leads to the metabolic resulted from the changes in the patients with autism. This was recently shown by Subhadeep *et al* (2017), in a research on the comparison of the relative concentration of necessary fatty acids (linoleic and linolinic alpha), the long unsaturated fatty acids and phospholipids in the plasma of the patients with autism in Saudi Arabia with the control group of the same age. They reported the remarkable modulation of fatty acid metabolism that was evaluated by the change of the ratio among necessary fatty acids, long unsaturated fatty acids and omega 3, omega 6 fatty acids and the decrease of the circulation level of phospholipids. The authors have put a special emphasis on the phosphatidyl ethanol amine, phosphatydil serine and phosphatidyl lipid species that can be used as potential biological markers for the treatment strategies or future preventions (Subhadeep *et al*. 2017).

MICROBIAL, PROBIOTIC AND ATOPIC DISEASES

Human Skin Microbial and Atopic: Human skin contains several dominant bacterial species in different places and they are *Propionibacterium*, *Eubacterium*, *Staphylococcus* and *Streptococcus* and a fungoid species called *Malaseziya*. The investigations that focused on the particular parts of the body, have determined the important properties of colonization in healthy people. *Corinobacterium* is the most common bacterial species in anterior nasal cavities and *Staphylococcus aureus* pathogen exists in healthy people. Dermatitis atopic *Staphylococcus* that includes *Staphylococcus aureus*, *Staphylococcus epidermidis*, has an important role in worsening the disease. Unfortunately, for the patients, the identification of probiotic strains that may have useful effects on human skin has not been determined (Johnson *et al*. 2016).

Microbial Airways: Asthma and Atopic: The changes in the human microbial and pathogens have had influences as the possible reasons of asthma. In a research on the healthy infants and infants with asthma, there was no

important change in the bacterial fila found in alimentary canal and the dominant fila in both groups having *Bacteroides*, *Freiquito* and *Proteobacteria*. While healthy infants were determined by the *Streptococcus pre vetal*, *Veillonella* and *Fusobacterium*. *Haemophilus* species asthmatic group was relatively abundant. In haemophilus species, pathogenic haemophilus influenza was considered as the asthma initiator. Although the future probiotic solutions may be applied by oral and inhaled indication, there might be gaps in our understanding of airway microbial, effective probiotics and their effects on the asthma and allergic diseases (Johnson *et al.* 2016).

The outbreak of atopic diseases such as exema, asthma and food allergies increases and it is related to the change of the intestinal microbiota. Health assumptions made by Strachan in 1989 showed that non-infection in early infancy causes the increase of atopic disease. Since the mutual effect of immune cells with microbial antigens is essential and important for the function and development of adaptive immune response, non-stimulation of immunity during the early life in the developed countries can cause the increase of immunity disorder which has been observed in asthma and atopic diseases.Delay or change in the main microbial can have an influence on the development of immunity response. Epidemiological data provide more evidence that shows the babies born by cesarean section, in comparison with the babies born through the vagina, develop atopic diseases such as asthma and type 1 diabetes and nutritional allergies more, (Versalovic *et al.* 2017).

OBESITY AND ITS RELATION WITH MICROBES

Obesity Metabolic Syndrome

The decrease of physical activity and the increase of energy consumption, particularly in the West, disrupts the energy balance in human beings and can lead to a complicated disease symptom that indicates in all a metabolic syndrome. The key characteristics of metabolic syndrome include obesity, the lack of glucose control, increased blood fat and blood pressure (Han and lean, 2016).

Intestinal Microbial Flora and Modulation of Inflammation: Obesity and insulin sensitivity are related to the enhanced modes of inflammatory symptoms in adipose tissue and the increase of pre-inflammatory cell level. It is getting more obvious that the low level of metabolic inflammation creates a causal relation between the obesity and insulin sensitivity. Human gut lumen acts as the source of LPS that is the main part of the outer membrane of gram negative bacteria. Several investigations have shown that the amount of this LPS in the blood of healthy people was obtainable; this indicates that LPS was continually absorbed by the intestine at low speeds. Interestingly, the circulation of LPS is related to the amount of insulin and in patients with type 2 diabetes, the amount of LPS circulation is higher. Sicard and *et al*, showed that there was a direct relation between the increase of LPS circulation and metabolic diseases and causes the increase of insulin sensitivity (Sicard *et al.* 2017).

Microbial Signaling through the Immune System: Alimentary canal has been covered with a number of epithelial cells which have a defensive role and is among the bacteria that are located in the alimentary canal lumen with under space; this secretory dam that consists of secreted and muscosal glycoprotein and black goblet cells is reinforced and pent cellulose cells cause the secretion of antimicrobial peptides such as lysozyme, spla2, RegIIIβ. The task of M cells is sampling from the lumen space and they sample microbial antigens and send them to dendritic cell and they go towards the inflammatory and non-inflammatory responses and active B cells for the secretion of IgA. All these factors cause the body to be in a balanced mode of its own microbiota and have a balanced mode of microbium in the stimulation of immune system (Spiljar *et al.* 2017).

Intestinal microbiota and some proteobiotics can increase the development of intestinal dam. They have changed some of the special species of probiotic expression of strong binding proteins including acladine, sinolin, cladin-2 and ZO-2 (Greenhalgh *et al.* 2016). The consumption of high-fat diet is also related to the increase of gram-negative bacteria than pram-positive. These findings are also the indication of this view that intestinal microbiota can be the change factor in the metabolic mode and lead to the endotoxemia and metabolic diseases. The treatment of the mice which have high-fat diet with antibiotic, decreases the normal amount of LPS plasma and as a result, the outbreak of the inflammation of adipose tissues, oxidative stress and macrophage markers decrease. Besides, it prevents from the hypertrophy of adipose cell and improves the metabolic parameters of diabetes and obesity in the mice with high-fat diet. Therefore, the fat in the diet can be related to the increase of LPS absorption and this occurs with changes in intestinal microbiota; therefore, the gram negative bacteria like *Bacteriomate* in *Eubacterium*, rectal *Clostridium cocoides* and also *Bifidobacteria* decrease (Petersen *et al.* 2017).

Gut Permeability and Metabolic Endotoxemia: High fat diet, increases the LPS plasma and is defined as “metabolic endotoxemia” and we found out that fat consumption changes the bacterial population (i.e. the decrease of *Bifidobacterium*) and finally, the fat consumption and obesity increase the gut permeability. We show that intestinal microbiota has an important role in the

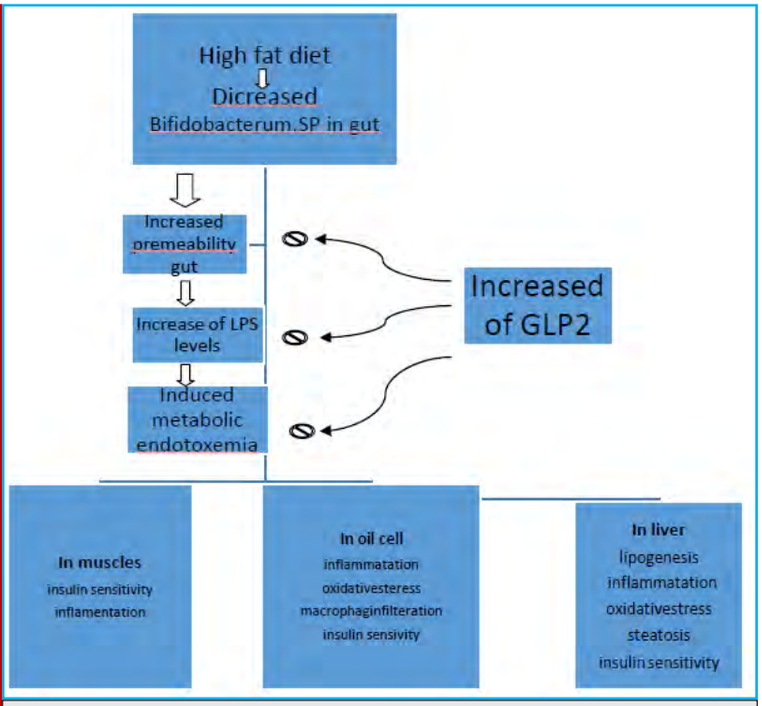

FIGURE 1. Changes in intestinal microbiota (after high-fat diet or obesity) improves the gut permeability, increases the metabolic endotoxemia and causes the development of metabolic disorder (Greenhalgh *et al.* 2016). For example, high-fat diet consumption changes the intestinal microbiota composition with a complicated method and the decrease of bifidobacterium spp. (Miliani *et al.* 2018) this phenomenon is accompanied by permeability that leads to the higher LPS levels of plasma (metabolic endotoxemia). (Petersen *et al.* 2017) Metabolic endotoxemia also increases the metabolic disorder resulted from low grade inflammation (insulin sensitivity, diabetes, obesity, steatosis, oxidative stress and adipose tissue macrophage infilteration. The increase of endogenous GLP-2 production causes the maintenance of bowel dam function, the decrease of metabolic endotoxemia and the development of metabolic disorder (Pflughoeft and Versalovic, 2012).

metabolic endotoxemia development and metabolic diseases along with obesity. For example, we found out that the remarkable changes in intestinal microbiota increase the insulin sensitivity completely through the antibiotic treatment, the related metabolic endotoxemia and metabolic disorders, i.e. glucose intolerance and insulin sensitivity (Haghes *et al.* 2017).

The various reports have shown that obesity because of diet manipulation (high-fat diet consumption) is determined by changes in intestinal microbiota towards the decrease of *Bifidobacteria*. In this research, in probiotic fed rats, the amount of intestinal *Bifidobacteria* was maintained and the effect of metabolic endotoxemia resulted from high-fat diet and inflammatory disorders, decreased. We have recently found out that the intestinal microbiota modulation has controlled and increased the production of intestinal inotropic peptide endogenous, i.e. glucagon-like peptide 2 (GLP-2) and subsequently, has improved the bowel dam function by a GLP-2 based mechanism (figure 1,2).

Contagious and Cardiovascular diseases: From the early half of the 19th. Century, contagious diseases have been known as the factor of artery hardening that with the changes including the increase of cholesterol absorption and lipoprotein improved by low density and the increase of the pressure of adhesion molecules and inflammatory cytokines lead to the vulnerability of the blood plaques along with the artery hardening. Epidemiological investigations have shown that there is a relationship among the inflammation of the gum, chlamydia pneumonia and contagion of helix pylori bacteria and the hardening or arteries. Indeed, chlamydia pneumonia can lead to the formation of sponge cells in blood vessel walls and the

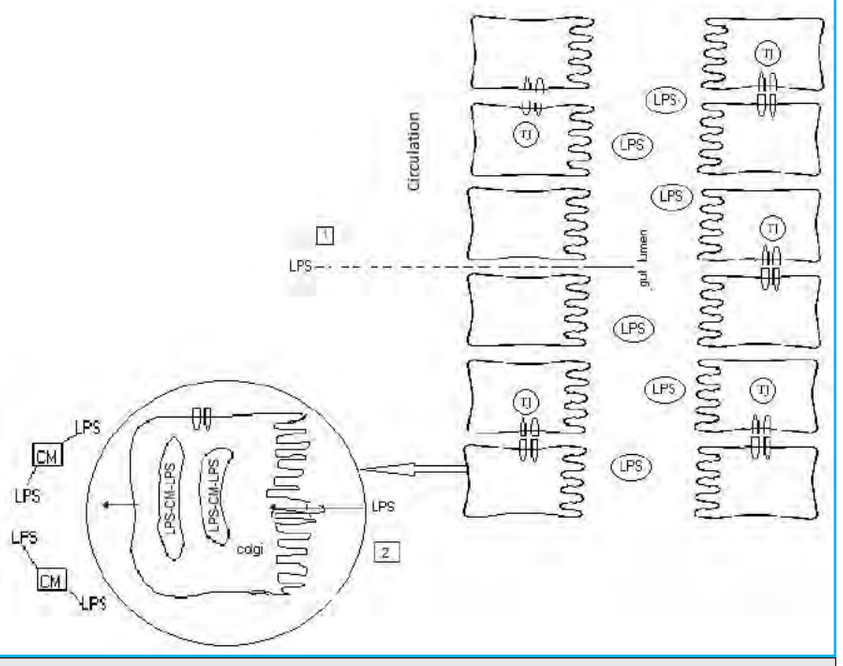

FIGURE 2. The intestinal absorption mechanism of lipopoly saccharide (LPS) are two mechanisms for the transfer LPS from inside of the intestinal cover tissue have been suggested: (Greenhalgh *et al.* 2016) The intracellular transfer through the easily facilitated enterocytes with cilomicrons (cm) and (Miliani *et al.* 2018) The intercellular secretion from inside of the tight joint (TJ) sites in the brain lining.The increase of intestinal integrity is related to the prevention treatment that seems to include glucagon peptide-2 (Kleerebezem, 2015). All of these together indicate that the changes in the tight joint sites and their secretion by the diet and microbial flora composition can increase endotoxemia. However, it is important to mention that it is not directly proven that LPS, in fact, can be transferred from inside of the tight joint sites (Kleerebezem, 2015).

bacterial DNA can be found in 50% of blood plaques. It is possible that chlamydia pneumonia can infect the macrophages and lymphocytes in the lungs and move to aortic vascular walls through the cells. Since there is a close relation between cardio-vascular disease and contagious disease, attempts have been made to treat the hardening of arteries by antibiotics (Kleerebezem, 2015). The conflict of inflammatory paths and metabolic in the hardening of arteries. The activation of LXR in macrophages lead to the cholesterol efflux from the cell and the activation of TLR4 by bacterial LRS or OXLDL, however, prevents LXRs and stimulates metalloproteinase (MMP4), (Kleerebezem, 2015).

Oral and Intestinal Natural Microbial Flora and Hardening of Arteries: The incomplete health of the teeth is related to the increase of the blockage of heart vessels and the result of several investigations show that there is an oral source for the bacterial plaque. To confirm these findings, oral pathogen can often be found in plaques and the direct control of *P.gingivalis* shows that this bacterium can lead to the increase of artery hardening. Insufficient oral sanitation has been recently attributed to the cardiovascular damages. Interestingly, it is related to the inflammation low intensity that is related to the cardiovascular advances. This information shows that bacterial oral cavity can increase the inflammations with low intensity and cause the hardening of arteries, (Kleerebezem, 2015).

PROBIOTICS AND PREBIOTICS

Probiotics and prebiotics may be useful in keeping the suitable microbial balance of intestinal microbiota of the elderly and help decrease the harmful effects of antibiotic consumption and less nutrition. Probiotics are alive microbes and when they are used in sufficient amounts, they are useful for the host. Prebiotics are the indigestible ingredients including various inulins and oligosaccharides that stimulate selectively the growth

of bacterial population in colon. *Bifidobacterium* and lactobacillus in the elderly often decrease with the disruption of health (Amy *et al*. 2015).The investigations show that probiotic consumption that includes these strains can increase their abundance remarkably along with the decrease of pathogenic micro-organisms in the intestine. A recent investigation in the lab environment showed that the intestinal microbiota of the elderly can be improved by suitable probiotic. *Bifidobacterium* and lactobacillus species along with the two prebiotics were added to the stool culture of the elderly participants. The addition of useful bacteria increases *Bifidobacteria* and decreases *Bacteroides*. The probiotic-prebiotic composition also increases the amount of *Bifidobacterium* and *Lactobacillus*. These all show that the main change in the intestinal microbiota for colon is healthier. Of course, prebiotic can alone improve health and change the intestinal microbial composition of healthy population (Caesar *et al*. 2016).

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Recent updates on the economic use of poly hydroxy butyrate (PHB): A green alternative to plastics

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ABSTRACT

Excessive uses of polymer plastics in boundless spheres have resulted in waste accumulation. There is an urgent need for switching over to compostable and biodegradable plastics that too from renewable resources. Polyhydroxybutyrate (PHB) is a unique biopolymer which is biodegradable and most close to plastics which is miscible with the conventional plastic. The adaptation and commercialization of PHB depend upon its ready availability in a pocket-friendly manner. The following presentation attempts to discuss economic strategies to reduce the production costs of PHB as well as its expeditious production from Agri-byproducts and its applications in various fields. Various sources like microorganisms, genetically modified bacteria, plants and different methods are being looked into, so that quality, quantity and financial side of PHB production can be controlled. It is presented here that PHB production is a natural capability of microbes exploited recently. This potential in the form of new technology is contributing to fulfil the need of plastic along with the environmental beauty and cleanliness. Novel bacterial fermentation strategies of polyhydroxybutyrate production, for improved productivity and quality of PHB for commercial production are summarized here.

KEY WORDS: PHB; RENEWABLE RESOURCES; BIOPLASTICS; PHB-PRODUCING BACTERIA AND BIOSYNTHESIS

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INTRODUCTION

The demand of plastics is increasing with the increasing population and synthetic plastics provide a range of utilities in the civilization of mankind, at the same time accumulation of these non-degradable plastics in the environment is a menacing drawback. Plastic is a synthetic polymer with several advantages because its structure can be operated chemically to have varied shape and durability. But microbes in the environment are not able to degrade plastic because of chemical complexity and large molecular size (Atlas & Bartha, 1993, Andrady, 1994; Zheng *et al.,* 2005; Mueller, 2006; Tokiwa *et al.,* 2009, Andrady, 2015; Barth *et al.,* 2016; Wei *et al.,* 2017).

Recently, plastic industries of many countries are taking interest in the replacement of non-biodegradable by degradable plastics so that environmental issues can be fixed. Because of resistant and elastic in nature, bioplastics are used in various studies, disposable commodities and as a packing material. The most studied is polyhydroxybutyrate (PHB), an eco-friendly, green plastic and a striking alternative to petrochemical plastic. Although lots of outlooks have been tied up with bioplastic but a lot has to be improved to make it commercially available. Most important among all is cost feasibility and a production cycle or the time taken by the bacteria for PHB production (Grothe, 1999; Gurieff, 2007; Cesário, 2014; Haas 2015). So, there is an urgent need to develop a cost effective and expeditious protocol for maximization of PHB production. With the advancement of science, it is suggested that this trouble can be swamped by using natural substrates like molasses and simple production procedures rather going for complex ones. This article reviews brief introduction about PHB and novel fermentation strategies for isolation and screening of efficient and important PHB producing strains. Also, some explanation about tools and technique for PHB optimization and identification are given. After that, molecular characterization of PHB genes is described. The present communication Present communication reviews the application, future trends and expression of PHB in alternative host.

Polyhydroxybutyrate (PHB), a family of bio polyesters with diverse structures, is the only bio plastics completely synthesized by microorganisms. These are natural, thermoplastic, aliphatic biopolyesters that completely meet the terms like *bio based*, *biodegradable*, *compostable* or *biocompatible to qualify them as Green plastics*. All bioplastics have a unique property of being completely degraded to CO_2 and H_2O by PHA hydrolases and PHA depolymerases (Jendrossek & Handrick 2002; Choi *et al.,* 2004). PHA production is mainly based on renewable resources (Braunegg *et al.,* 2004, Bharti & Shweta 2016; Chandani, 2018). Agricultural biproducts are used for fermentative PHA production (Gasser *et al.,* 2009; Hamiesh *et al.,* 2015; Getachew & Woldesenbet 2016). Biosynthesis and degradation of PHA is entirely attuned to carbon cycle (fig. 1). These are the only polymer plastic miscible and industry compatible products which do not necessitate the creation of any additional machining capabilities.

STRUCTURE OF POLYHYDROXYBUTYRATE (PHB)

Polyhydroxyalkanoates (polyesters) are formed by linear polymerization of hydroxy acyl units. The 2-, 3-, 4-, 5- or 6- hydroxy acidsjoin to form homo-polymers or copolymers (Chen, 2010). On the basis of number of carbon atoms present in the monomeric form of polyhydroxyalkanoates (PHAs) these are classified as short chain, medium chain (3 to 5 carbon atoms) and long chain (6 or more) PHAs (Pan and Inoue, 2009). The third main class of P(3HA)s is the SCL/MCL-P(3HA)s, which is a copolymers made up of SCL and MCL-3HA monomers consisting of 3–14 carbons. LCL–PHAs have properties in between the SCL-P(3HA)s and MCL-P(3HA)s dependent on the mole ratio of SCL to MCL monomers, but the nature and proportion of these monomers are being influenced by the type and relative quantity of carbon sources supplied to the growth media. In spite of a large number of polyhydroxyalkanoatepolymers, only a few of them are employed for large scale production which includes: P(3HB); poly-(3-hydroxybutyrate-co-3 hydroxyvalerate) and poly-3-hydroxybutyrate-co-3-hydroxy hexanoate (Asrar & Gruys, 2002; Chanprateep, 2010; Chandani, 2018).

POLYHYDROXYBUTYRATE PRODUCER

Microorganisms, as well as transgenic plants, are used for PHB production (fig. 2). Among the more than 250 different natural PHA-producers, only a few bacteria have been engaged for the biosynthesis of PHB. They include hydrocarbon degraders, halophiles, photosynthetic bacteria, plant growth promoting bacteria etc. as shown in fig. 2. *C. necator* has been the most extensively studied and commonly used bacterium for PHB production. Imperial Chemical Industries (ICI plc) firstly used this bacterial strain for the production of PHBV copolymer with a trade name was Biopol. In recent times, Metabolix Inc. (USA) obtained the Biopol patents. For industrial purpose, it is wanted to grow strains that can attain high final cell density in short period of time and high PHB production from undemanding and inexpensive substrates. Thus, genetic engineering serves as a powerful tool in the development of microbial strains that can produce PHA from cheap renew-

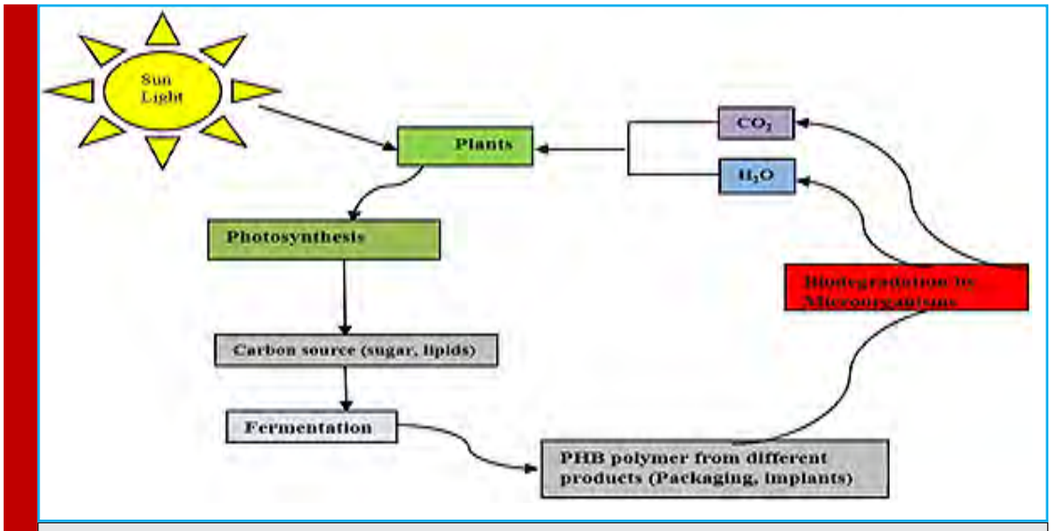

FIGURE 1. Life cycle of polyhydroxybutyrate

able resources efficiently. Transgenic plants are also very good alternative host for the production of PHB. But nowadays, the research on halophilic bacteria for PHB production has attracted scientist because these bacteria have advantage of unsterile and continuous fermentation process. However, few reports have been published to explore the potential of PHB production using marine bacteria and seawater as nutrient sources (Liu *et al.*, 2016).

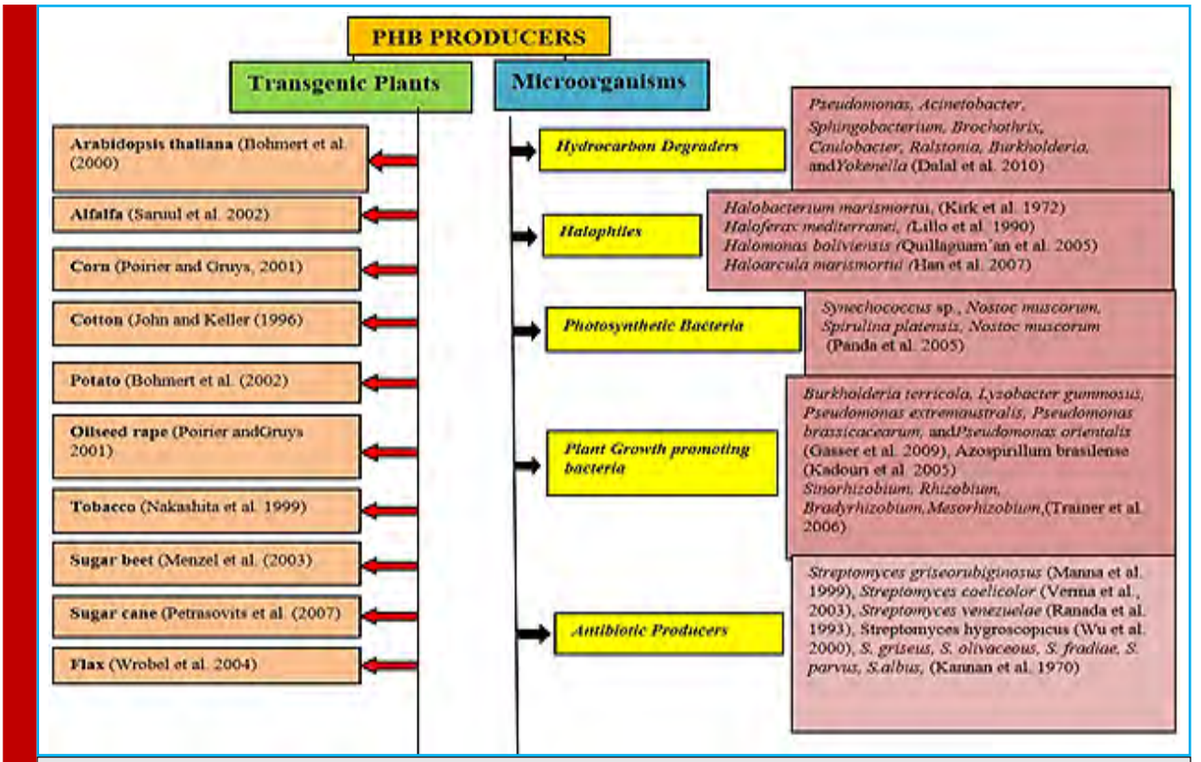

FIGURE 2. Different plants and bacteria capable of Polyhydroxybutyrate production

GENERAL PROPERTIES OF PHB

PHB a poly-3-hydroxybutyric acid homo-polymer has a melting point of 179 ^{0}C and is extremely crystalline (80%) in nature. Temperature above melting point leads to degradation of PHB. Some of the properties of PHB are similar to polypropylene with three unique characteristics: thermoplastic process ability, complete water resistance and complete biodegradability (Hrabak, 1992). General properties of PHB are: water insoluble, relatively resistant to hydrolytic degradation, good oxygen permeability, resistant to UV, susceptible to acids and bases, soluble in chlorinated hydrocarbons including chloroform, non-toxic, biocompatible, high tensile strength (40MPa), sinks in water facilitating anaerobic biodegradation and less sticky when melted making it potentially good material for clothing in future. PHA polymers with different subunit compositions and hence different physical and thermal properties can be tailor-made using novel PHA synthase for specific applications (Sharma *et al.,* 2017)P. putida LS46123, was able to synthesize polyhydroxyalkanoate (PHA.

IMPORTANCE OF PHB TO MICROBES

PHB is very important for microbes. Under nutrient stress, intracellular accumulation of PHB accumulates up to a level of as high as 90% of the dry cell weight (Madison & Huisman 1999). When the availability of nutrient sources like nitrogen and phosphorus is less and carbon is in excess, PHB acts as energy storage house. PHB act as a redox regulator within the cell and in some bacteria like *Ralstonia eutropha* and *Rhodospirillum rubrum,* it is involved in pyridine nucleotide dependent reduction of acetoacetyl-CoA. (Senior & Dawes, 1973; Steinbüchel, 1991). Encystment of *Azotobacter* cells is also associated with PHB accumulation. During starvation, presence of PHB protects the cellular components like RNA and proteins (Vinet & Zhedanov 2010) and it plays an important role in sporulation. Obruca *et al.,* (2016which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.Accumulation of polyhydroxybutyrate (PHB) investigation suggested the potential protective mechanisms of PHB when bacterial cells are exposed to freezing and thawing. Pavez *et al.,* (2009) studied that PHAs apply a protective effect against freezing in *Sphingopyxis chilensis*. Several PHA-producing bacterial strains have also been isolated from Antarctic freshwater and Antarctic soil which indicates that PHA accumulation is a common metabolic strategy adopted by many bacteria to deal with cold environments and other stresses (Galia, 2010; Nielsen *et al.,* 2017) (fig. 3).

GENES AND ENZYMES INVOLVED IN BACTERIAL PHB SYNTHESIS PATHWAY

Different genes are involved in PHA biosynthesis in various pathways and in some bacteria, they occur together as clusters. The organization of the genes involved in PHA biosynthesis is varied among the organisms and the difference in gene organization makes the different PHA operon. Molecular genetic studies have expanded the knowledge available since 1987 about the various metabolic, biochemical and physiological processes taking place in PHB synthesis. Various microorganisms have been characterized depending upon the genes and enzymes they utilize for PHB synthesis and degradation. Now it is clear that several pathways for PHB formation have been evolved by nature according to the requirements of a particular niche. Three different enzymes are involved in PHB biosynthesis pathway (fig. 4). New *phaC* genes that encode PHA synthase enzymes, produce PHAs with novel monomer compositions, have been isolated from different bacteria in various environments (Sasidharan *et al.,* 2016; Ling *et al.,* 2011; Ng & Sudesh, 2016)Malaysia. It is a rod-shaped, gram-negative bacterium with high sequence identity (99%. DNA extracted from a soil microbial community was used to construct metagenomic libraries, which were then screened for novel *phaC* genes (Cheng *et al.,* 2014).

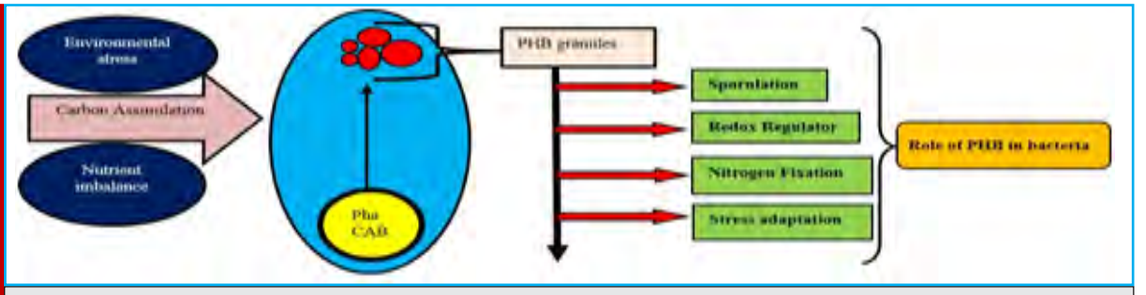

FIGURE 3. PHB formation under stress and its importance for bacteria

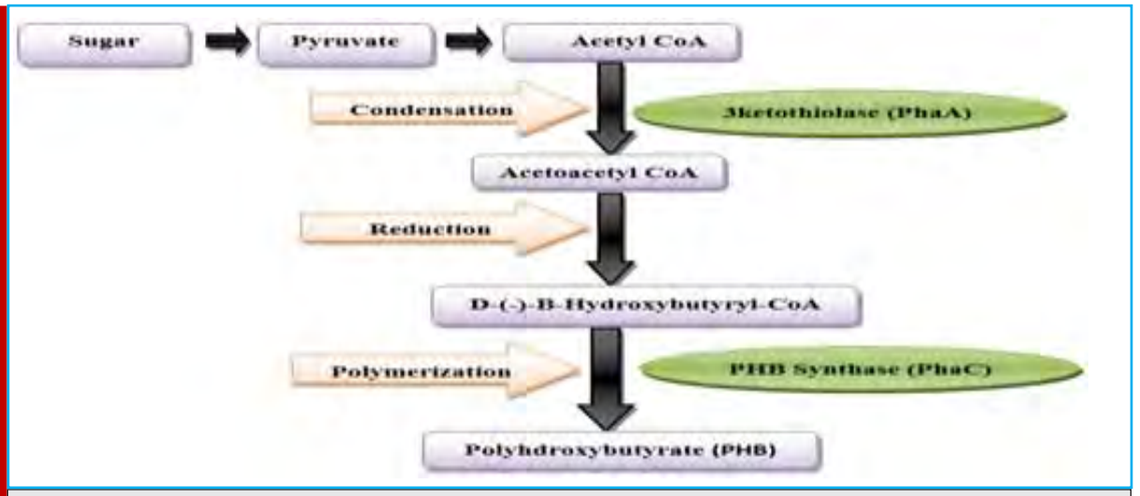

FIGURE 4. Biosynthetic pathway of PHB

SCREENING AND IDENTIFICATION METHODS FOR PHB ACCUMULATION IN BACTERIA

Screening of a broad collection of bacteria in short period is very crucial. Several methods given below are on hand for screening the PHB producing bacteria. Staining and microscopic visualization methods include the use of different stains like Sudan Black B and Nile Blue A, specific for detecting PHB granules. For the rapid screening, viable colony staining technique has been recommended. Sudan black B was first advocated by Hartman (1940), for use as bacterial fat stain. Later on, Burdon revised the procedure for identifying bacterial intracellular fat in 1942. They prepared Sudan B stained bacterial slides, counterstained with safranin but it is considered as the preliminary test. Ostle & Holt, (1982) and Spiekermann *et al.,* (1999) suggested another procedure.

Nielsen *et al.,* (2010) studied a combination of fluorescence in situ hybridization (FISH), with staining techniques for cell viability, PHB accumulation and polyphosphate in microorganisms. A number of Spectrophotometric and analytical methods have been developed to determine the PHB content in the microbial cells. A method developed by Williamson & Wilkinson, (1958) measures the turbidity of PHB produced by the digestion of microbial cells with sodium hypochlorite solution. Jüttner *et al.,* (1975) told about Infrared Spectroscopy which is based on the information that the PHB molecule gives a strong carbonyl absorption peak at 1728 cm^{-1} in the infrared spectrum. PHB is extracted from dried cells with chloroform and its content determined from the absorbance of carbonyl bond. Hong *et al.,* (1999) introduced Fourier transform infrared spectroscopy (FTIR) which is a speedy method for detecting bacterial polyhydroxybutyrate in intact cells. Ion-exclusion high-pressure liquid chromatography (HPLC) was used by Karr *et al.,* (1983) to quantitate PHB in different microorganisms like Rhizobium and japonicum bacteroids. Isotope dilution mass spectrometry was used by Alvarez *et al.,* (2017) to quantify PHB in prokaryotes at microscale level. The gas chromatographic (GC) determination of PHB in microbial cells was first described by Braunegg *et al.,* (1978). NMR techniques have been applied fruitfully to resolve the composition of the hydroxybutyrate units in a copolymer (Bloembergen *et al.,* 1986as determined by NMR, ranged from 0 to 47 mol % /3-hy- droxyvalerate (HV; Caballero *et al.,* 1995; Gross *et al.,* 1989). Srienc *et al.,* (1984) used flow cytometry to clearly demonstrate potential advantage of single cell light scattering measurement by flow cytometry for analysis and control of fermentation processes. Flow cytometry cell sorting technology should find significant application in strain improvement and mutant selection. Takeuchi *et al.,* (2016)natural seawaters were cultured in nutrient-rich medium for purple non-sulfur photosynthetic bacteria, and twelve pink- or red-pigmented colonies were picked up. Gas chromatography mass spectrometry analysis revealed that four isolates synthesized PHA at levels ranging from 0.5 to 24.4 wt% of cell dry weight. The 16S ribosomal RNA sequence analysis revealed that one isolate (HM2 isolated pigmented bacteria under nutrient-rich conditions as PHA-producing purple non-sulphur photosynthetic bacteria from natural sea waters. Low cost substrates were used by Takshshi *et al.,* (2017) to produce PHA from selected marine strains.

SUBSTRATES AND GROWTH CONDITIONS FOR PHB PRODUCTION

Nutrient limitation directly increases PHB production but high nitrogen state leads to augment biomass growth with no PHB production (Albuquerque *et al.*, 2010). 66% of PHB on dry weight basis was produced by *Pseudomonas* sp. when feed on methanol as primary carbon source (Suzuki *et al.*, 1986). Carbon/nitrogen (C/N) ratio is a very crucial variable to be considered during PHB production. When C/N ratio increased to 28.3, PHB production also increased by 1.8-folds, where sucrose was carbon source, ammonium sulphate was nitrogen source, optimum pH of 6.5, and temperature of 33 °C were provided in batch culture of *Alcaligenes latus* ATCC 29713 (Grothe *et al.*, 1999). It was found that decrease in dissolved oxygen content, decreases biomass accumulation and PHB production. Apart from it, Study by 13C NMR spectroscopy showed that feeding strategy greatly affects the monomers compositions and production of PHB produced (Ivanova et al., 2009). HV contents of PHA increased by 8% when fermented molasses as source of VFAs was fed continuously (Albuquerque *et al.*, 2011). Increase by 4.8-fold was reported when whey, a dairy waste product rich in sugars, as substrate was fed intermittently with ammonium sulphate in fed-batch culture of Methylobacterium sp. ZP24 under oxygen limiting conditions (Nath *et al.*, 2008). Gumel *et al.*, (2014) suggested that the monomer composition of the PHA ranges from C4 to C14, and were strongly influenced by the type of carbon substrate fed. Interestingly, an odd carbon chain length (C7) monomer was also detected when C18:1 was fed. Polymer showed melting temperature (Tm) of 42.0 (0.2) °C, glass transition temperature (Tg) of -1.0 (0.2) °C and endothermic melting enthalpy of fusion (Hf) of 110.3 (0.1) J g-1. The molecular weight (Mw) range of the polymer was relatively narrow between 55 to 77 kDa. PHB output from Methylobacterium SPV-49 was studied byGhatnekar *et al.*, (2002). They studied various carbon sources and found that PHB accumulation was highest when glucose was the carbon source. PHB accumulation was also there when sucrose and lactose was used as carbon source. Sudan black B was used to isolate 37 mutants of Azotobacterchroococcum for PHB production (Parshad *et al.*, 2001). PHB production was maximum with 2% glucose and 15mM/l ammonium acetate at 36 and 48 hours of growth under submerged and stationary culture respectively. They also found that sucrose and commercial sugars were better carbon source than glucose and mannitol as PHB accumulation was higher with sucrose and commercial sugars. Growth culture parameters for R. eutropha NRRL814690 were optimized by Khanna & Srivastava, (2005b) to reduce PHB production cost. In 48 hours 3.25g/l of biomass was produced with 1.4g/l of PHB concentration and fructose and ammonium sulphate were used as carbon and nitrogen source respectively. Alcaligeneseutrophus MTCCC1285 was used to study the effect of pH on yields of PHB (Kumar & Prabakaran, 2006). Two pH levels viz. 6.9 and 8.0 were used to study PHB production. At pH 6.9 the amount of PHB produced was 0.8, 0.5, 0.4 and at pH 8.0 it was 1.1, 0.65, 0.55 and 1.0 g/ml-l in glucose, sago, thippi and molasses substrate based media respectively. Wei *et al.,* (2011) shown that the production of PHB is maximum at pH 7.0 and temperature 30˚C. Operational condition at neutral pH 7 resulted in PHB accumulation up to 25% of CDW when compared to basic pH 9 (8.5%) and acidic pH 6 (15%). Acetic acid and propionic acid mixture in different proportion was used as carbon substrate to enrich the culture with organic load of 8.5 g COD L^{-1} day maintained at pH ranging from 7.7 to 9.5. Enriched culture showed high production rate and yields (389 mg PHA g^{-1} of nonpolymeric biomass) in SBR if maintained at pH of 7.5 with *Lampropedia hyalina* as dominant bacterial species (Villano *et al.,* 2010). Growth of bacterial strain and PHB production is also determined by agitation rate. Agitation rate of 150-200 rpm is best for PHB production and production decreases as the rate exceeds 200 rpm (Wei *et al.,* 2011). Different agitation rates were opted by different scientists according to the type of microorganism and culture conditions (Castillo *et al.*, 2016; García-Torreiro *et al.,* 2017).

OPTIMIZATION OF CULTURE CONDITIONS FOR MAXIMUM PHB PRODUCTION USING STATISTICAL TOOLS

Fermentation has to be optimised to increase the yield and productivity of various bioprocesses. Conventionally, one component change was used to optimise fermentation but this is time consuming (Khanna & Srivastava 2005a). In recent years statistically optimised methods have been used to replace this technique. Pandian *et al.,* (2010)it was suggested that strain SRKP-3 was similar to Bacillus megaterium. A four-factor central composite rotary design (CCRD isolated a gram-positive bacterium SRKP-3 similar to *Bacillus megaterium* that potentially accumulated polyhydroxyalkanoates (PHAs). A four-factor central composite rotary design (CCRD) was employed to optimize the medium and to find out the interactive effects of four variables, viz. concentrations of dairy waste, rice bran, sea water and pH on PHB production and a yield of 6.37 g/L of PHB dry weight was achieved by the optimized medium at pH 9. Muralidharan and Radha adopted a two-stage cultivation strategy for PHB production. Culture conditions for *Bacillus subtilis* MTCC 9763 were optimised

carbon, N_2 source, temperature, pH and incubation time. 55.0 mg PHB/g cell weight was obtained with glucose as carbon source, 17.2 mg/g cell weight with peptone as N_2 source, pH 7 yielded 35.43 mg/g cell weight and 35^0C yielded maximum production i.e. 52.2 mg/g cell weight (Muralidharan & Radha, 2014). Batch kinetics can be used for model development, which will make possible simulation of nutrient limited cultivation(s) for over accumulation of PHB (Aswathy 2015).

COST EFFECTIVE AND EXPEDITIOUS PHB PRODUCTION PROTOCOL– NEED OF THE HOUR

The high cost of PHB production is the major limiting factor for its commercial use. The factors behind the increased cost is the substrate used and downstream processing. This high cost can be reduced by use of cheaper carbon sources. The precursor molecules for PHB production are volatile fatty acids which can be obtained from various sources including municipal waste, banana pseudo stem, spoiled cereals, pea covering, apple pomace, effluents from vegetable oil mills, potato peels, rice bran, groundnut oil molasses, cotton and mustard cake. Lower volatile fatty acids are utilized by several bacteria like *A. utrophus, B. megaterium, P. oleovorans, R. bejjerinckia* and *Nocardia* to produce PHB (Kalia *et al.,* 2000). Various agricultural and dairy wastes have been used to produce PHB with or without ammonium sulphate supplementation. Valentin & Dennis, (1997) accounted that genetically modified *E. coli* was efficient of using molasses as carbon source to produce PHB. The net dry cell mass, PHB concentration and efficiency of PHB production were 39.5 g/l/h, 80%, (w/w) and 1g/l/h respectively. Among all the waste used, anaerobically treated waste gave the maximum PHB production. Kumar *et al.,* (2004)the production of biodegradable plastics using the sludge has been proposed. Storage polymers in bacterial cells can be extracted and used as biodegradable plastics. However, widespread applications have been limited by high production cost. In the present study, activated sludge bacteria in a conventional wastewater treatment system were induced, by controlling the carbon: nitrogen ratio to accumulate storage polymers. Polymer yield increased to a maximum 33% of biomass (w/w used sludge from waste water treatment plants to produce bioplastic. They found that when C:N ratio was raised from 24 to 144 the polymer yield was increased to a maximum of 33% of biomass (w/w). Mayeli *et al.,* 2015 used petrochemical wastewater as the source of carbon for the production of polyhydroxyalkanoates (PHA) in an effort to decrease its cost of production. Getachew & Woldesenbet, (2016) isolated *Bacillus* sp. which can be used for feasible production of PHB using agro-residues especially sugar cane bagasse. These protocols were able to solve two big environmental problems, first were the utilization of industrial waste and second were reduced cost of PHB production.

PHB EXTRACTION AND RETRIEVAL

In addition to maintenance of pure culture, PHA retrieval process and high substrate cost are some other factors that add to the high cost of PHB production. Several retrieval processes have been looked into and studied, over the past years to develop an economical protocol for large scale PHB production. PHB. Chloroform extraction method was depicted in 1990 (Doi, 1990). Hot chloroform was used to extract PHA in a Soxhlet apparatus and PHA so obtained was sorted from lipids by salting out with $(C_2H_5)_2O$, C_6H_{14}, CH_3OH or C_2H_5OH. At last, PHA was purified by re-suspending in $CHCl_3$ and by salting out with C_6H_{14}. PHA was retrieved from *R. eutrophia* by digesting with hypochlorite pre-treated with surfactant (Ramsay *et al.,* 1990). Three different chlorinated solvents ($CHCl_3$, CH_2Cl_2 and $C_2H_4Cl_2$) were used to retrieve PHA and it was found that biomass pre-treated with acetone was best to retrieve PHA (Ramsay *et al.,* 1994). A system for retrieving PHB from *E. coli* expressing lysozyme gene from T7 demonstrated by (Fidler & Dennis, 1992). Lysozyme causes the release of PHB granules by penetrating the cells and causing cell lysis. The cells are harvested and suspended into chelating agents like EDTA at the end of accumulation phase. This leads the release of PHA granules through cell disruption by the activated lysozyme. Dispersion with NaClO and $CHCl_3$ method was advocated by Hahn *et al.,* 1995. They claimed the removal of almost all of the cellular components from PHA during hypochlorite digestion (Hahn *et al.,* 1995; Gamal *et al.,* 2013).

MOLECULAR CHARACTERIZATION OF PHB GENES FOR THE MAXIMUM PHB PRODUCTION

The introduction of molecular techniques has boosted the biopolymer research that has enabled to understand various regulations in the PHA synthesis in vivo to produce PHB in an efficient way. The isolation of the genes involved in PHA biosynthesis resulted in heterologous expression of the cloned PHA genes in suitable host organisms and metabolic engineering for higher and safer productivity. Different strategies have been applied to identify and isolate PHA biosynthesis genes from various natural microorganisms. Colony PCR and semi-nested PCR techniques were used by Sheu *et al.,* (2000), to screen polyhydroxyalkanoates (PHAs) producers, isolated from different environments. Multiple sequence alignment was used to design degenerate primers for use in PCR to identify PHA synthase genes. For *Ralstonia*

eutropha the sensitivity limits of colony PCR were 1 x $10^5$and the results advise the use of this PCR in identification of PHB fabricators from environment. Bacterial strains isolated from varying environment were studied for the presence of PHA synthase using colony PCR (Sujatha et al., 2005)totally biodegradable plastics. The cost of these biopolymers is 25% more than the synthetic polymers that prevents their usage in wider range of applications. In order to reduce the cost, much effort has been made to screen the promising indigenous PHB producing strain in the present study. As a first step, among thirty scl (Short-Chain-Length.

Genomic DNA isolated using Sambrook *et al*., (1989) protocol was used as the template to carry out colony PCR using three primers phacF1, phaF2 and phaCR4 to detect PHA synthase genes. The PHA operon was first characterized in *R. eutropha* by Peoples & Sinskey, (1989) that made available the use of heterologous probe to isolate PHA genes from a number of organisms. Most of the PHA genes reported are isolated from various bacteria through constructing genomic DNA library and screening with probes. Rehm & Steinbüchel, (1999) cloned and characterized the polyhydroxyalkanoic acid synthase gene from *Chromobacteriumviolaceum* (phaCCv). A 6.3-kb BamHI fragment was found to contain both phaCCv and the polyhydroxyalkanoic acid (PHA)-specific 3-keto thiolase (phaACv). E. coli strains harboring this fragment produced significant levels of PHA synthase and 3-keto thiolase. Three genes are considered to be essential in the PHB biosynthetic pathway are phbA (β-keto thiolase), phbB (acetoacetyl coenzyme A reductase) and phbC (PHB synthase), were identified in *Azospirillum-brasilenses* Sp7. The phbA, -B, and -C genes were found to be linked together and located on the chromosome. In order to reduce the cost, much effort has been made to screen the promising indigenous PHB producing strain Pseudomonas LCD-5. This strain was selected for further characterization (Sujatha et al., 2005).

Preethi *et al*., (2012) isolated, characterized and screened PHA producing bacteria from soil by Nile Blue staining method. Screened organisms were subjected to fermentation with glucose as carbon source and low-cost raw material like jambul seed. The strain SPY-1 showed higher PHA accumulation when compared to the other strains and the reference strain *R. eutropha*. Berekaa, (2012) designed and applied a pair of specific PCR primers for genotypic detection of phaC synthase gene in eight strains. Approximately, 760 bp DNA fragment was successfully amplified in the eight strains. The sequence showed 99% identity to phaC gene for polyhydroxyalkanoate synthase of many *B. megaterium* strains deposited in Genbank. While, showed 73% and 72% identity to synthases of *Bacillus mycoides* and Bacillus sp. INT005, respectively. The PHB synthases obtained from Chromobactariumvoilaceum belongs to class 1 PHA synthases. This study seeks to investigate the structural and functional properties of PHB synthase (*pha*C) by predicting its three-dimensional structure using bioinformatics methods (Blessia *et al*., 2012; Bresan *et al*., 2016).

PHB PRODUCTION IN ALTERNATIVE HOST (HIGHER ORGANISMS)

PHB biosynthesis studies from yeast and insects can be used to transform plants for PHB accumulation. PHB synthase gene from *R. eutropha* expressed in *S. cerevisiae* has been used to manifestate PHB biosynthesis (Leaf *et al.,* 1996). A yeast plasmid was constructed using the *phaCRe* gene from *R. eutropha,* which enabled functional synthase enzyme expression in *S. cerevisiae*. A very less amount of PHB (0.5%) accumulation was there in these cells (Poirier *et al.,* 2005). *Spodoptera frugiperda* cell lines accumulated PHB upon simultaneous transfection with mutant rat fatty acid synthase and PHB synthase from *R. eutropha*. One liter of cultured cells produced 1 mg of PHB (Williams *et al.,* 1996). Expressing PHA biosynthesis genes in plants is need of hour. PHA biosynthesis genes have been expressed in *A. thaliana* and several agricultural crops like *B. napus, G. hirsutum, N. tabacum, S. tuberosum* and *Z. mays* (John & Keller, 1996; Hahn *et al.,* 1999;retained their catalytic activity, and reacted with peroxisomally available precursors because PHB synthesis in transgenic plant cells was localized to peroxisomes. Up to 2 mg/g fresh weight PHB was produced in suspension cultures of Black Mexican Sweet maize cells after biolistic transformation with three peroxisomally targeted bacterial genes. An equilibrium effect is proposed to explain the unexpected existence of (R Houmiel *et al.,* 19991994, Proc Natl Acad Sci USA 91: 12760-12764; Nakashita *et al.,* 1999; Steinbüchel, 2001; Bohmert-Tatarey *et al.,* 2011; Petrasovits *et al.,* 2012).

TACTICS TO AMEND PHB PRODUCTION

Generally, non-genetically engineered bacterial strains are used in PHB production systems but efforts have been made to enhance PHB production through genetic engineering. Most the modifications include changes in regulatory systems and transgenic favouring PHB synthesis. Deeper insights into regulatory pathways have led to the construction of mutants with improved PHB production. Moreover, few transgenic strains have exhibited to produce ample of PHB for mass scale production. The growth in fermentation approaches has also exhibited hope for improvement. In general, the exploitation of recombinant bacterial strains in combination with continuous and multi-stage fermentation techniques

and cheaper raw material could be a particular way to improve PHB production at mass scale.

APPLICATIONS OF PHB AND CURRENT COMMERCIAL STATUS OF BIOPLASTICS

Apart from the common applications such as production of poly bags, receptacles, instrumentation and decanters for soft drinks and dairy products, drugs can be made with the help of bioplastic. With the advancement of knowledge and technology, this biomaterial will mediate the manufacturing of products like cellular phones, cameras, medical equipments and automobile parts. The application of bioplastics in tissue engineering as a scaffolding material is presently gaining interest (Misra *et al.,* 2006; Luklinska & Bonfield, 1997). They can be a replacement of body devices as they are immunologically inert and degraded slowly inside human tissue. These are used by scientist to make medical devices like artificial bone implants, pacemaker, valves and bio-resorbable surgical sutures (Fedorov *et al.,* 2005; Rossiiskaia akademiia nauk *et al.,* 1995). They can also be used as drug delivery agents for slow and constant release of drug inside the body for a longer time period (Chen & Wu, 2005; Grage *et al.,* 2009).

Studies related to use of bioplastics in medical science are in preliminary phase and a lot of improvements have to be done. PHB based microcapsules have many applications in pharmacology such as a controlled release of water soluble drugs, various types of proteins, peptides and nucleic acids. Controlled release Methylene green drug is encapsulated with PHB microcapsules is an example of biodegradable microcapsules (Bonartsev *et al.,* 2007). Since the large-scale production of bioplastics at industrial scale is very expensive so it has not been used extensively. During 20th century, the bioplastics production was mainly dominated by the developed countries like North America, Japan, and Western Europe etc. Bioplastic market is at its initial stages of development in South East Asia. In the recent years biopolymers are becoming a crucial part of Food Science and Technology, Nanotechnology, Chemical Sciences, Medical and Agricultural Sciences. Nearly 20% increase in bioplastic production was observed in this year. The production of bioplastic has increased for 1.5 million tons in 2011 to 6 million tons in 2016 (five-fold increase). By 2020 bioplastic production will touch a mark of 12 million tons (International Conference and Exhibition on Biopolymers and Bioplastics, 2015).

CONCLUSION AND FUTURE OUTLOOK

As the public concerns are increasing about the hiking cost of petroleum and damaging effects of the petroleum based plastic materials in the environment, this work was focused on the production of biodegradable plastics polyhydroxybutyrate from low-cost and easily available raw materials. PHB is a biopolymer which can be completely degraded into H_2O and CO_2 by soil microorganisms. This review paper was aimed to provide an overview of bacterial polyhydroxybutyrate. This review covers the characterization, biosynthesis, identification and quantification, biotechnological strategies for improvement of PHB production, and its application in different fields. Presently their production is pricey and laborious, so profit making from bioplastics is at initial stages. Advance investigations on high PHB producing novel bacteria novel bacteria, recombinant microbial strains, mixed cultures, competent fermentations, recovery, purification and the use of low-cost and economical substrates like Agri-byproducts can significantly help to trim down the production cost.

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On the relationship between spiritual care competence of nurses working in Shafa Hospital with their quality of working life

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ABSTRACT

Spiritual care is one of the basic principles of nursing practice that addresses the spiritual needs of patients. According to the World Health Organization, this dimension has been neglected. This study was conducted in 2016 to evaluate the correlation between spiritual care Competences of nurses working in Shafa hospital of Ahvaz with their quality of working life.The research type was descriptive and co relational. Population of study includes all nurses working in the Shafa hospital of Ahvaz. One hundred nurses who had the criteria for the study were selected by convenience sampling. These nurses have answered the demographic questionnaire, the spiritual care Competence questionnaire and the quality of working life questionnaire. Data were analyzed using SPSS22 software and descriptive statistics and inferential statistics such as Pearson correlation and multiple regressions.The results showed that there is a significant positive correlation between the spiritual care with quality of working life of nurses (p=0.02), being professional (p=0.02), personal support (p=0.001), referral to specialists (p=0.02), attitude to patient's religious spirits (p=0.02) and relationships (p=0.01). The results of regression analysis indicated that the variable of personal support can significantly anticipate the quality of working life of nurses. (p=0.001). Based on the results of this study, the relationship between spiritual well-being and quality of work life in nurses, especially the role of later personal support as a predictor of quality of working life, and its positive impact on improving patient care services and, consequently, patient's satisfaction of care, it is recommended that relevant authorities in macro planning to motivate and improve the quality of life of personnel on the merits of spiritual care and special attention support.

KEY WORDS: SPIRITUAL CARE COMPETENCE, QUALITY OF WORKING LIFE, NURSES

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INTRODUCTION

One of the most important dimensions of the human being's existence is spirituality, which includes a set of values, attitudes and hopes that connect one to a superior being and binds to health, goodness, and healing (Ross, et al. (2014). Today, the role of spirituality in promoting the health of the patient has been more concerned and helping to meet the spiritual needs of patients and their families is considered as the main element of clinical care (McSherry and Cash. (2004 Usha Devi 2015).

Despite the fact that spiritual care is an essential component of nursing practice, and any health care system that seeks to provide comprehensive care, it considers spiritual care as an essential part of care (Chan et al. 2006). But the World Health Organization emphasizes that this aspect of care to meetthe needs of the patients' spiritual needs has been neglected (Chan et al. (2006, Mazaheri, et al., 2008). While Mazaheri and colleagues found in their study that nurses had a positive attitude toward spirituality and spiritual care, (Mazaheri,et al., 2008.) On the other hand, Strong et al. noted that, although 87% of nurses believed that they had to pay attention to the spiritual needs of patients, only 42% of them thought that these measures would be done in their parts (Strang, Strang, and Ternestedt,2002Colichi et al., 2017).

This is while spirituality and spiritual care of nurses help them in their duties, especially in dealing with patients (Gholamali et al., 2007), because spiritual care has a significant contribution in achieving preservation and promotion of health, preventing the disease, and eliminating ill health and discomfort (Farahani et al. (2005).Therefore, meeting the spiritual needs of patients should be an integral part of nursing interventions (Cavendish et al., (2003).

In this regard, Florence Nightingaleemphasizes: "The nurse plays an important role in improving the patient's health by taking into account the spiritual and psychological aspects of the patient" (Macrae, (2001). The American Nursing Association also considers the spiritual dimension as a center of nursing attention in expressing clinical practice standards, (ANA,2007). Therefore, comprehensive nursing care can be considered as body care, mind and soul, as an essential and vital part of caregiver and is a unique aspect of care, and a nurse who claims to provide comprehensive care should have the Competence of spiritual care (Newman et al., (1991). In other words, this competence can affect the provision of optimal services and quality of care.

Spiritual care is especially important for cancer patients, since cancer diagnosis and treatment is associated with a high degree of physical and psychological disturbances (Stein, et al., 2008). Apart from the stage of cancer, patients experience a great deal of fear and are uncertain about the side effects and benefits of treatments and how these factors affect their future lives (Kazer et al., 2013,Borneman, et al., 2014). Therefore, in the long run, cancer and its treatment can reduce the well-being of patients not only in the physical and psychological aspects, but also in the spiritual dimension (Conway, 2010). Therefore, specialists in this field should be equipped in the spiritual domain and related issues to meet such needs of cancer patients.

Studies have also emphasized the positive impact of attention to spiritual issues in improving the quality of care and thus the health of patients. For example, in a study by Rahnema et al. in 2014, with the purpose of designing a spiritual care model in the rehabilitation of cancer patients found that identifying and addressing the spiritual needs of patients through a systematic approach, development of knowledge and sensitivity of nurses to the spiritual needs of patients, spiritual deserving and empowerment of nurses, team performance enhancement and family support to provide spiritual care for patients during rehabilitation are essential (Rahnama et al., 2014). In addition, the positive effects of religious worship on the desire for life, the feeling of goodness and happiness, the reduction of anxiety and tension and the strengthening of other therapeutic processes are documented in most cultures and religions, (Dilshod 2016, Colichi et al., 2017).

The results of these studies indicate that spirituality is one of the factors affecting the quality of working life (Shojaee and Khazaee 2011 and Usha Devi 2015). Despite the fact that employee satisfaction surveys have long been of concern to organizations providing health services, and the managers of these organizations have used the results to solve organizational problems, today, a new concept of job satisfaction, entitled "quality of workinglife" is intended for managers, (Dalagas 2016). Quality of working life is a comprehensive and inclusive plan that is aimed at promoting the satisfaction of staff and is necessary for each organization to attract and retain employees (Bredle, et al. (2011). This program has a number of specific features, such as meeting standards in work, giving people equal opportunity to hire and provide fair payrolls for employees, amenities, health and safety considerations, participation in decision making, management practices, diversity and richness of businesses (Donald et al., 2011) and Beaudoin, and Hassles, 2003). In relation to nurses, due to their job nature, quality of working life is influenced by four factors of social, executive, managerial and cultural conditions that the occurrence of problems and deficiencies in this field causes job dissatisfaction and, as a result, psychological exhaustion and job loss (Taghavi 2004).

There are few studies on the relationship between the quality of working life and the Competence of spiritual care among nurses, although the findings are still consistent. The results of van der Walt &tKlerk (2014).), Ming Chia (2012) Asaroodi and Akbari (2011); Jafari and Khodayari (2012) and Nejad, and Ghalavandi 2012) have shown that there is a significant relationship between the subscales of spiritual care with quality of working life. For example, in a study conducted by Sharpe (2016)entitled the role of religion and ethics in balancing work and life of employees, he concluded that those who have high levels of spirituality and morals comparing those who have lower spiritual levelscan better balance between their work and life.The balance between work and life is one of the subscales of variable of the quality of working life (Sharpe 2016). Also, it was found that structured education based on spirituality increases nurses' quality of working life (Sydkavvsy and 2015).

Regarding the fact that spiritual care, more than the technical aspects of care, will lead to patient satisfaction as well as special attention of Ministry of Health and Medical Education to the category of spirituality, which is considered to be the required duties of treatment groups, the limitedresearches in this field in the field of nursing and work researcher's experience in the care of cancer patients, the researchers attempted to determine the relationship between the spiritual carecompetence of nurses working in the Shafa hospital with the quality of their working life in 2016.

MATERIAL AND METHODS

The research method is descriptive-analytic and a correlationtype (registration code: B-9542). The statistical population consisted of all nurses working in different parts of ShafaHospital of Ahvaz University of Medical Sciences (which is a special wardfor cancer) in 2016. The total number of nurses was 120 and 104 of them had inclusion criteria (voluntary consent, being employed in one of the wards of the Shafa hospital and having at least 6 months of work experience), 100 of them were selected by census method . Using ethical considerations (code of ethics: IRAJUMS.REC.1395559), 120 questionnaires were submitted to the nurses to be completed, of which 100 questionnaires were completedwith no defect completed.

RESEARCH TOOLS

A) Spiritual Care Competence Questionnaire: This questionnaire was designed by Van Leone et al. in 2009. The questionnaire consists of six dimensions related to nursing Competence in providing spiritual care as follows: "Spiritual Care"; related to assessment and implementation of this dimension refers to the ability to identify spiritual needs and planning for spiritual care (6 questions). "Professionalism and improvement of the quality of spiritual care" includes the part of nurses' activities aimed at ensuring the quality of care and extending policies in the field of spiritual care (6 questions). "Personal support and counseling with patient ", which is the heart of spiritual care, is the actual provision and evaluation of face-to-face care for the patient and his relatives (6 questions). Referral to specialists is related to other areas of health care, and the religious advisor with the expert in providing religious counseling is clearly its point of view (3 questions). "Attitude towards the religious beliefs of the patient", in which the individual factors related to the provision of spiritual care are categorized (4 questions). "Communication" is the contact between the nurse and the patient (2 questions). Students were asked to identify their level of Competence for providing spiritual care on a five point Likert scale (Koren et al. (2009),Khalaj et al (2103)) in their research found that the Cronbach's alpha coefficient for the whole tool is 0.77 and for subcategories is between 0.65 and 0.85. All questions had a significant intra-questions correlation. The results of the test-retest showed the stability of the questionnaire and its subcategories. The exploratory factor analysis with Varimaxrotation, created 6 factorswithEigengreater than one, describing 63.18% of the variance. The subcategories showed good homogeneity, with mean intra-correlation of questions greater than 0.35 and the reliability coefficient of the test-retest test. The confirmatory factor analysis showed acceptable fitness for the 6-factor model.

B) Quality of working life questionnaire: This questionnaire contains 39 items in the form of a 5-point Likert scale, which a score of 1-5 is assigned for each item, so that the score of 5 is considered for totally agree and the score of 1 for totally disagree. This scale has four subscales in assessing the nurses' quality of working life, including: 1. Working life-home life; 2. working plan; 3. working background; 4. working world. The first subscale is working –home life or the interface between working and home life of nurse. Since nurses are mostly women, this dimension shows the role of mother (child care), daughter (caring for elderly parents), and wife (family needs) of nurses in contrast to the working environment. Theworking plan subscale is the combination of nursing work and describes the real work that nurses do. The working background subscale includes functional environments in which the nurses work and examines the impact of the work environment on the patient and nurse.

This dimension represents the human and environmental factors related to the field of work, in other

words, the conditions and opportunities for training to improve the performance of work and the interpersonal relationships of colleagues. Obviously, improving the quality of the field of work (working background) provides the conditions necessary to increase the effectiveness of the staff. The working world subscale reflects the social and occupational contexts and the social feedback of the job position in the individual in the form of job satisfaction. This dimension has a social impact on the performance of nurses. The internal reliability of the questionnaire has been reported by Brooks et al. in various dimensions ranging from 0.56 to 0.85. Khani and his colleagues reported the reliability of the above questionnaire in the research between 0.75 and 0.93.

The collected data were analyzed using SPSS software version 22 by use of descriptive (mean and standard deviation) and inferential (Pearson correlation coefficient and multiple regression with simultaneous method) statistics.

RESULTS AND DISCUSSION

Data analysis showed that 24% of subjects were male and 76% were female. Also, the mean age of the experimental group was 36.65 ± 7.49. Most of the research subjects (73%) were married. In terms of education, the majority of subjects were undergraduates (48%) and graduate students (48%) and the rest (4%) were associate degree students. Finally, the highest working experience was 19 years and the least 2 years. The mean and standard deviation of spiritual careCompetence and quality of working life subscales are presented in Table 1.

The Pearson correlation coefficient was used to measure the relationship between spiritual careCompetence-and quality of working life, as presented in Table 2. As can be seen, the correlation coefficients between spiritual care Competence (evaluation and implementation of spiritual care, professionalism, personal support, referral to specialists, external attitude and communication) and quality of working life was 0.22, 0.22, 0.41, 0.22, 0.22 and 0.23, respectively, which are statistically significant.

In order to predict the quality of working life, simultaneous multiple regression analysis was used, as shown in Table 3.

Table 3 shows that, among the six subscales of spiritual care, only personal support provides a significant prediction of the quality of working life score. That is, if the personal support variable increases a standard deviation, the quality of working life score will be increased equal to 0.35 (beta value) ofstandard deviation. The remaining variables do not predict quality of working life significantly.

The results of the above table show that the modified value of R in the predictive model predicts 16% of the variance in the quality of working life score.

The aim of this study was to investigate the relationship between spiritual careCompetenceof nurses working in Shafa hospital and their quality of working life in 2016. The results of this study showed that there is a positive and significant relationship between spiritual careCompetencesubscales (assessment and implementation of spiritual care, professionalism, personal support, referral to specialists, external attitude and communication) with quality of working life. This means that with the increase in spiritual care, quality of working life increases, and with its decline, the quality of working life decreases. This finding is consistentwith the results of the researches conducted byseveral others and shows that paying attention to spirituality in the sphere of standard health care will increase the quality of working life.

For example, in a research in 2015, Usha Devi showed that components of spiritual leadership, including altruistic love, hope, faith, contact, membership, organizational commitment, and quality of working life positively affect each other. Sharp's (2016) study also showed that the existence of high levels of spirituality and ethics among employees leads to a balance between work and life as one of the indicators of the quality of working life. In

Table 1. Mean and standard deviation of spiritual careCompetence and quality of working life subscales

Variable	Number	Mean	Standard deviation
Evaluation and implementation of spiritual care	100	20.16	4.39
Professionalism	100	21.93	3.89
Personal support	100	20.22	4.60
Referral	100	11.15	2.35
external Attitude	100	14.68	2.88
Communication	100	6.96	1.90
Overall Score of Spiritual Care Competence	100	67.03	13.10
Quality of working life	100	95.10	13.16

Table 2. Pearson correlations of spiritual care Competencesubscales with quality of working life

Variable	Quality of working life	
	Correlation coefficient	Significance level
evaluation and implementation of spiritual care	0.22	0.02
professionalism	0.22	0.02
personal support	0.41	0.001
referral to specialists	0.22	0.02
external attitude	0.22	0.02
Communication	0.23	0.02

Table 3. Simultaneous multivariate regression analysis to predict the quality of working life through the variable levels of spiritual care Competence in nurses

Predictive variables	B	Standard error	Beta	T value	Significance level
Fixed number	28.13	8.96		3.13	0.002
personal support	1.007	0.30	0.35	3.30	0.001
evaluation and implementation of spiritual care	0.43	0.29	0.14	1.50	0.13
professionalism	0.29	0.37	0.088	0.79	0.43
referral to specialists	0.25	0.67	0.045	0.37	0.70
external attitude	0.24	0.56	0.05	0.44	0.66
Communication	0.34	0.72	0.04	0.46	0.64

fact, people who have a high understanding of morals and spiritual principles, try to comply with the professional principles in dealing with the needs of their family members or in dealing with hospital patients. Also, conflicts between family life and working life of nurses are sometimes considered as an important source for their performance. Nurses who experience severe conflicts have a lower quality of working life and this relationship has been studied in various researches. The spiritual well-being and the high spiritual potential in them will enable them to manage and balance the stresses from the sources of family resources and work resources.

Regression analysis to determine the predictive value of quality of working life from the six subscales of spiritual careCompetence showed that among the six subscales of spiritual care Competence, merely the personal support had a positive and significant prediction of quality of working life variance in such a way that by increasing one unit in the personal support score, a difference of 0.35 in the standard deviation of the quality of working life of the nurses is obtained. Seyed-kavousi and Nasr-Esfahani, in 2015, conducted a studyaimed at influencing the components of spiritual leadership on the quality of working life of nurses.

Table 4. Summary of the results of the predictive power of the spiritual careCompetencefrom variable of quality of working life innurses

Model	R	R-square	R
1	0.46	0.21	0.16

The findings showed that the levels of productivity with a significance level of 0.064, work outlook of 0.045 and faithfulness of 0.033 had the highest effect on quality of working life, and the remaining variables of spiritual leadership had a relatively weak linear relationship, but no significant level, with quality of working life (Koren et al 2009).

The findings of this study, consistent with the present study, have shown that structured education based on spirituality increases nurses' quality of work.In explaining the findings of the research, it can be said that the spirituality in the workplace and the familiarity of nurses with the principles of spiritual care will make nurses look better at the patients. For example, Chia states in his research that the principles related to the spirituality and familiarity of nurses ultimately correlate with the observance of their professional ethics.

In explaining the relationship between professional ethics of spirituality, they argued that nurses, with the understanding of the patient's needs, responsibility, and a strong relationship between medical staff and patients, lead to a climate of high responsibility and commitment that are indicators of the quality of working life of nurses. Bell 2006 in their research have shown that nurses who are religiously healthy tend to recognize the

spiritual needs of patients. Nurses may not be able to communicate with the patient due to lack of familiarity with the study of religious needs and lack of knowledge and skills in providing religious care, and sometimes the patient may think that the nurse is in fact intending to interfere in his religious affairs, while if a nurse is familiar with the way the patient's religious assessment is considered and is part of his/her career, as well as the ability to communicate with the patient appropriately, he/she will be able to identify the spiritual needs of the patient and provide the same as other physical and mental needs.Thus, considering the importance of the spiritual dimension in nursing for providing spiritual care to the patient along with physical and mental care and the necessity of practicing this skill in nurses, they ultimately improve their services to patients and increase their quality of work. .

LIMITATIONS

The psychological and fatigue effects of completing the questionnaires and the focus of the study on nurses, which limit the generalization of the findings to other team members, are among the limitations of this study.

CONCLUSION

In this research, the relationship between spiritual care and its subscales with quality of working life was investigated. Findings showed that there is a positive and significant relationship between the level of spiritual care, professionalism, personal support, referral to specialists, attitude towards the patient and the level of communication of nurses with quality of working life, and even the quality of life score can be predicted through the spiritual care score. In general, factors such as understanding patients and their needs, observing ethics, responsibility and professionalism in performing nursing duties are considered as spiritual care features that have a very effective role in increasing the quality of working life of nurses. Considering the importance of paying attention to spiritual needs, especially in patients with incurable diseases such as cancer, education and promotion of spiritual care in the treatment team, especially nurses, will be accompanied by the well-being and physical and mental health of the patients. On the other hand, reducing job burnout and psychological distress leads to increased spiritual well-being and quality of working life for nurses.

CONFLICT OF INTEREST

The authors have no conflict in writing this article.

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Effect of different exposure settings on the diagnosis of vertical root fractures on cone-beam computed tomography images

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ABSTRACT

By considering the importance of vertical root fractures (VRF) and the known complications of incorrect diagnoses, the present study aimed to evaluate the effect of different kilovoltage peak (kVp) and milliampere (mA) settings in cone-beam computed tomography (CBCT) on the diagnosis of VRFs in endodontically treated single-canal premolars. Eighty intact human premolars were endodontically treated and coded. The roots of half of the samples were fractured, and CBCT images were taken by using four different combinations of maximum (max) and minimum (min) kVp (60-86) and mA (6-10) settings under the same conditions. The images were randomly observed twice by an experienced oral and maxillofacial radiologist over a two-week interval. The results were analyzed by Chi-square test. In the kVp max/mA max group, none of the images were diagnostic. In the kVp max/mA min group, 62 images (77.5%) were nondiagnostic. The results of these two groups showed significant statistical differences with the other two groups ($P<0.001$). Incorrect diagnoses were equal to 22.5% in the kVp min/mA min group, and 16.2% in the kVp min/mA max group with no significant statistical differences ($P<0.4$). It seems that the best view for identifying VRFs on CBCT images in axial sections can be obtained with kVp min/mA max setting. It can be concluded that kVp min/mA max and kVp min/mA min settings are suitable for the diagnosis of VRFs. Also, a lower kVp renders a lower patient radiation dose.

KEY WORDS: CONE-BEAM COMPUTED TOMOGRAPHY, TOOTH FRACTURES, DIAGNOSTIC IMAGING

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INTRODUCTION

Vertical and horizontal root fractures (VRF/HRF) have always been regarded as diagnostic predicaments since they are difficult to detect on intraoral radiographs. Conventional and digital intraoral radiographic techniques are the most common methods for detecting root fractures. These fractures can also be assessed by routine dental examinations (Özer, 2011; Edlund et al. 2011). Endodontic therapy is one of the major causes of root fractures (Taramsari et al. 2013; Uzun et al. 2015). VRFs are more prevalent in the patients over the age of 40 years and in maxillary and mandibular premolars and in the mesial roots of mandibular molars. In molars, VRFs usually occur in a buccolingual direction in separate roots, while mesiodistal fractures are less common. In anterior teeth, the fracture usually extends in a buccolingual direction (Li et al. 2002). The most common radiographic findings in root fractures include an increased thickness of the periodontal ligament (PDL), a deep local or vertical bone loss, and peri-radicular bone loss (Shi et al. 2009). Root fractures have become diagnosable since 1896 when two-dimensional (2D) radiography was introduced to dentistry (Li et al. 2002). However, if the angle of the projection is not perpendicular to the fracture line, these fractures may remain undiagnosed (Salineiro et al. 2015).

For a more accurate diagnosis, the conventional 2D radiography can be completed with a third dimension. Cone-beam computed tomography (CBCT) was introduced in 1998 for dento alveolar imaging (da Silveira et al. 2013). Many studies have proven the efficacy of this system in the detection and diagnosis of root fractures (Taramsari et al. 2013). The old techniques used for the diagnosis of VRFs are unreliable due to the presence of nonspecific signs and symptoms which lead to unnecessary tooth extractions. Moreover, VRFs may extend from the internal part of the root in buccal, lingual, apical, or cervical directions. In these cases, the induced inflammation will lead to bone loss and may create a radiographic view similar to that of periodontal diseases. Therefore, an accurate diagnosis of VRFs prevents the erroneous extraction of treatable teeth (Taramsari et al. 2013; Chang et al. 2016).

The X-rays projected from CBCT units are influenced by different factors such as the voxel size, field of view (FOV), degree of rotation, condition of the tube, and voltage, which can change the image quality, and therefore, should be selected carefully in different diagnostic cases (Jones et al. 2015; Neves et al. 2014; Kamburoğlu et al. 2013). In 2015, Jones et al assessed the range of eleven different CBCT parameters in the diagnosis of HRFs. The effect of these factors on the diagnosis of VRFs is still a matter of debate as the studies on the diagnosis of VRFs with the use of CBCT have focused on the effect of root filling materials (Taramsari et al. 2013; Neves et al. 2014), different image modes such as resolution, zoom, speed, or different voxel sizes (Taramsari et al. 2013; Neves et al. 2014, Uzun et al. 2015; Chang et al. 2016).

Therefore, an information gap is observed among these studies. The present study was performed to assess the effect of different kilovoltage peak (kVp) and milliampere (mA) settings of a CBCT unit on the detection of VRFs in endodontically treated single-canal premolars at the dental branch of Islamic Azad University, Tehran, Iran during 2015-16. The null hypothesis was that the changes in the kVp and mA of CBCT units have no effect on the detection of VRFs.

MATERIAL AND METHODS

In this in-vitro diagnostic study, 80 extracted single-canal human premolars without any cracks or fractures were selected by using target-based sampling method. The surfaces of the teeth were cleaned of debris and tissue remnants.

Root canal treatments: Access cavities were prepared in all the teeth. A #20 K-file (Dentsply Maillefer, Ballaigues, Switzerland) was selected as the initial file. A #35 K-file (Dentsply Maillefer, Ballaigues, Switzerland) was selected as the master apical file (MAF). The root canals were widened to the #60 K-file (Dentsply Maillefer, Ballaigues, Switzerland) via step back technique. All the root canals were obturated by using #35 gutta-percha points (DiaDent, Burnaby, BC, Canada) and AH26 endodontic sealer (Dentsply DeTrey, Konstanz, Germany).

Formation of VRFs in the dental roots: In the roots of half of the teeth, a VRF was created by exerting a mechanical force by using a dental plugger, while the other half were left intact. For ensuring the presence of fractures, the teeth were stained with methylene blue dye, and then, they were coded. Also, the fracture site was covered with red wax. Afterwards, the teeth were kept in physiologic serum until the experiment.

Placing the teeth in dental sockets: The coded teeth were covered with a layer of red wax to compensate for the thickness of the PDL, and then, they were placed in the dental sockets of a dried human mandible. The longitudinal axes of the teeth were positioned perpendicular to the horizon.

CBCT imaging: CBCT images were taken of all the teeth by the use of the Rotograph Evo 3D Machine (Villa Sistemi Medicali, Milan, Italy) with four different combinations of maximum (max) and minimum (min) kVp (60-86) and mA (6-10) settings under similar conditions in terms of the distance, resolution, etc.

First group: kVp min(60)/mA min(6) Second group: kVp max(86)/mA min(6)
Third group: kVp min(60)/mA max(10) Fourth group: kVp max(86)/mA max(10)

Image observation: The CBCT images were saved and assessed by using the OnDemand3D software program (version 1.0, Cybermed Inc., Seoul, Korea). All the images (a total of 320 images in the four groups) were randomly put in the Viewer file such that the observer was unaware of the order and distribution of the images. The observer was an oral and maxillofacial radiologist experienced in interpreting CBCT images. A dental root was considered vertically fractured when a vertical radiolucent line was detected on both axial and coronal views. The results were entered in a data sheet. After two weeks, the observer reevaluated the images, and the results were registered in separate data sheets.

Statistical analysis: The cases of correct diagnoses (true positive (TP) + true negative (TN)), incorrect diagnoses (false positive (FP) + false negative (FN)), and nondiagnostic cases were determined. The data were entered into SPSS version 21 software program (IBM Co., Chicago, IL, USA) and were analyzed according to Chi-square test.

RESULTS AND DISCUSSION

This study was performed on 80 endodontically treated, single-canal mandibular premolars divided into two groups (40 teeth with and 40 teeth without VRFs), and the images were obtained by using four different combinations of mA min, mA max, kVp min, and kVp max exposure settings.The distribution of the dental specimens according to mA=6 are presented in Table 1 categorized in two columns of the real fractures and the fractures observed on CBCT images. The images of 62 dental samples (38.7%) which had been exposed with mA=6 did not have the required quality for observation and detection of fractures and were excluded from the study. This table shows that the numbers of the correct diagnoses including the positive and negative cases were equal to 72 cases (73.4%), while the numbers of the incorrect diagnoses including the positive and negative cases were equal to 26 cases (26%).

Table 1. Distribution of the evaluated teeth according to the real fractures and the fractures observed on CBCT images (mA=6)

Real vertical fracture / Detection of fracture on CBCT images	No	Yes	Total
No	46	22	58
Yes	4	26	30
Total	50	48	98

Table 2. Distribution of the evaluated teeth according to the real fractures and the fractures observed on CBCT images (mA=10)

Real vertical fracture / Detection of fracture on CBCT images	No	Yes	Total
No	38	11	49
Yes	2	29	31
Total	40	40	80

The distribution of the dental specimens according to mA=10 are presented in Table 2 categorized in two columns of the real fractures and the fractures observed on CBCT images. Images of 80 dental specimens (50%) which had been exposed with mA=10 were nondiagnostic and were excluded. This table shows that the numbers of the correct diagnoses including the positive and negative cases were equal to 67 cases (83.5%), while the numbers of the incorrect diagnoses were equal to 13 cases (16%).

The distribution of the dental specimens according to kVp=86 are presented in Table 3 categorized in two columns of the real fractures and the fractures observed on CBCT images. Images of 142 dental samples (88.7%) which had been exposed with kVp=86 did not have a suitable quality for the detection of fractures and were excluded. This table shows that the numbers of the correct diagnoses including the positive and negative cases were equal to 18 cases (100%) with no incorrect diagnoses.

The distribution of the dental specimens according to kVp=60 are presented in Table 4 categorized in two columns of the real fractures and the fractures observed on CBCT images. Images of 160 dental specimens (100%) which had been exposed with kVp=60 were nondiagnostic and were excluded. This table shows that the numbers of the correct diagnoses including the positive and negative cases were equal to 129 cases (80.6%), while the numbers of the incorrect diagnoses were equal to 31 cases (19%).

Table 3. Distribution of the evaluated teeth according to the real fractures and the fractures observed on CBCT images (kVp=86)

Real vertical fracture / Detection of fracture on CBCT images	No	Yes	Total
No	10	-	10
Yes	-	8	8
Total	10	8	18

Table 4. Distribution of the evaluated teeth according to the real fractures and the fractures observed on CBCT images (kVp=60)

Real vertical fracture Detection of fracture on CBCT images	No	Yes	Total
No	74	25	99
Yes	6	55	61
Total	80	80	160

The distribution of the samples according to the correct and incorrect diagnoses categorized by the exposure settings is presented in Table 5 which shows that when the kVp max/mA max setting was used, none of the images were diagnostic, and when the kVp max/mA min setting was used, 62 images (77.5%) were nondiagnostic. In terms of the diagnostic ability, these two exposure settings showed significant statistical differences with the other two settings (P<0.001). When the kVp min/mA min setting was used, incorrect diagnoses (FP + FN) were made with regard to 18 images (22.5%), while when the kVp min/mA max setting was used, incorrect diagnoses (FP + FN) were made on 13 images (16.2%); Chi-square test showed no significant differences between these two exposure settings (P<0.4).

The present study showed that in the mA min(6)/kVp min(60) group, the observer was able to make correct diagnoses in 62 cases (77.5%), while in 18 cases (22.5%), the diagnoses were incorrect. In the mA min(6)/kVp max(86) group, the observer was able to make correct diagnoses in 18 cases (22.5%), while no incorrect diagnoses were made in this group, and also, 62 images (77.5%) were nondiagnostic. In the mA max(10)/kVp min(60) group, the observer made correct diagnoses in 67 cases (83.7%), while the diagnoses were incorrect in 13 cases (16.2%). In the mA max(10)/kVp max(86) group, the observer was unable to make a diagnosis with regard to any of the cases, and all the 80 images were nondiagnostic. Therefore, the kVp max/mA max and mA min/kVp max showed significant differences with the other two exposure settings (P<0.001), while the mA max/kVp min and mA min/kVp min exposure settings showed no significant statistical differences according to Chi-square test (P<0.4).

Therefore, it seems that in the diagnosis of VRFs in endodontically treated teeth on CBCT images, the changes in the kVp are more effective than the changes in the mA since the diagnoses have been more realistic with the kVp min/mA max exposure setting.

In 2015, Jones et al evaluated the effect of different exposure parameters of CBCT units on the detection of simulated HRFs. The kVp was set at 90 kV, while the size of the FOV and scan speed were constant, focusing only on the mA changes. Also, two different exposure times were used. The results showed that the highest levels of sensitivity and specificity were calculated when mA max was used. Also, the highest level of positive predictive value (PPV) and negative predictive value (NPV) was achieved in the cited study at mA max with an acceptable significance level. The results of the present study are similar to those of the cited study with regards to the effect of the mA and maintaining the mA max to achieve the best image quality, although, in the above-mentioned study (Jones et al. 2015), the kVp remained fixed. The present study showed that keeping the kVp at the minimum possible level with a fixed mA results in the best image quality.

Pauwels et al (2015) evaluated the improvement in the quality of CBCT images after reducing the mA. The results demonstrated that at low levels of mA and at mA min, the image quality degrades due to increased radiographic noise and the consequent increase in the contrast-to-noise ratio (CNR). Therefore, the cited study is in line with ours in terms of confirming a better image quality when using higher mA settings. In a study by Palomo et al, a reduced kVp and a fixed mA were used to decrease the patient dose. They demonstrated that by

Table 5. Distribution of the evaluated samples according to the correct and incorrect diagnoses categorized by the studied groups

Definite diagnosis Detection of fracture on CBCT images	TP+TN (%)	FP+FN (%)	Nondiagnostic (%)	Total (%)
mA min/kVp min	62(77.5)	18 (22.5)	0(0)	80(100)
mA min/kVp max	18(22.5)	0 (0)	62(77.5)	80(100)
mA max/kVp min	67(83.7)	13(16.2)	0(0)	80(100)
mA max/kVp max	0(0)	0(0)	80(100)	80(100)

TP+TN=true positive + true negative, FP+FN=false positive + false negative

a decrease in the kVp and changing the other exposure parameters, high-quality images can be obtained at a reduced patient dose. Therefore, the results of the mentioned study are in agreement with ours in terms of the use of kVp min settings to reduce the patient dose. Kim et al (2010) compared different exposure settings of different CBCT units and concluded that to achieve the best image quality, the mA can be increased up to 10 times with a constant kVp. It seems that the overall results of the cited study are in agreement with ours.

The results of the study by Neves et al in 2014 on the evaluation of the effect of different CBCT imaging parameters on the diagnosis of HRFs with different obturation materials showed that parameters such as the resolution and obturation materials in endodontic therapy are more important; however, they limitedly investigated the effect of the mA and kVp. Therefore, although their results are not directly comparable to ours, they are indicative of the importance of factors such as the resolution. In our study, the changes in the mA and kVp directly influenced the image resolution.

Although in the diagnosis of root fractures, which is among the most difficult diagnoses in dental imaging, CBCT can be used with the minimum patient radiation dose in comparison with other imaging modalities such as medical CT, by considering the "as low as reasonably achievable" (ALARA) principle, even in such cases, it is attempted to deliver a minimum radiation dose to the patient. In the present study, we adhered to the ALARA principle by minimizing the kVp and maximizing the mA to achieve high-quality images with the best resolution and the minimum patient radiation dose. The in-vitro design of the present study was a limitation since due to the presence of the PDL around natural teeth in vivo, they may show a different behavior than those under laboratory conditions. The positive points of the present study included its novelty, the simultaneous analysis of two exposure parameters, and a large sample size.

Since the X-rays projected from CBCT units are influenced by factors such as the voxel size, FOV, degree of rotation, and tube status, which can influence image quality, future studies to assess the effect of these parameters on the detection of VRFs are recommended for the accurate and definite diagnosis of this type of root fracture.

CONCLUSION

The results of the present study showed that the best views for the detection of simulated VRFs on CBCT images in axial views are achieved with the mA max/kVp min exposure setting. Also, the patient radiation dose can be reduced by minimizing the kVp.

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Nutritional Communication

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Physical and milling properties of chickpea, *Cicer arietinum* influenced by seed characteristics

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ABSTRACT

Physical and mechanical properties of food gains is important during designing, improvement and optimization for separation and cleaning. More than 190 varieties of chickpea belonging to both *kabuli* and desi types have been released in India on the basis of yield and disease reaction, ignoring the miller's and traders' preferred processing traits. There is dearth of information about milling performance of chickpea varieties cultivated in India. The objective of this work was comparing some physical properties of two *kabuli* and two *desi* varieties of chickpea seeds (Kripa, RVKG 101 and JAKI 9218, JG 130). Although kabuli type chickpea varieties are mostly consumed as whole seed, yet they were included in the study for comparing their milling potential with desi types. Milling quality of the seeds was also found to be affected by their physical properties and varietal differences were also observed. The maximum values of seed weight, volume and bulk density among the varieties were observed in kabuli type variety Kripa. The kabuli varieties, exhibited better *dal* recovery (70.69 -71.04%) than *desi* (66.12- 66.38%) along with lesser husk content (5.32-5.77%) than desi (8-47-9.74%) due to their thinner seed coat. The *dal* recovery was positively correlated with 100-seed weight and volume but negatively correlated with true density. *Dal* recovery among *desi* varieties can be improved by reducing seed coat thickness.

KEY WORDS: PHYSICAL PROPERTIES; MILLING CHARACTERISTICS; BULK DENSITY

INTRODUCTION

Food legumes including beans and chickpea are important food crops because of their nutritional quality for supplementation of protein in vegetarian diet. They are rich sources of complex carbohydrates, proteins, vitamins and minerals (Wang *et al*., 2010). Legumes have been considered a rich source of protein throughout the world and contain approximately three times more proteins than cereals. Chickpea (*Cicer arientum* L.) is considered as the fifth valuable food legume in terms of worldwide economical standpoint. It has been used for the preparation

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of various traditional foods such as ingredient in bakery products, imitation milk, infant food formulations and meat products (Ravi and Suvendu, 2004, Ashok Kumar *et al,* 2015*;* Jukanti, *et al* 2012). Different traditional oriental foods are prepared using chickpea flour both at household and industrial levels. Dried legume seeds generally promote slow and moderate postprandial blood glucose increase. They are also a source of high-quality protein and have been known as "a poor man's meat" (Isabel and Garmen, 2003; Rincon *et al*., 1998; Taylor *et al*, 2016; Fabri *et al,* 2016; Carmo *et al,* 2017).

India is the largest producer (22.95 m tonnes), consumer (22.49 m tonnes) and importer (4.67 m tonnes) of pulses in the world (DES, 2017-18). It is also the largest pulses processor owing to poor pulse processing facilities in the major exporting countries like Pakistan (21.6%), UAE (10.6%), Algeria (11.6%) and Saudi Arab (9.5%) (DAC&FW 2017). Chickpea is the most important pulse crop in India accounting for nearly 40% (9.33 m tonnes) of the total pulse production (22.95 m tonnes) and 64% of total pulse export during 2017-18 (DAC&FW, 2017).

It is a good source of carbohydrates and protein which accounts for about 80% of the total dry seed mass (Geervani 1991, Chibbar *et al*. 2010) and constitutes an important component of diet of largely vegetarian Indian masses. Chickpea seed has high digestible protein and complex carbohydrate with low glycemic index and is relatively free from anti-nutritional factors (Muzquiz and Wood 2007, Wood and Grusak 2007; Riberro *et al*, 2017).

Chickpea protein complements cereal based diet with several essential amino acids.

Information on physical properties of byproducts is needed in designing and adjustment of agricultural machineries (Ghamari, 2012; Alexander *et al* 2017). The geometric properties such as size and shape are the most important physical properties considered during the separation and cleaning of grains (Nalbandi *et al.,* 2010; Meng *et al* 2010; Wood *et al*, 2017). In view of this, several studies have been conducted on the physical properties such as size, weight, volume, bulk density, true density of different crops. Because of varietal variability in chickpea seeds, understanding of physical properties of different varieties is necessary. Milling characteristics are important for *dhal* processing units where whole dhal recovery is an important factor for *dhal* processors. The dhal processing of legumes are mainly influenced by the size of the grain, the husk, the adherence of the husk to the seed and cotyledon texture properties. When there is a strong adherence of seed coat to the cotyledons, it hinders in the milling and whole dhal recovery is affected. The objective of this work was to study some physical properties of four varieties of chickpea seeds (Fig.1) to develop appropriate technologies in designing and adjustment of machines used during harvesting, separating, cleaning, handling and storing of them.

MATERIAL AND METHODS

Four released varieties of chickpea (kabuli and desi) were procured from Department of Plant Breeding and Genetics, R.A.K. College of Agriculture, Sehore, Madhya Pradesh. Grains of all the varieties were thoroughly cleaned and stored in airtight containers before analyzing.: Bengal Gram or Gram- in Hindi Chana are the local names and scientifically called chickpea has the botanical name *Cicer arietinum* (L.). Broadly, chickpea is categorized into two type- Desi and kabuli. Desi chana has dark seed, rough (puckered) seed coat while kabuli type is smooth and light coloured seed coat. Adherence of seed coat to the cotyledons in desi type is tight while it is loose in kabuli. Kabuli chickpeas are mainly used for table purpose as a whole grain while desi type is mainly used for making dhal. The scientific names of both type is same-*Cicer arietinum*.

All the observations were taken in triplicates and mean values are used for further analysis. The various physical properties like 100-grain weight, 100-grain volume and bulk density were determined by standard method and true

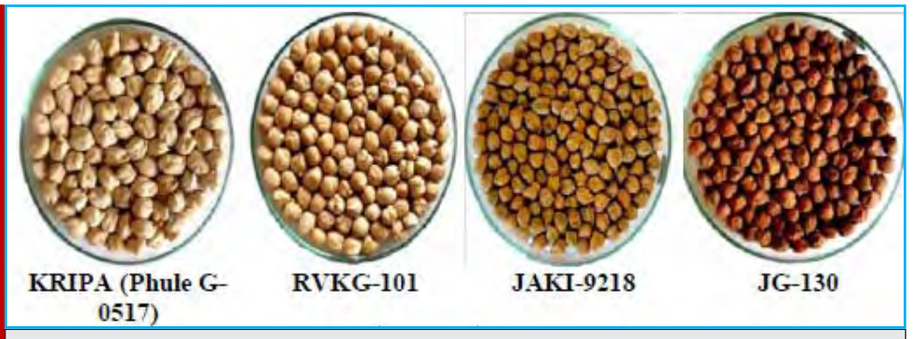

FIGURE 1. Pictorial view of grains of chickpea (*Cicer arietinum* (L.)-Kabuli and *Cicer arietinum* (L.)-Desi) varieties

Table 1. Physical characteristics of seeds of gram varieties

Variety	100 Seed weight (g)	100 Seed volume (ml)	Bulk density (g/ml)	True density (g/ml)
Kripa	56.74	70.67	0.80	0.83
RVKG 101	45.88	61.67	0.74	0.80
JAKI 9218	24.79	32.67	0.75	1.33
JG 130	25.62	33.33	0.76	0.87
Mean	38.26	49.58	0.77	0.96
SEm	0.45	1.24	0.019	0.022
C.D.5%	1.57	4.31	0.066	0.077

density by Bhattacharaya *et al.,* (1977). One kg of grain was milled in CFTRI Dhal making machine and the splits, broken grains and husk were weighed separately to estimate the Dhal recovery of grains (Agrawal and Singh 2003).

Three measurements were taken on each analysis and the results were expressed as the mean of those values ± standard deviation. Significance was tested using the Duncan's Multiple Range Test at 5% level of probability.

RESULTS AND DISCUSSION

Seed weight, seed volume, bulk density and true density characteristics differed significantly among the varieties except bulk density (Table 1 and Fig. 2). 100 seed weight ranged from 24.79 to 56.74 g; seed volume from 32.67 to 70.67 ml and both physical properties were observed highest in kabuli gram variety Kripa (Phule G-0517). All the varieties under evaluation have more or less parallel bulk density ranging from 0.75 to 0.80 ml. Highest true density was observed in desi variety of gram *i.e.* JAKI 9218 (1.33 g/ml) followed by JG 130 (0.87 g/ml) and least in kabuli gram variety RVKG 101 (0.80 g/ml). It revealed that the particles of the desi variety of gram have been densely packed. Agrawal & Singh (2003) reported that 100 seed weight varied from 23.12 to 25.15 g in chickpea varieties.

MILLING CHARACTERISTICS

Dhal recovery ranged from 66.12-71.04 per cent; Kripa registered high value (71.04%) and least was found in

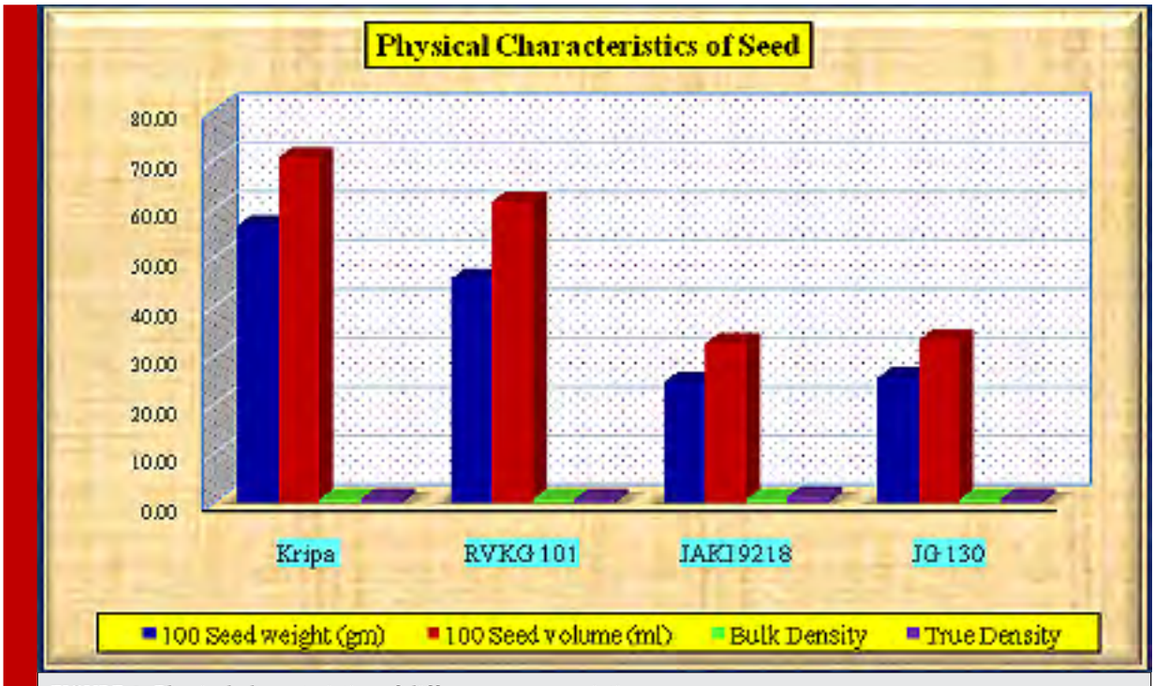

FIGURE 2. Physical characteristics of difference gram varieties

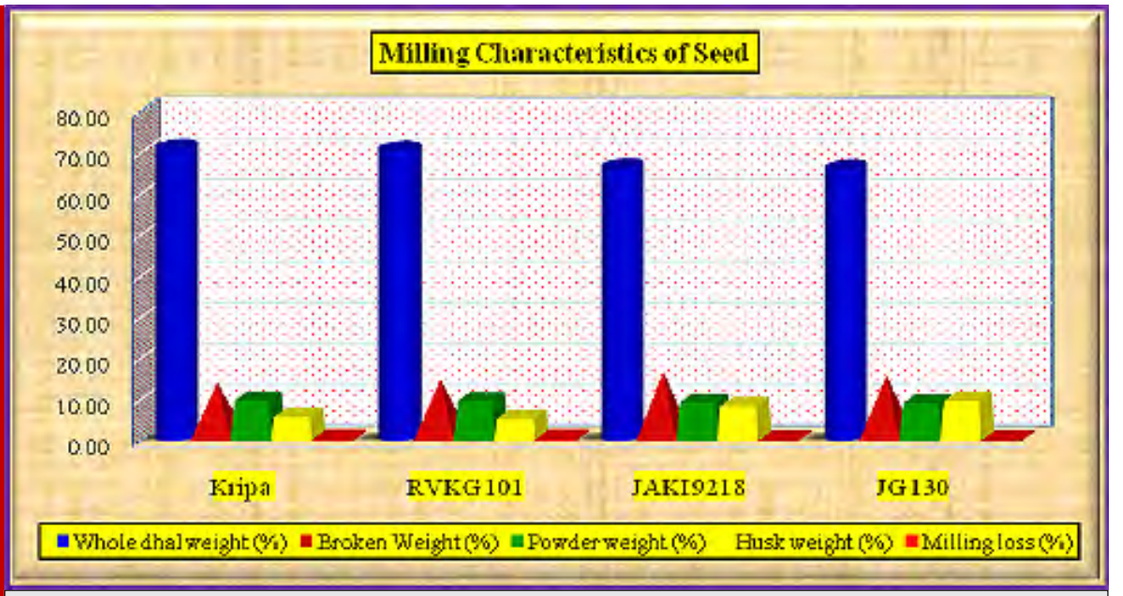

FIGURE 3. Milling Characteristics of different gram varieties

Table 2. Means of Milling Characters and cooking quality of dhal of gram varieties

Variety	Milling characters (Percent recovery- Weight basis)				
	Whole Dal	Broken Dal	Powder	Husk	Milling loss
Kripa	71.04	13.07	9.77	5.77	0.36
RVKG 101	70.69	13.93	9.66	5.32	0.40
JAKI 9218	66.38	15.72	9.32	8.47	0.11
JG 130	66.12	14.83	9.14	9.74	0.17
Mean	68.56	14.39	9.47	7.32	0.26
SEm	0.442	0.319	0.115	0.208	0.049
C.D.5%	1.530	1.106	0.399	0.720	0.172

JG 130 (66.12%) as kabuli gram have thinner testa as compared to desi and its adherence to cotyledons is also loose. Therefore, removal of testa becomes easier in kabuli gram. The brokens and powder varied from 13.07-15.72 and 9.14-9.77 per cent respectively. Amount of husk is supposed to be proportional to the thickness and mass of husk over the cotyledon. Therefore, kabuli gram varieties have lower husk recovery than desi type. The husk varied from 5.32-9.74 per cent whereas JG 130 (9.74%) recorded higher value and RVKG 101 (5.32%)

Table 3. Correlations of physical & milling characteristics in kabuli and desi gram varieties.

	100 Seed weight (g)	100 Seed volume	Bulk density	True density	Whole Dal (%)	Broken Dal (%)	Powder (%)
100 Seed Volume	0.995						
Bulk density	0.582*	0.501*					
True density	-0.634*	-0.648*	-0.288				
Whole Dal	0.971**	0.989**	0.372	-0.624*			
Broken Dal	-0.954**	-0.943**	-0.631*	0.814**	-0.899**		
Powder	0.955**	0.964**	0.419	-0.433	0.972**	-0.825**	
Milling loss	0.909**	0.941**	0.242	-0.779*	0.963**	-0.897**	0.874**

had least one. Milling loss ranged from 0.11-0.40 per cent, RVKG 101 recording higher (0.40%) values and least was found in JAKI 9218 (0.11%). There was significant difference in all the milling characteristics.

Bindu & Kasturiba (2017) reported that husk varied between 8.45-10.07 per cent and *dhal* recovery between 76.55-78.55 per cent. Less than 10 per cent of the grains were collected as brokens which is unavoidable in milling of pulses. In general bold-seeded varieties produced slightly higher per cent of powder than small-seeded varieties. Other reported a dal yield ranging from 83.1-87.8% in kabuli types and 61.3-82.6% in desi chickpea types (Shrivastava *et. al.,* 2017).

Correlation of Physical and Milling Characteristics

The whole dhal recovery is higher where 100 seed weight and 100 seed volume is more but it also caused more milling loss as well as more powder. It was also evidenced from positive correlation of 100 seed weight with powder and milling loss. It is also observed that true density has negative correlation with whole dhal recovery, powder and milling loss signifying the vice-versa relationship amongst them while, broken dhal is positively correlated with the true density (Table 3). Therefore they should have been the balanced of 100 seed weight and true density to minimize the milling losses, broken dhal to recover maximum whole dhal. 100 seed weight and true density also contributed to affect the milling properties of chickpea (Deshpande *et al.,* 1993, Nimbalkar 2000; Ravi and Harte, 2017; Alexander *et al*, 2017).

CONCLUSION

Kabuli gram (Kripa) had significantly higher seed weight of 56.74 ± 1.57 followed by RVKG 101 (45.90 ± 1.57). Desi gram had average 100 seed weight, 100 seed volume (70.67 ml), while, JAKI 9218 occupies only 32.63 ml volume. Among the varieties used in the study kabuli gram variety Kripa had the highest dhal recovery of 71.04% in overall kabuli gram have higher dhal recovery than desi gram. The milling recovery of whole dhal was also found higher in kabuli gram varieties than that of desi ones, most preferable by the millers.

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Comparative analysis of green diesel versus petro-diesel in compression ignition engine

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ABSTRACT

Biofuel based energy is likely to be promoted to reduce the dependency on fossil fuels. The most common alternative fuel is FAME biodiesel that is produced by trans esterification. But some disadvantages are also associated with biodiesel; new technologies are under investigation for the production of other biofuels. In the present study green diesel was produced by hydro processing of waste cooking oil. The performance and emission characteristics of green diesel were investigated in single cylinder CI engine. The results were also compared by the corresponding results obtained by conventional diesel fuel. The engine was tested in a series of steady state operating conditions at different load by keeping constant speeds of 1500 rev/min. The performance parameters like brake thermal efficiency (BTE %), specific fuel consumption (SFC in kg/h) and emissions parameters like HC, CO_2, CO and NOX emissions were investigated. It was observed that BTHE of green diesel is 23% and 21.76% for petro diesel at 20% load, at 40% load it is 34.24% for green diesel and 25.8% for petrol diesel at 60% load it is 35.93% for green diesel and 31.74 for petro diesel at 80% load it is 38.24 for green diesel and 31.66 % for petro diesel at 100% load it is 31.6% for both green diesel and petro diesel These results show that BTHE increases for green diesel fuels at all load except full load condition. From the results, it was found that for green diesel BSFC(kg/kwh) is1.83 for green diesel and 2.57 for petro diesel at no load condition and at full load it is 0.267for green diesel and 0.270 for petro diesel which is less as compare to petro diesel. It was observed that CO (%VOL) at load (%) 20,40,60,80,100 for green diesel are 0.06, 0.03,0.02,0.02,0.03 and for petro diesel are 0.06,0.04,0.03,0.03 and 0.04 respectively. It is observed that HC (PPM) emission at load (%) 20,40,60,80,100 for green diesel are 14, 21,20,18,25 and for petro diesel are 27, 29.25,24 and 32 respectively. It was observed that NOx (PPM) emission at load (%) 20,40,60,80,100 for green diesel are 217,511,846,1047,978 and for petro diesel are 256,533,994,1129 and 1114 respectively. It was observed that smoke (%VOL) at load (%) 20,40,60,80,100 for green diesel are 7.3,8.7,10.7,20,35 and for petro diesel are 21.2,28.5,42.2, 54.7and 69.1 respectively. From the emission results it is concluded that green diesel (i.e. CO emission hydrocarbon emissions, smoke density and nitrogen oxides emissions) are better than the conventional diesel.

KEY WORDS: GREEN DIESEL, HYDRO PROCESSING, ENGINE PERFORMANCE, EMISSIONS

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INTRODUCTION

Energy plays an important role in the growth of any country. In the transportation, agriculture, industrial sector and to fulfill the basic human needs a huge amount of energy is required. About 81% of total energy demand of the world is fulfilled by non-renewable sources - coal, petroleum-based products, and natural gas, (Prabhahar et al. 2012). But due to the limited availability and harmful effects of fossil fuels on environment, it remains an opinion that alternative fuel could be the indispensable component in the future. A few alternative sources of energy are already exists such as nuclear, wind, solar and geothermal, (Frederica et al. 2016 and Vijander et al. 2018).

But some challenges such as portability, storage, capital, efficiency etc. make them inadequate and sometimes non-viable also. For internal combustion engine, biofuel based energy is likely to be promoted to reduce the dependency on fossil fuels. The world looks towards such a bio-fuel substitute which promises to preserve the environment, and also help in the sustainable and inclusive growth of the country. In last decade biodiesel, ethanol and green diesel were emerging alternative fuels. Biodiesel occupies a prominent position in the field of alternative fuels for CI engine till date, (Prabhahar et al., 2012) invested the performance and emission characteristics of CI engine using Pungamia methyl ester (PME) diesel blend. The experimental results show that BSFC slightly increased and brake thermal efficiency decreased for pungamia methyl ester blends as compared with petroleum diesel fuel. It also found that emission of CO and smoke are decreased about 34% and 25% respectively for B20 at full load, NOx emission increased by 8.5% for B20 blend (Sotelo-Boyás et al. 2010). However, there is an issue of concern about whether is it reliable to use 100% biodiesel without any engine modification. In this regard, some manufacturer is showing caution about honoring warranties on an engine if biodiesel is used (Labecki et al. 2012).

After looking behind these facts, importance of green diesel increases as an alternative biofuel. In this context, Green diesel is emerging as most promising biofuel because it has better fuel properties as compared to conventional diesel, and biodiesel. It was found that cetane number, oxidation stability, cold flow properties and cloud point are better in case of green diesel as compared to petroleum diesel and biodiesel (Kalnes et al. 2008).

In comparison to petroleum diesel and biodiesel, the energy density of green diesel is also greater. The cetane number of green diesel lies between 80 to 99 which is much higher than diesel standard, (Fahmi et al. 2015). The density of green diesel lies in the range of 0.77-0.83 g/ml which also meets the biodiesel and diesel standard. In case of green diesel the net heating value lies between 42 and 44 MJ/kg, which is also almost comparable to the of petroleum diesel, (Kennedy et al. 2015). In the present study green diesel was produced from waste cooking oil (WCO) by hydro processing method. Hydro processing is mainly comprised of two steps hydrocracking and hydro treating (Eduardo et al. 2017). Hydro processing occurs at high temperature (about at 400°C). Due to this large amount of heat is required to transfer, therefore large amount of energy input is required in this process which significantly increase the cost of green diesel.

MATERIALS AND METHODS

Hydrocracking: It is a destructive hydrogenation process, in which heavy molecules (having higher molecular weight) broken down to lighter molecules (shown in figure 1), (Ramakanta et al. 2017). The conversion occurs at higher temperature and demands use of hydrogen gas at higher pressure to slow down the condensate chain polymerization reactions. (Toshiyuki et al. 2012). So, that high temperature and high pressure (HTHP) reactor is required for this process. The external features of the reactor are shown in figure 2. In this process sodium carbonate ($NaCO_3$) is used as catalyst to optimize the production. In this experimental investigation H_2 was supplied up to 5.5 bar pressure and sample was heated continuously until temperature rise to 400°C. To ensure the proper mixing of H_2 and feedstock stirring was also continue during this process. When the temperature rise to 400°C, heating is continued for about one hour so that complete cracking of heavy molecules take place. The maximum pressure in this process rises up to about 150 bar. This process leads to the production of C_{15}-C_{18} hydrocarbon molecules, i.e. a mixture in the boiling temperature range of diesel, which is generally known as "green diesel" and its chemical nature is similar to conventional diesel (Huber et al. 2010).

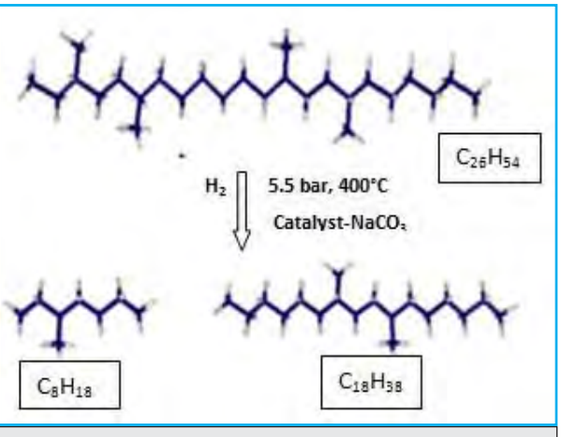

FIGURE 1. Hydrocracking on a bifunctional catalyst

FIGURE 2. True boiling point (TBP) distillation unit.

In hydro processing about 85% (by vol.) bio crude is obtained while the rest 10% escapes in the form of non-condensable gases and 4-5% remains at the bottom of collecting flask in the form of water.

Bio crude distillation: Fractional distillation is a technique by which different components are separated on the basis of difference in their boiling points (www.researchgate.net). Bio crude is a mixture of different liquid hydrocarbon, and these hydrocarbons have different properties, (Linghong et al. 2011). This process enables the separation of different hydrocarbon compounds by evaporation and subsequent condensing of vapours in the collecting tubes of the fractional receiver. The original set-up used in this experiment consist of a 15 theoretical plate column, and having 2 Lt pot flask capacity. In this process a heating mantle is used to heat up the pot flask. The temperature of the pot and column head is measured with the help of thermocouples. The reflux ratio of the condenser was maintained 15:1. The valve fitted at the portion of the TBP column allow the vapours to exit the column, and then they condensed into the condenser. From the condenser liquid proceed to the collecting tubes of the fractional receiver. An automatic turning device makes it possible to collect different fractions in the fractional receiver. The whole arrangement is shown below in figure 2

Fuel properties: The performance and emissions characteristics of fuel depend upon physical and chemical properties of the fuel (Parvaneh et al. 2017). The compatibility of the fuel to the engine also assured by the fuel properties. The fuel properties help us to explain the behavior of fuel. The important fuel properties such as API density, kinematic viscosity, net calorific value and flash point etc. were measured in laboratory. The comparison of some important fuel properties for green diesel and petro-diesel are shown in the table 1.

Table 1. Comparison of fuel properties

Fuel property	Green diesel (WCO)	Petro-diesel
Density(kg/m3)	857	830
Viscosity(centi-stoke)	3.0934	2.9
Flash point(°C)	115	70
Net calorific value KJ/KgK	38778	42878

FIGURE 3. External features of the HPHT reactor

Engine Specification: Here, to investigate performance and emissions from green diesel single cylinder CI engine is use the specifications are shown in table 2

Table 2. Specifications for diesel engine.

Engine Make	Kirloskar
Type	1 cylinder, 4 stroke Diesel
Power	5.2 kW at 1500 RPM
Stroke	110 mm
Bore	87.5 mm. 661 cc
Compression ratio	CR 17.5

Experimental setup and procedure: The steady state engine test runs were carried out on a single cylinder Kirloskar TV1 CI engine having power 5.2 KW running at 1500 RPM speed. The test engine and dynamometer were controlled by a microprocessor system equipped with data acquisition and logging. Sensors were fitted to the engine and the dynamometer, to measure relevant parameters and send the data to the control system. The

sensors measured engine load, engine speed, inlet air temperature, exhaust gas temperature, lubrication oil temperature, fuel consumption and the cooling water temperature. The engine was tested in a series of steady state operating conditions at engine speeds of 1500 rev/min and engine loads of 0%, 20%, 40%, 60%, 80% and 100% load. At each of these conditions the engine was allowed to settle and warm up for about 4-5 minutes.

During the experiments, the cooling water and engine oil temperatures were constant at about 27°C, and the laboratorytemperature was within 25-30°C. The gas analyzers and the measuring equipment were calibrated before each experiment. Experiments were initially carried out on the engine using diesel as fuel in order to provide base line data. The green diesel were prepared and made to run on the engine. The engine was started using neat diesel and allowed to run for at least 10-15 minutes before taking observations. After engine conditions stabilized and reached to steady state, the base line data were taken. Load was varied (Zero load & full load condition) using the alternator load bank and the same was recorded. Gaseous emissions, fuel consumption were also recorded from the respective sensor.

The quantitative analysis of exhaust gas was carried out by using AVL DIGAS ANALYSER and SMOKE METER. In the present study emissions of CO, HC, CO_2 and NO_X are taken into account for emissions analysis, (Kasiraman et al. 2012).

RESULTS AND DISCUSSION

The variation of Break Thermal Efficiency (BTHE) with different loads for both fuels is shown in figure 4. The BTHE of the engine increases with increasing load for diesel and green diesel both. It is observed that BTHE increases due to increase in power developed with increasing the load. It is observed that BTHE of green diesel is 23% and 21.76% for petro diesel at 20% load, at 40% load it is 34.24% for green diesel and 25.8% for petrol diesel at 60% load it is 35.93% for green diesel and 31.74 for petro diesel at 80% load it is 38.24 for green diesel and 31.66 % for petro diesel at 100% load it is 31.6% for both green diesel and petro diesel. The results show that BTHE increases for green diesel fuels at all loads except full load condition at full load remains almost same. The BTHE increases with load because as load is increases, the peak temperature increases which promote the better combustion (Phoona et al. 2017).

The higher BTHE of green diesel may be attributed to its high density and combustion phasing relative to TDC (Muralidharn et al. 2011). From figure it is found that a large part of fuel burns just after the TDC. It is favorable with concerned to better performance that large fraction of fuel burns in the proximity of TDC. These two factors believed to supersede the effect of its lower heating value.

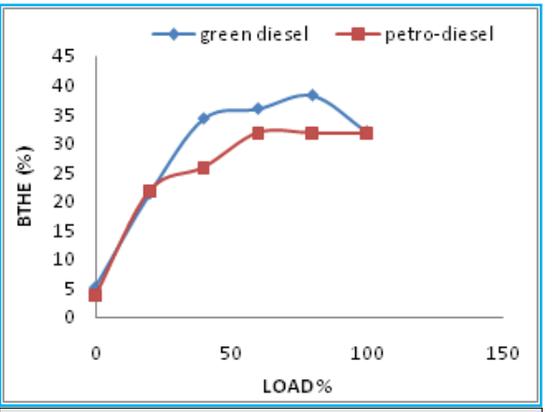

FIGURE 4. Variation of BTHE at different load

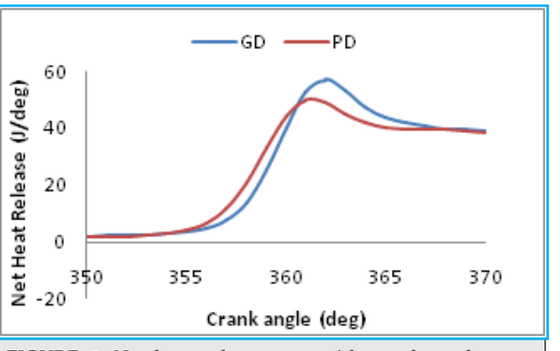

FIGURE 5. Net heat release rate with crank angle near the TDC

From the results, it was found that for green diesel BSFC (kg/kwh) is 1.83 for green diesel and 2.57 for petro diesel at no load condition and at full load it is 0.267 for green diesel and 0.270 for petro diesel which is less as compare to petro diesel. Figure 6 illustrates the brake specific fuel consumption (BSFC) of the fuel samples

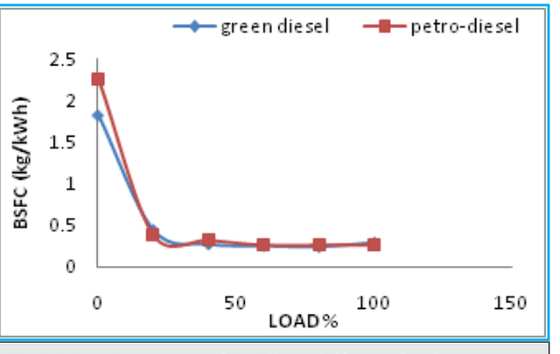

FIGURE 6. Variation of BSFC at different load

tested with respect to different loads. BSFC is defined as the fuel consumption rate divided by its corresponding engine power output, (Prabhahar et al. 2012) The low BSFC of green diesel is due to it is high calorific value (John et al. 2015)

Figure 7 illustrate that mechanical efficiency slightly increases when engine was run on green diesel as compare to petro-diesel. The reason behind this may be a little higher viscosity of green diesel as compare to petro-diesel. As viscosity increases, the lubricating capacity also increases which results in the less frictional losses, (Saravanan et al. 2010). This behavior of green diesel was shown at all load conditions.

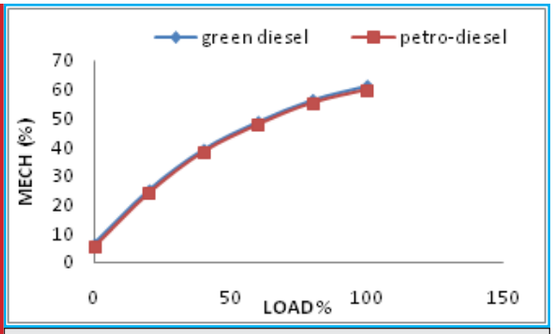

FIGURE 7. Variation of MECH at different load

From the result it was observed that CO (%VOL) at load (%) 20,40,60,80,100 for green diesel are 0.06, 0.03,0.02,0.02,0.03 and for petro diesel are 0.06,0.04,0.03,0.03 and 0.04 respectively. The variation of CO emissions with loads is shown in figure 8. The figure shows that CO emission decrease with engine load. CO is a product of incomplete combustion, thus at higher engine loads, higher combustion temperature promotes more complete combustion and hence less CO emission. High CO emissions were observed at lower loads. In addition, at lower loads CO emissions are increased due to incomplete combustion (Mathur 2014).

It can be seen that CO emissions for green diesel were lower as compared to petroleum diesel which might be due to its short combustion tail during the late stage as shown in pressure vs. crank angle curves in figure 9. The lower CO emissions for green diesel indicate the better combustion than petroleum diesel at all load stage. This shows that there is better utilizations fuel energy in case of green diesel.

From the experiments it was observed that HC (PPM) emission at load (%) are 20,40,60,80,100 for green diesel are 14, 21,20,18,25 and for petro diesel are 27, 29.25,24 and 32 respectively The variation of HC emissions with loads is shown in Figure 10. The figure shows that HC emission decrease with engine load but slightly increases

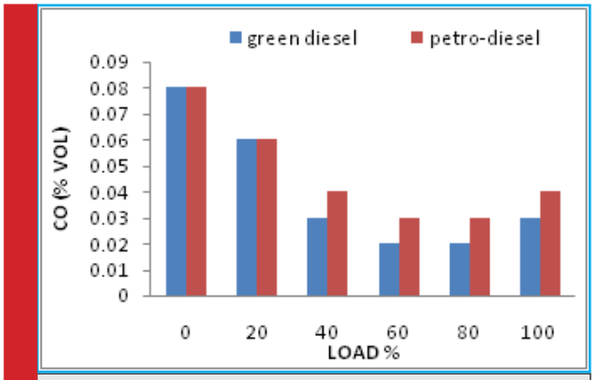

FIGURE 8. Variation of CO at different load

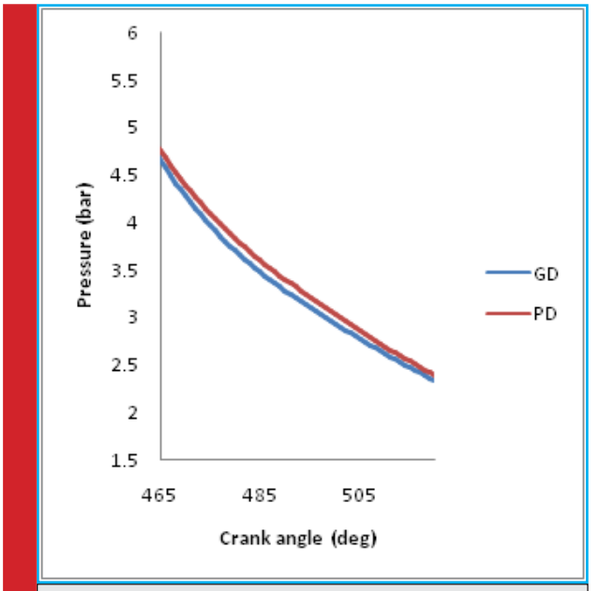

FIGURE 9. In cylinder pressure variation with crank angle in large view (last stage of combustion)

at full load. HC is a product of incomplete combustion, thus at higher engine loads, the higher combustion temperature promotes more complete combustion and hence less HC emission (Ganeshan 2006). It can be seen that there was decrease in ppm of HC when the diesel engine fuelled with green diesel at all loads stage. The results show that HC emissions decreases by 25-30% in case of green diesel at all load stages except idle stage. That is due to the better air-fuel mixing in case of green diesel. The high peak of net heat release rate is an indication of better combustion. From above observations we can says that there is less HC emissions problem with green diesel. These results also show the better combustion quality of green diesel.

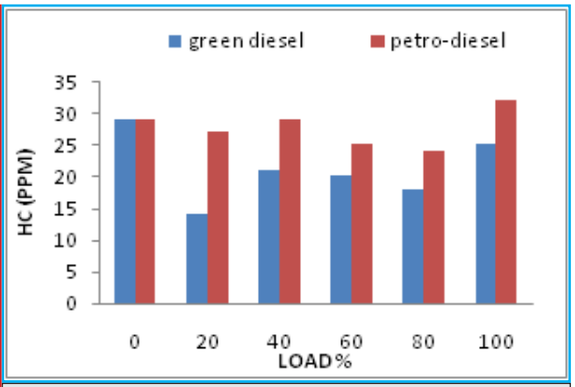

FIGURE 10. Variation of HC at different load

From the experiment it was observed that NO_x (PPM) emission at load (%) 20,40,60,80,100 for green diesel are 217,511,846,1047,978 and for petro diesel are 256,533,994,1129 and 1114 respectively. Figure 11 shows the oxides of nitrogen (NOx) emission of different fuel samples tested at different load conditions. In general, the formation of NOx is affected by the peak flame temperature, the high burning gas temperature, the ignition delay and the content of nitrogen and oxygen available in the reaction mixture (Imdadul et al. 2017).

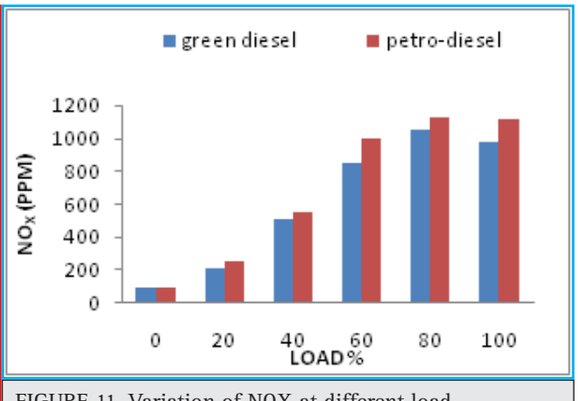

FIGURE 11. Variation of NOX at different load

It was observed that as the load increased, the temperature in the cylinder also increased and thus higher absolute NO_x (ppm) formation. The results obtained from experiment also shows same trend for both fuels. The emissions of NO_X from green diesel is very less as compare to conventional diesel. The emissions of NO_X in ppm at idle stage are almost same for green diesel and conventional diesel. The results shows that NO_X emissions decreases about 10% in case of green diesel as compare to petro-diesel. This may be due to the less availability of nitrogen and oxygen mixture content, (Aatola et al. 2008).

From the results it was also observed that CO_2 (%VOL) at loads (%) 20,40,60,80,100 for green diesel are, 2.7, 3.5, 5.1, 5.9, 6.3 and for petro diesel are 2.7, 3.4, 4.8, 5.7 and 6.3 respectively The experimental results of CO_2 emissions are illustrated in figure 12. There is a general trend that as emissions of CO decreases then CO_2 emissions increases, (Ganeshan 2006). Because decrease in CO emissions indicates that combustion quality is improved, that results increase in the CO_2 emissions. The results show that emissions of CO_2 increases slightly for green diesel as compare to petroleum diesel.

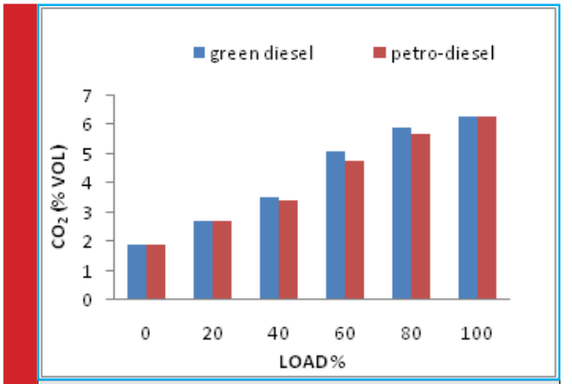

FIGURE 12. Variation of CO_2 at different load

From the present data it is apparent that that smoke (%VOL) at load (%) 20,40,60,80,100 for green diesel are 7.3,8.7,10.7,20,35 and for petro diesel are 21.2,28.5,42.2, 54.7and 69.1 respectively. Smoke formation occurs primarily in the fuel rich zone of the cylinder, at high temperature and pressure. Smoke formation can be controlled by applying partially oxygenated fuel, which reduces locally over-rich regions (it is the region where the fuel is more than required). When the engine runs under-load at WOT (Wide open throttle), maximum fuel is injected to supply maximum power, which is a rich mixture. Thus at higher load, an increased fuel-air ratio fuel is injected in large quantities and much of the unburnt fuel escape with the exhaust resulting in maximum smoke emission, (Rajput 2012).

Other reason may be the difference in chemical structure of the green diesel. Under less than 75% load condition, smoke formation was less and it increases with the increase in load. Also, the difference in smoke level of the green diesel and diesel is more significant at all load. The smoke reduction with pure green diesel was around 70-80% as compared diesel. Also, The average smoke density in percentage were 17.2, 21.2, 28.5, 42.2, 54.7 and 69.1 for the diesel while for green diesel the smoke

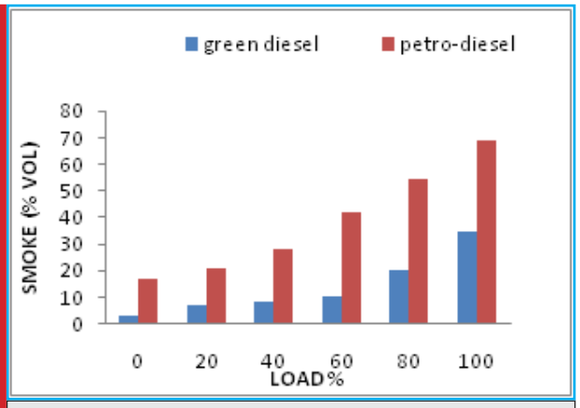

FIGURE 13. Variation of smoke at different load

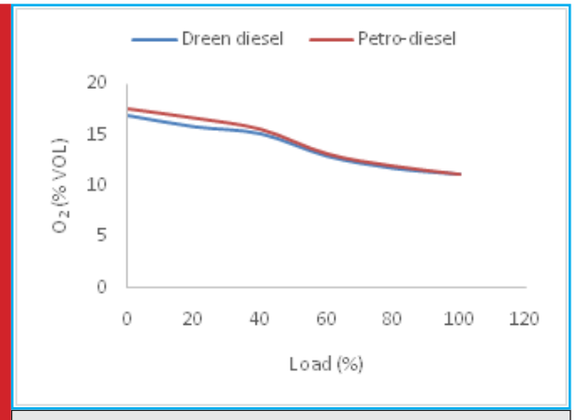

FIGURE 14. Variation of oxygen emissions with load

densities were 3.5, 7.3, 8.7, 10.7, 20 and 35 at no load, 20, 40, 60, 80 and 100% load condition. The green diesel have a narrow range in regards to length of hydrocarbons which promote the air – fuel mixing (Kalens et al. 2008). The better air – fuel mixing improved the combustion quality which causes low smoke emissions.

From the results it is observed that O_2 (%VOL) at load (%) 20,40,60,80,100 for green diesel are 15.8, 15.1, 12.9, 11.7, 11.1 and for petro diesel are 16.7, 15.6, 13.2, 12and 11.2 respectively. Figure 14 shows that emissions of oxygen slightly decreases for green diesel as compared to petro-diesel. That might be due better combustion of green diesel. That results shows the better performance and emissions characteristics of green diesel. The results are as per expected because carbon dioxide emissions are high for green diesel fuel and follows general trend, (Saravanan et al. 2010). The variation of oxygen emissions with load for green diesel and petro-diesel are shown in figure below.

CONCLUSION

The performance and emission characteristics of green diesel were investigated by varying load and keeping compression ratio fixed in CI engine. The results are also compared by the corresponding results obtained by conventional diesel fuel. The following results are concluded from experimental work and analysis: The performance results shows that brake thermal efficiency and brake specific fuel consumption both are better in case of green diesel fuel. The emissions results of green diesel (i.e. hydrocarbon emissions, smoke density and nitrogen oxides emissions) are too much better than the emissions results of conventional diesel. So, green diesel is better than conventional diesel as per pollution concern. The fuel properties like density and kinematic viscosity of both fuels are almost comparable so, there may not be cold starting and fuel injection problem.

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Sucralose and maltodextrin-An altrernative to low fat sugar free ice-cream

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ABSTRACT

In recent past; change in lifestyle has paved the way for many diseases like obesity and diabetes with huge demand for reduced calories low fat sugar free products. Therefore, to meet the demand of the current market a low-fat sugar free ice-cream was prepared. The per kg final formulation provided by response surface methodology (RSM) to prepare desirable low-fat sugar free ice cream is 751ml skim milk, 31gm cream, 65gm SMP, 18.2ml sorbitol, 30gm maltodextrin, 70gm polydextrose, 12.5gm WPC-70, 0.009gm sucralose and 5gm emulsifier. RSM was used to investigate the influence of predictor variables (sorbitol and sucralose) on ice-cream color and appearance, body and texture, flavor and taste and melting resistance. 1-3% level of sorbitol and 0.005-0.015% sucralose was varied. Finally, 2.33% sorbitol and 0.009% sucralose were obtained as optimum levels. Available literature indicated levels of Maltodextrin as fat replacer was found effective at 3% and polydextrose at 7% in providing bulk to the ice-cream without affecting the sensory attributes and Physico-chemical parameters. Low calorie sweeteners sorbitol and sucralose on comparison to sucrose were effective in imparting sweetness without adding calorie to the prepared ice-cream. Prepared ice-cream was effective in mimicking mouth feel of full fat ice-cream. The Optimized ice-cream was analyzed for various parameters including Total solids, fat, protein, moisture, titrable acidity, ash, carbohydrate and overrun as 30.44, 2.40, 4.69, 69.56, 0.135, 1.34, 22.01 and 61.6g/100 g respectively. The total plate count (TPC) of freshly prepared ice cream was 1.5×103, yeast &tmould was absent and coliform count was found nil. The calorific value of ice-cream was reduced from 200 kcal to 116.74 kcal/100 gram

KEY WORDS: ICE CREAM, LOW FAT, RSM, SORBITOL, SUCRALOSE

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INTRODUCTION

Ice-cream is a frozen dairy product obtained by appropriate mixing and processing of cream and other milk food along with sugar and flavor, in presence or absence of stabilizer or color and with absorption of air during freezing (De, 2015). Ice-cream is loaded with high fat, protein and carbohydrate which add to its calorific value. On average the calorific value of Ice Cream is 200 kcal/100g (Pinto and Dharaiya, 2014). The fat rich diet has been recognized as alarming reason for high energy intake, positive energy balance and a major cause of obesity. These troubled eating habit coupled with lack of physical activity and stressed life has resulted in various health issues across globe especially among the natives of Asian continent (Thomas et al.,1992; Siggaard et al.,1996; Wylie-Rosett, 2002). In India alone about 48.14 percentage residents consume high fat diet (Chatterjee, 2007). Survey of Indian Council of Medical Research affirmed that in urban locality 49 percentage of female and 36 percentage of male population suffers from obesity. Obesity can be explained as a store of several other health issues like hyperlipidemia, hypercholesomia, diabetes, hypertension, cancer and gallstones. WHO has estimated a world-wide heavy upsurge in the diabetic cases, expected to raise by 57.2 million till 2025 in sharp contrast to the diabetic cases of 19.4 million as in 1995. India has already become the home to diabetes with 69.1 million patients and which is expected to surge by 79.4 million till 2030, (Mohan et al., 2010 and Kaveeshwar et al., 2014 Sonwane and Hembade, 2014 Tripathy et al., 2017).

In recent years, with growing demand of low calorie, low fat products the dairy industry has come up with numerous low-fat and fat-free ice cream products. With increased consumer attentiveness for improved and efficient foods various new technologies has come to the fore for manufacture of such products. Ice cream is one of the most served and loved desserts but is high in fat content (10–14%) and sugar (30%) therefore; formulating its low fat and sugar free version will serve in good cause for reducing the extra-calorie intake and make it healthier. Removal of sugar to prepare sugar free ice cream counts for some defects like adjustment in total solids and loss in freezing point depression. The prior can be compensated by using bulking agent like polydextrose and later by adding freezing point depressant like sorbitol (Tharp, 1991, Pinto and Dharaiya, 2014; Patil and Banerjee, 2017).

Bulking agents impart creaminess, smoothness, improve texture and provide a mouth feel and protection against temperature fluctuation to please customers, (Goff and Jordan, 1985). Sorbitol or mannitol containing Sugar-free products contain low glycemic index (GI). Low GI foods are important in dietary management as they allow slow movement of glucose into the blood resulting in very low rise in blood glucose and insulin levels. The use of artificial sweeteners in food are very useful as it imparts sweetness without adding sugar which result in calorie reduction, helps in weight loss and diet control. Artificial sweeteners are considered safe as some of them are not digested by our body like sucralose. Fat has a major part in the structure of ice cream and its removal from the ice cream lowers the characteristics of the product which requires a substitute to be added. The substitute on addition preserves the characteristics of ice cream known as fat replacer. Fat mimetics are indigestible, low calorie and posses' dissimilar chemical structure than fat. These are of different types of carbohydrate or protein-based. Physiological characteristics and desirable eating qualities like viscosity, mouth feel and appearance of fat are copied by these fat mimetics.

In the wake of outlined research prospect, the present study was carried out with the comprehensive objective to prepare low fat sugar free ice-cream and to understand the role of various artificial-sweeteners, fat replacers and bulking agenton Physico-chemical, textural and microbiological properties of ice-cream.

The major highlight of the study is that the calorific value of the prepared ice-cream is 116.74 kcal/100 gmuch lower as compared to average calorific value of 197.13 kcal/100 gfor an average ice cream.

MATERIAL AND METHODS

Ice-Cream Formulation: The Central Composite Rotatory Design (CCRD) of Response Surface Methodology (RSM) was used to obtain 13 different combinations of sorbitol and sucralose to prepare the experimental low-fat sugar free ice-cream. Identical composition of Maltodextrin, Polydextrose and WPC were used in all the thirteen different combinations. Fat and total solid were used as per the standards given by Food Safety and Standards Authority of India (FSSAI), 2006 (Table I). Ice-cream with 13 different compositions was processed using the method given by Arbuckle, (2013).

In this table (Table I) CCRD design is presented with 13 different experimental runs of independent variables (Sorbitol) Factor A and (Sucralose) Factor B and its coded level. All the responses are shown with sensory score allocated by the judges.the independent variables and their decoded or actual levels are depicted below as note.

Note:

S. No.	Variables Actual	Coded	Levels -1.41	-1	0	+1	+1.41
1.	Sorbitol	A	0.58	1	2	3	3.41
2.	Sucralose	B	0.0029	0.005	0.01	0.015	0.017

Table I. Experimental Design Matrix and Sensory Score of the Low-Fat Sugar Free Ice-cream

Std	Run	Factor A Sorbitol (%)	Factor B Sucralose (%)	% Maltodextrin	% Polydextrose	% WPC	% Fat	% Total Solids	Color and Appearance	Body and Texture	Flavor and Taste	Melting Resistance
10	1	0	0	3	7	1.25	2.5	28.14	8.5	7.5	7.5	7.89
6	2	1.41421356	0	3	7	1.25	2.5	28.14	7.44	7.5	7.5	7.25
1	3	-1	-1	3	7	1.25	2.5	28.14	7.38	6.5	7	7.5
2	4	1	-1	3	7	1.25	2.5	28.14	7.5	7.03	6.37	6.75
7	5	0	-1.414213562	3	7	1.25	2.5	28.14	6.5	7.33	6.81	7.01
13	6	0	0	3	7	1.25	2.5	28.14	8.01	8.25	8.25	7.5
12	7	0	0	3	7	1.25	2.5	28.14	8.05	8.7	8.15	8
9	8	0	0	3	7	1.25	2.5	28.14	7.95	8.5	7.98	7.69
3	9	-1	1	3	7	1.25	2.5	28.14	7.5	6.5	7.25	7.25
4	10	1	1	3	7	1.25	2.5	28.14	7.38	7.16	7.34	7.14
8	11	0	1.414213562	3	7	1.25	2.5	28.14	7.25	6.88	7.01	7.01
11	12	0	0	3	7	1.25	2.5	28.14	8.25	8.5	8.5	8
5	13	-1.41421356	0	3	7	1.25	2.5	28.14	7	7.16	7.31	6.98

The sensory analysis was done by the panellist on sensory score card.Numerical scores were allocated for color and appearance, body and texture, flavor and taste and melting resistance of the ice cream based on the nine - point's hedonic scale. The numerical score was used as an indication of the quality. These scores were further used in the RSM to obtain the optimum levels of sorbitol and sucralose. For obtaining the optimum levels of artificial sweeteners to be used maximum desirability value solution was preferred. Response surface plots were used to explain the effects of independent factors on the response variables. The study was replicated three times to minimize the chance of errors and results were statistically analyzed. All the responses were numerically optimized by maximizing within the critical limits using Design Expert Software. The independent factors were set within the experimental range.

The detailed processing of final ice-cream is mentioned in Fig I.

Physico Chemical Analysis: The prepared ice-cream was subjected to Physico-chemical analysis. Fat content; total solids, total ash, moisture content and titrable acidity of the ice-cream were calculated using FSSAI Laboratory Manual 1. Protein content of the frozen mixture was determinedbyKjeldahl method.Total Carbohydrate was calculated from the difference of proximate composition. Overrun was calculated as per the method of Sommer (1951).For Microbiological analysis, Total Plate Count was performed as per IS 5402:2012, Yeast and Mold was performed as per IS 5403:1999 (RA2013) and Coliforms Test was done using IS 5401(P1):2012. Calorific value of ice cream was calculated using formula given below

Calorific Value = % Carbohydrate × 4 + % Fat × 9 + % Protein × 4

RESULTS AND DISCUSSION

The sensory data of the experimental Ice cream along with the CCRD matrix is summarized in Table I. The obtained sensory data was subjected to evaluation by quadratic model and the statistical significance of the terms in the regression equation was examined by analysis of variance (ANOVA) as presented in Table II.

The color & appearance score of the experimental ice cream ranged from 6.5 to 8.5 (Table I). The minimum score was obtained for 2% sorbitol and 0.002929% sucralose treatment combination and the maximum score was obtained for 2% sorbitol and 0.01% sucralose treatment combination. Lack of fit was not significant,

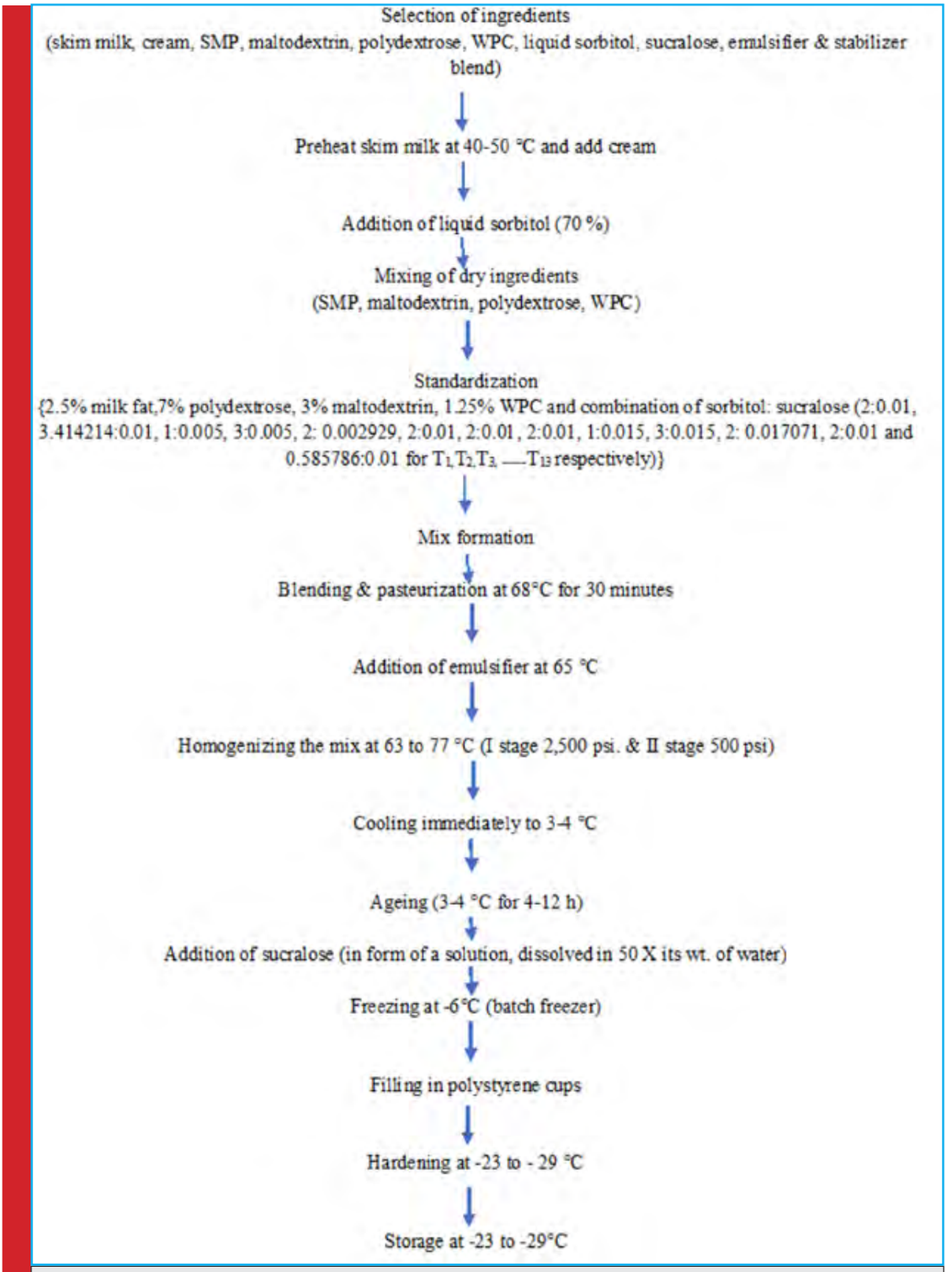

FIGURE 1. Flowchart for preparation of low-fat sugar-free ice-cream

Table II. Regression Coefficients of Polynomial Model for Sensory Response of Low Fat Sugar Free Ice cream

Factor/ Term	Color and Appearance	Body and Texture	Flavor and Taste	Melting Resistance
Intercept	8.15	8.29	8.03	7.82
A-Sorbitol	0.078	0.21	-0.034	-0.060
B-Sucralose	0.13	-0.063	0.19	0.018
AB	-0.060	0.033	0.18	0.16
A2	-0.37	-0.59	-0.38	-0.33
B2	-0.54	-0.70	-0.62	-0.38
R2	0.8036	0.8004	0.8237	0.8058
APV	5.907	5.279	6.581	5.778
Lack of Fit	NS	NS	NS	NS
P value	0.0203**	0.0214**	0.0143**	0.0196**

(F_{cal}>F_{tab}) confirming the significance of model at 5 per cent level of significance. The model terms for sorbitol and sucralose was highly significant in color and appearance score at linear as well as quadratic level. The coefficient estimates of color and appearance score model shows that levels of different variable had significant effect on the score. The linear and quadratic effect of Sorbitol and Sucralose is expressed in graph obtained in Fig II (a). The response surface equation derived for predicting color and appearance score could be given as:

$$+8.15+0.078*A+0.13*B-0.060*AB-0.37*\ A^2-0.54*\ B^2$$

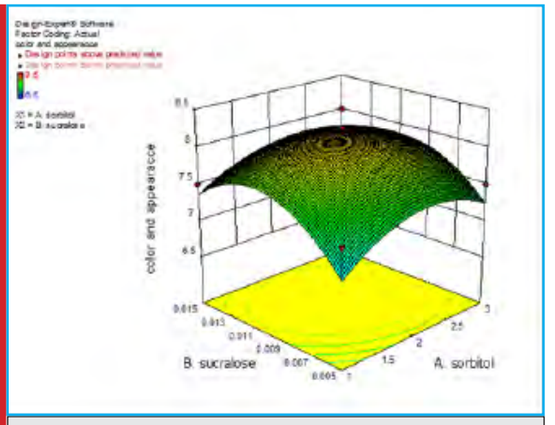

FIGURE 2. (a) Response surface 3D plot showing effect of sorbitol and sucralose on color and appearance

The score for Body Texture of the experimental ice cream ranged from 6.5 to 8.5 (Table I). The minimum score was acquired for 1% sorbitol and 0.05% and 0.015% sucralose treatment combination and the maximum score was acquired for 2% sorbitol and 0.01% sucralose treatment combination. The regression coefficient data presented in Table II revealed that the coefficient of determination was 0.8004. Further, it can be figured that the significance of model at 5 per cent level of significance as Lack of fit was not significant, (Fcal>Ftab). The model terms for sorbitol and sucralose was highly significant in body and texture score at linear as well as quadratic level in Fig II (b). The response surface equation estimating Body and Texture score could be given as:

$$+8.29+0.21*A-0.063*B+0.033*AB-0.59*\ A^2-0.70*\ B$$

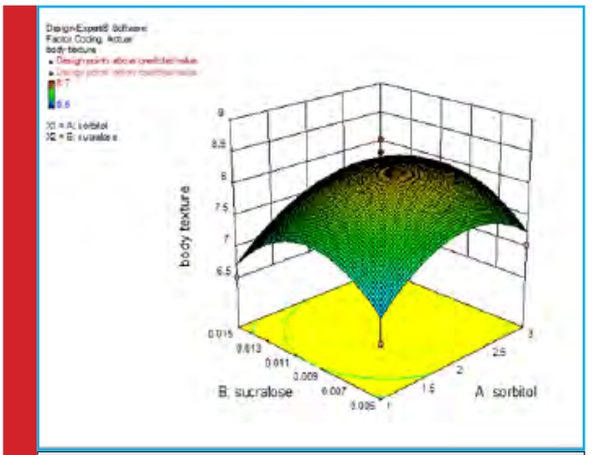

FIGURE 2. (b) Response surface 3D plots showing effect of Sorbitol and Sucralose on body and texture

The Flavor & Taste score of the experimental ice cream ranged from 6.75 to 8 (Table I). The minimum score received was for 3% sorbitol and 0.005% sucralose treatment combination and the maximum score received was for 2 % sorbitol and 0.01 % sucralose treatment combination. The coefficient of determination was 0.8237 as shown in regression coefficient data presented in Table II. Rathod et al. (2013) has also reported almost similar result (i.e. 0.89) while optimizing levels of artificial sweetener for preparation of sugar free ice-cream. The Lack of fit was not significant, (Fcal>Ftab) confirming the significance of model at 5 per cent level of significance. The model terms for sorbitol and sucralose were highly significant in flavor score at linear level in Fig II (c). The response surface equation for Flavor and Taste score could be given as:

$$+8.08-0.034*\ A+0.19*B+0.18*\ AB-0.38*\ A^2-0.62*\ B^2$$

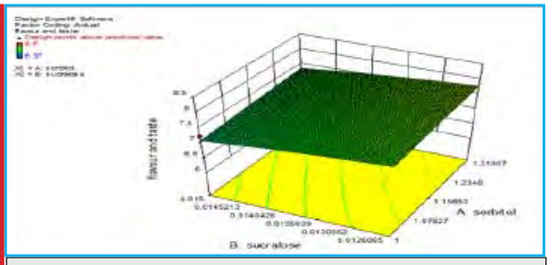

FIGURE 2. (c) Response surface 3D plots showing effect of Sorbitol and Sucralose on flavour and taste

The melting resistance score of the experimental ice cream ranged from 6.37 to 8.5 (Table I). The minimum score was for 3% sorbitol and 0.005% sucralose treatment combination and the maximum score was for 2% sorbitol and 0.01% sucralose treatment combination. The coefficient of determination was 0.8058 as depicted in regression coefficient data presented in Table II. The model terms were significant at 5 per cent level of significance as the Lack of fit was not significant, ($F_{cal}>F_{tab}$). The model terms for sorbitol and sucralose was highly significant in melting resistance score at linear and quadratic level in Fig II (d). The Melting Resistance score can be calculated by the response surface equation mentioned below:

$$+7.82-0.060*A+0.018*B+0.16*AB-0.33* A^2-0.38* B^2$$

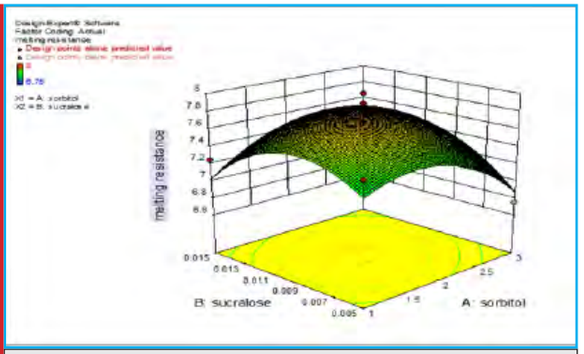

FIGURE 2. (d) Response surface 3D plots showing effect of Sorbitol and Sucralose on melting resistance

Physico-chemical Analysis

Ice cream was prepared with the finally optimized formulations and was subjected for analysis of parameters like fat content, total solid, protein, total ash, moisture, titrable acidity, overrun and carbohydrate. The total fat content was found to be 2.4 g / 100g. As per the FSSAI standards, the total fat content in low fat ice cream should not be more than 2.5 percent. The total solid content (TSC) in the final formulated ice-cream was reported to 30.44 g / 100g which is as per the standards of FSSAI. However, reduction in TSC can be attributed to the fact that sucrose is replaced by high intensity artificial sweeteners required in very small amount as against sucrose which is used in concentration of 15 % in normal ice cream. The minor diminution in the total solid content can be attributed to the moisture content of dry ingredients i.e. polydextrose and maltodextrin as reported by (Pinto and Dharaiya, 2014).

The Protein content was found to be 4.69 g /100g. The protein content as per FSSAI Standards should not be less than 3 percentages. The final formulated ice cream was enriched with 1.25 % WPC-70. Whey Protein Concentrate (WPC) is rich in essential amino acids such as lysine, tryptophan, cysteine and methionine. Whey solids possess nutritionally and functionally biologically active superior proteins (Steinholthand Holth, 1999)and their incorporation in the ice cream mix would result in superior product in terms of increasing the protein content of the ice cream (Vulnik, 1995). The total ash and total moisture in the ice cream was recorded to be 1.34 g/100g and 69.56 g / 100g respectively. The increase in the ash content is due to addition of fat replacer (Murtaza et al., 2004).

According to FSSAI standards Titrable acidity of ice cream should not be greater than 2.2g/100 g and the titrable acidity in the ice cream was found to be 0.135 g / 100g. The decreased level of fat per cent in ice cream affects titrable acidity content of ice cream.

As level of fat content decreases Titrable acidity also decreases (Chavan et al.,2014).The Over-run in the ice cream was estimated to be 61.6%. In a similar study by Pon et al. (2015)on textural and rheological properties of sugar free ice cream they calculated an average overrun as 65.12%. This estimated value was in concordance with the present study. However; it is also cited that high overrun is difficult in batch-type freezer (Dervisoglu et al. 2005; Guven et al. 2003).The literature also establishes that increased sugar concentration has important role in high overrun (Akin et al. 2007; Guven and Karaca 2002). Schmidt et al.(1993)in his study also indicated that carbohydrate-based fat replacers like polydextrose and maltodextrin, used in the formulated ice cream under study has led to decrease overrun and; thisstudy was further supported by Jamshidi et al. (2012).The Carbohydrate in the ice cream was found to be 22.01 g / 100g.Hence, it can be stated that the physicochemical analysis of formulated "Low Fat Sugar Free Ice Cream" satisfies the standards and claims for low fat sugar free ice-cream.

The final product obtained was subjected to microbiological analysis for parameters like Total Plate Count, Yeast and Mould and Coliform. The Total Plate Count in the optimized low-fat sugar free ice cream was cal-

culated to be $1.5*10^3$ cfu / gm. The permissible standard for TPC as laid down in FSSAI for ice cream is $2*10^5$ cfu/ gm. The Yeast and Mould in the optimized low-fat sugar free ice cream was absent. Yeast and Mould in ice cream should be absent as per the standards laid down by FSSAI. The Coliform count in the optimized low-fat sugar free ice cream was found to be NIL. The permissible standards for Coliforms as laid down in FSSAI for ice cream is 50cfu / gm.

In the present study, 13 treatments of low fat sugar ice-cream were prepared with fat being replaced by maltodextrin at 3%.Removal of fat causes such body and textural problems as coarseness and iciness, crumbly body, shrinkage and flavor defects (Berger 1990; Marshal and Arbuckel 1996). Samples with reduced fat and sugar showed a higher intensity of bitter aftertaste, adhesiveness, and firmness, and lower intensity of creaminess (Cadena et al. 2012). In a similar study, Verma (2002) observed an increasing trend in the sensory score with increase in maltodextrin level with respect to flavor, body and texture, melting quality and overall acceptability. Fat replacers and substitutes are extensively used in the preparation of fat-reduced foods (Rolon et al. 2017). Carbohydrate based or protein-based fat replacers could adequately mimic milk fat in terms of texture and flavor retention in ice creams (Roland et al. 1999; Prindivilleet al. 2000and Elango et al. 2017).

Hence, Maltodextrin effectively worked as fat replacer in low fat sugar free ice cream.

RSM was used for optimization of final formulation of low fat sugar free ice cream. The graphical representation [Fig II (b)]exhibited that high level of sucralose had negative effect on body and texture. Color and appearance and melting resistance graph [Fig II (a), (d)] shows quadratic increase followed by a decrease at certain level. With increasing level of sorbitol and sucralose flavor and taste will enhance [Fig II (c)]. After the optimization, low fat sugar free ice cream was formulated; sugar was substituted by artificial sweetener sucralose at 0.009% and sorbitol 2.33%. The experimental ice cream on consumption was not differentiated by any panelist as sugar free ice cream containing artificial sweetener. The after taste of the artificial sweeteners was effectively masked by flavor. Hence, sucralose and sorbitol were effective in replacing sucrose, as low-calorie sweeteners.

Ice cream, being a rich source of fat, protein and carbohydrate, contributes significantly towards calorific value. The average calorific value of ice cream is approximately 200 kcal/100g. The energy value was calculated by taking the energy value of fat, protein and carbohydrates as 2.4, 4.69 and 22.01 kcal/g respectively. The calorific value of prepared low-fat sugar free ice cream is 116.74 kcal/g much lower than the average calorific value of ice-cream.

CONCLUSION

The outcome of the present study establishesthat the combination of sorbitol and sucralose given by RSM are effective in preparing low fat sugar free ice cream. Maltodextrin at 3% level is effective as fat replacer, polydextrose at 7% level is effective in providing bulk to the ice cream and combination of sorbitol and sucralose provide sweetness without affecting the sensory attributes and Physico-chemical parameters apart from low total solids and low titrable acidity. Maltodextrin is effective as a fat replacer in mimicking the mouth feel of full fat ice cream. Sorbitol and sucralose effectively works as low-calorie sweeteners. Sucralose is effective sweetener as a substitute of sucrose. The calorific value of low fat sugar free ice cream was efficiently reduced to 116.74 kcal/100g due to addition of low calorie sugar substitutes and fat replacers.

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CONFLICT OF INTEREST

The authors do not have any conflict of interest.

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An efficient micropropagation protocol for direct organogenesis from nodal explants of medicinal climber, *Tylophora indica*

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ABSTRACT

This study aimed at in vitro multiplication of *Tylophora indica* (Burm F.) Merill.via direct shoot regeneration. Initially, screening was done for the analysis of different explants for their competence for direct shoot regeneration. The explants were cultured on media supplemented with eight different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mgl^{-1}) of each cytokinin separately and the concentrations of each cytokinin with highest responses were used in combination with IAA (0.1, 0.25 and 0.50 mgl^{-1}) and IBA (0.1, 0.25 and 0.50 mgl^{-1}). Nodal explants revealed the maximum capacity for direct shoot regeneration among all the explants tested. Following the direct shoot emergence, explants were shifted for subculturing on shoot proliferation media. The medium supplemented with TDZ (1.5 mgl^{-1}) proved effective for achieving optimum number of shoots with appropriate length. The rooting medium supplemented with 0.5 mg/l IBA resulted in maximum number of roots per shoot. Rooted plantlets were successfully hardened in the growth room and later established in the greenhouse. The survival rate of the plants was recorded as 90%. Plants showed no morphological variations.

KEY WORDS: ORGANOGENESIS, NODAL EXPLANTS, PLANT GROWTH REGULATOR, REGENERATION, TYLOPHORA INDICA

INTRODUCTION

In developing countries like India, medicinal plants continue to be the main source of medication. Indian subcontinent is a vast reservoir of medicinal plants that are used in traditional medical treatments (Chopra et al., 1986). Herbal medicines are fairly safe remedies and this approach of health care is increasing frequently as the products employed are non toxic with fewer side effects, better compatibility and affordability (Dubey et al., 2004; Sharma et al., 2008; Philomena, 2011). The demand of medicinal plants has necessarily increased due to resur-

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rection of public interest in plant-based medicines coupled with swift expansion of pharmaceutical industries (IMS, 2015).

The genus *Tylophora* comprises 60 species that are mainly distributed in tropical and subtropical Asia, Africa and Australia. *Tylophora indica* (Burm f.) Merill.is an important threatened medicinal plant commonly known as Antmul. Medicinally *Tylophora indica* is an important plant and very efficiently used in all the systems of medicine. It is a perennial, small, slender, much branched pubescent twining or climbing herb found in the sub-Himalayan tract from Uttar Pradesh to Meghalaya and in the central and peninsular India. It has also been reported from Eastern, North-East and Central India, Bengal and parts of South India (Gupta, 2003). It is also found in Ceylon, Malay island and Borneo. It is traditionally used as a folk remedy in certain regions of India for the treatment of bronchial asthma, inflammation, allergies, rheumatism and dermatitis. Its antitumor, immunomodulatory, antioxidant, antiasthmatic, smooth muscle relaxant, antihistaminic, hypotensive, antirehumatic activities are scientifically proven. In Ayurveda, the plant has been used in treatment of asthma, dermatitis and rheumatism (Anonymous, 1978 and Chopra et al., 1986).

The other reported activities include immune modulatory activity, antioxidant and free radical scavenging activity (Mohan et al., 2014, Ranemma et al., 2017), anti-inflammatory activity (Ravikumar and Abbulu, 2011), hepatoprotective activity (Mujeeb et al., 2009), antibacterial activity (Ranemma et al., 2017), anticancer activity (Vijayakumari et al., 2014), antianxiety activity (Rao et al., 2013; Mannikoth et al., 2016), anticonvulsant activity (Hafis et al., 2017), antiamoebic activity (Ganguly et al., 2001; Haung, 2004) and anti asthmatic activity (Umamaheshwari et al, 2017). The active constituents of *Tylophora indica* are alkaloids like tylophorine, tylophorinine, tylophorinidine and septidine and non-alkaloidal compounds like kaempferol, quercetin, α- and β-amyrins, tetratriacontanol, octaosanyloctacosanoate, sigmasterol, β-sitosetrol, tyloindane, wax, resin, and tannins (Rout et al., 2012; Saraswati et al., 2013; Mohan et al., 2014; Joy et al., 2017).

For in vitro regeneration of this plant, the effect of various growth regulators have been analysed from time to time and plant has been micropropagated successfully in different parts of the world (Nema et al., 2007; Sahai et al., 2007; Rani and Rana, 2010; Davendra et al, 2011; Kaur et al, 2011; Mohan et al., 2014). In India it has also been regenerated in in vitro conditions but still its micropropagation is in vague in some important regions of India. Particularly the plant being among the threatened plant species and in different regions where the physiological conditions are unusual, therefore, a protocol must be standardized to cope with the alarming situations. Direct regeneration has been reported to vary with concentrations and combinations of hormones, light and incubation, genotype and explant used (Parveen and Shahzad, 2014; Niedz et al., 2015).

Hence, it is important to standardize the culture conditions to achieve direct regeneration in desired genotypes. Therefore, the objective of the present study was to investigate the responses of *T. indica* nodal explants in vitro to growth regulators at a range of concentrations and in different combinations in order to identify optimum conditions for adventitious regeneration of shoots from nodal explants and acclimatize and transfer to green house.

MATERIAL AND METHODS

Collection of plant material: The plants were collected from Chambal eco-region and grown in the Medicinal Plant Garden, Jiwaji University Gwalior, Madhya Pradesh, India. Plant specimen prepared by standard method was submitted to Herbarium of Institute of Ethnobiology, Jiwaji University Gwalior, India, where it was taxonomically identified (IOE-235).

Surface sterilization: Healthy nodes were collected from field grown plants as an explant source. After washing with running tap water, the leaves were trimmed off and nodes were washed for 15 minutes under continuous stream of running tap water. Explants were soaked for 3 minutes in 1% Tween 20 detergent (polyoxyethylene sorbitan monolaurate; Sigma chemicals Co., St. Louis, MO, USA) and were rinsed with distilled water, then were treated with 2% Bavistin (Carbendazium 50% WP; BASF India Limited) for 5 minutes and finally surface sterilized with 0.1% $HgCl_2$ for 3 minutes and rinsed four times with autoclaved double distilled water under aseptic conditions in laminar chamber. Surface sterilized explants were transferred to sterilized petridishes. 0.5 cm long explants were cut and inoculated on culture media supplemented with different concentrations and combinations of growth regulators.

Culture medium: Surface sterilized explants 0.5 cm long were transferred to sterilized petri dishes in laminar chamber and inoculated on Murashige Skoog's (MS) medium containing3% sucrose and gelled with 0.8% agar supplemented with various concentrations auxins and cytokinins like BAP, TDZ, KIN, IBA and IAA for shoot and IBA and NAA for root initiation. The pH of the medium was adjusted to 5.75 using 0.1N NaOH or 0.1N HCl before autoclaving for 20 min at 121 °C and 15 lbs pressure.

Culture conditions: Cultures were incubated at (25±2) °C and 60%-70% relative humidity, and light intensity

2500 lux with a photoperiod of 16 h light and 8 h dark. Each experiment was conducted twice with 10 replicates per treatment. In the in vitro cultures of *Tylophora indica,* sub-culturing was done after every 30 days on the same media. All the cultures were observed regularly; contaminated cultures were discarded at the earliest and were autoclaved. The cultures were observed for the effect of media adjuvant with different growth regulators and organic supplements on in vitro regeneration. All the cultures established for in vitro shoot regeneration were monitored for percentage shoot induction, average number of shoots per explant and average shoot length.

In vitro rooting of shoots: For rooting of in vitro induced shoots, half strength MS media supplemented with indole-3-butyric acid (IBA) or α-naphthalene acetic acid (NAA) at 0.50, 1.00 and 1.50 mgl^{-1}, were used as rooting media. Shoots of 1.0–1.5 cm were excised from multiple shoots and then cultured in rooting media. Data were recorded on percentage of rooting and the number and length of roots/shoots after four weeks of culture. Rooted plantlets of about 6 cm in length were washed thoroughly in running tap water, and transplanted into autoclaved plastic pots filled with mixture of sand, soil and manure in 1:2:1 ratio under artificial light in 16 h/8 h photoperiod conditions covered with polythene bags to maintain humidity and then kept in the laboratory. After two weeks, the pots were transferred to a greenhouse under 30 °C with 60% relative humidity and were watered daily. The regenerated plantlets were acclimatized for 4-8 weeks and then successfully transferred to the soil under normal conditions.The percentage of survival explants, percentage of shoot response, shoot length, shoot initiation time, shoot number, root response percentage, root initiation time and number of roots were recorded after every 14 days. The data was analysed by using statistical ez Anova version 2. The data are presented as Mean ± Standard error of two replicates.

RESULTS AND DISCUSSION

Tissue culture acts as a tool for propagation and conservation of most of valuable plant species (Gantait et al., 2011; Khan et al., 2012). The microcolonal propagation of plants is influenced by some important and concomitant factors such as media composition, plant growth regulators, tissue sensitivity and culture environment. Using different combinations of growth regulators and appropriate growing environment, the important plant species can be regenerated. The aim of the present study was to investigate the effect of some important growth regulators on an important endangered medicinal plant species *Tylophora indica* under in vitro conditions with a view to develop an efficient, reliable and reproducible protocol for its clonal propagation. During the study, out of all the explants used, only nodes were able to show response of shoot induction and caulogenesis which is contradictory with the earlier reports (Thomas and Philip, 2005; Verma et al., 2010) where leaf explants showed best results of shoot induction response.

In the present endeavour there were three different cytokinins viz; Benzyl amino purine (BAP), Thidiazuron (TDZ) and Kinetin (Kn) and two auxins viz; Indole acetic acid (IAA) and Indole butyric acid (IBA) used to evaluate their effect on in vitro shoot regeneration. Eight different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mgl^{-1}) of each cytokinin were worked out separately and the concentrations of each cytokinin with highest responses were used in combination with IAA (0.1, 0.25 and 0.50 mgl^{-1}) and IBA (0.1, 0.25 and 0.50 mgl^{-1}).

Explants cultured on MS medium supplemented with different concentration of BAP showed an increase in the percentage shoot induction and average number of shoots per explants with increase in the concentration of BAP from 0.5mgl^{-1} to 2.5mgl^{-1} but the average shoot length showed continuous increase with the increase in BAP concentration. A maximum of 78.89 ± 1.11 % explants cultured on MS media supplemented with BAP

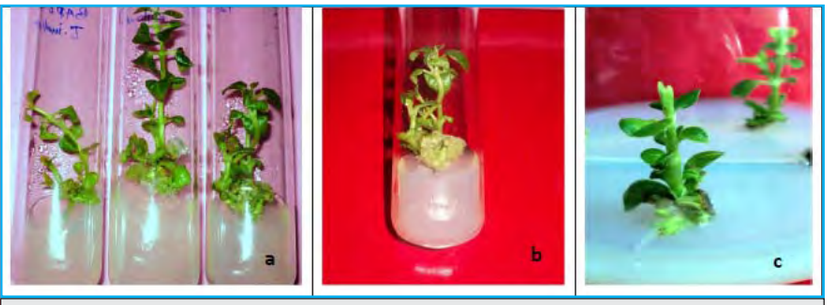

FIGURE 1. Shoot initiation from nodal explants on MS medium supplemented with various cytokinins. (a) BAP (b) Kn (c) TDZ.

FIGURE 2. Shoot initiation from shoot nodal explants of *Tylophoraindicaon* multiplication media (MS medium) supplemented with (a) BAP 1.5 mgl^{-1}+ IAA (b) BAP 1.5 mgl^{-1}+ IBA (c) Kn2.5 mgl^{-1}+IAA (d) Kn2.5 mgl^{-1}+IBA (e) TDZ 1.5 mgl^{-1}+IAA (f) TDZ 1.5 mgl^{-1}+IBA (g) Development of successfully acclimatizedplantlets.

(2.5 mgl^{-1}) responded for shoot induction, the highest number of shoots per explants being recorded as 3.60 ± 0.10 (Figure 1a). With further increase in BAP concentration, the percentage shoot induction decreased and average number of shoots per explants also declined. At 4 mgl^{-1} of BAP, the average number shoots were slightly increased up to 2.35 ± 0.05.No callus formation was observed (Figures 3-5).

Direct shoot development within 20-21 days of explant inoculation was observed in all the concentrations of Kn used. The highest percentage of shoot induction (74.44 ± 14.45 %) was observed in explants on MS media supple-

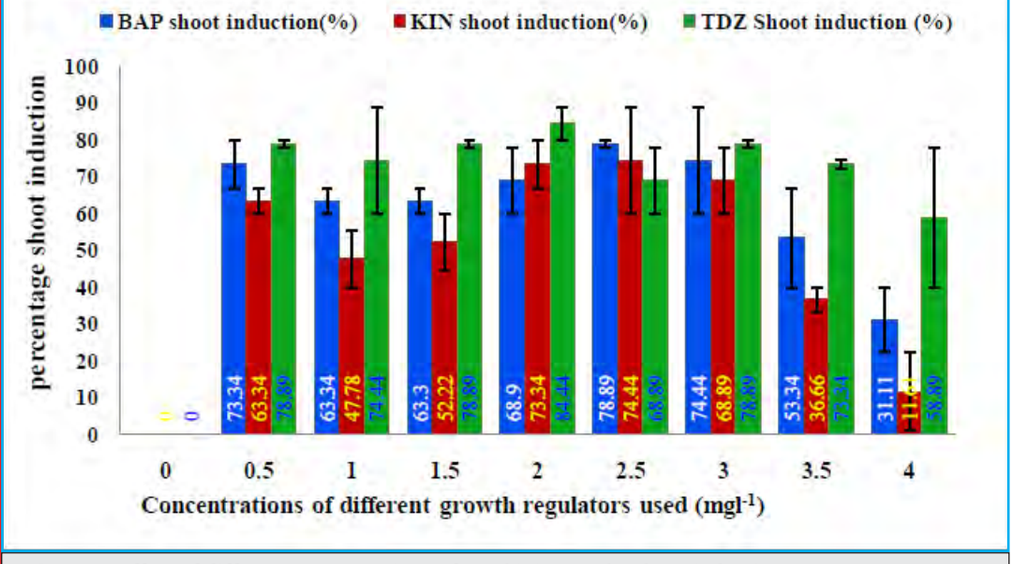

FIGURE 3. Effect of different concentrations of cytokinins on the shoot induction

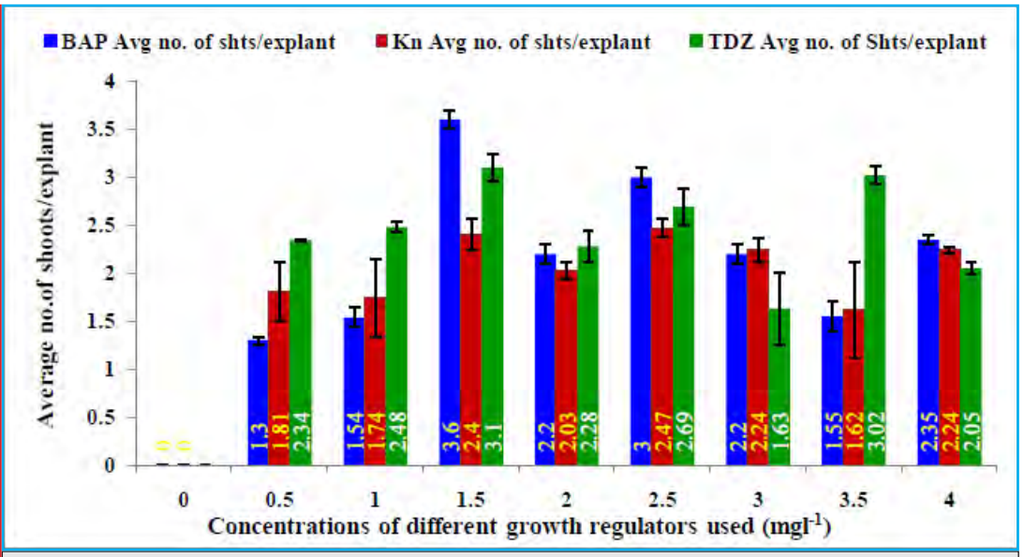

FIGURE 4. Effect of different concentrations of cytokinins on the average number of shoots per explant.

mented with 2.5 mgl^{-1} Kn, with average number of 2.47 ± 0.09 shoots per explants (Figure 1b). Maximum average shoot length 2.70 ± 0.40 cm was recorded at 3.5 mgl^{-1}. Increase in Kn concentration in the media results in the decrease of all the facets except the average shoot length which showed irregularity. An increase in the average number of shoots per explant was again observed at 4 mgl^{-1}. No callusing was observed (Figures 3-5).

Fresh explants cultured on MS media supplemented with different concentrations of TDZ responded well in all the facets with a shoot induction percentage upto 84.44 ± 4.45% at TDZ (2 mgl^{-1}). The explants exhibit

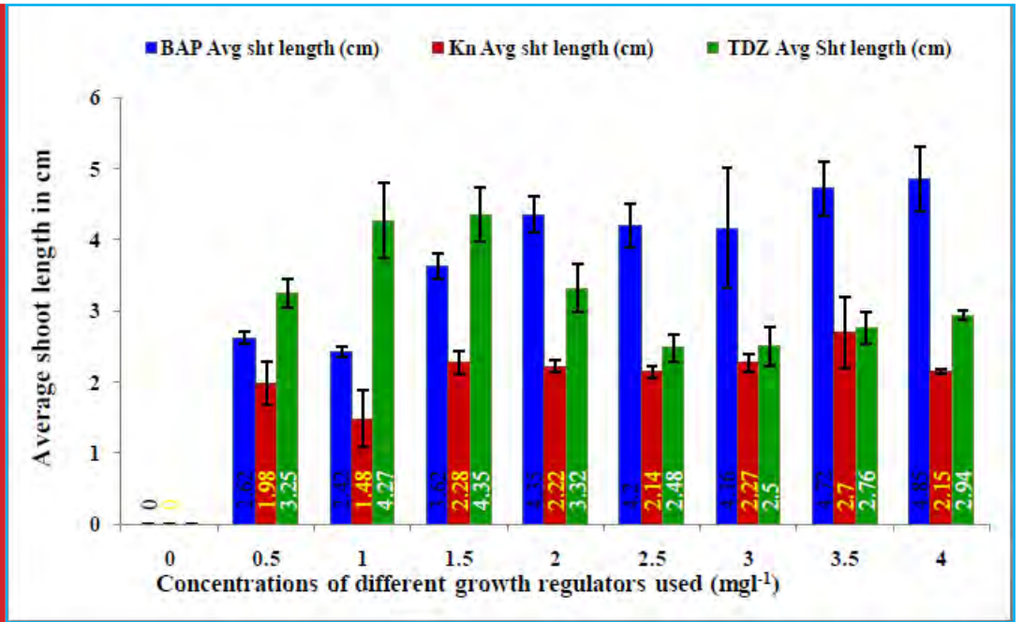

FIGURE 5. Effect of different concentrations of cytokinins on the average shoot length (cm) from the nodal explants *Tylophoraindica*

Table 1. Effect of cytokinins (BAP, Kin and TDZ) on the induction of shoots from the nodal segments in *T. indica*

Conc. of BAP (mg l^{-1})	Conc. of Kin (mg l^{-1})	Conc. of TDZ (mg l^{-1})	Shoot induction (%)	Number of shoots/ explants(Mean±S.E)	Average Shoot length (cm)
Control	-	-	0	0.0 ± 0.00	0
0.5	-	-	73.34 ± 6.6	1.30 ±0.04	2.62±0.08
1	-	-	63.3± 3.3	1.54±0.10	2.42±0.08
1.5	-	-	63.3 ± 3.3	3.60±0.10	3.62±0.18
2	-	-	68.9 ± 8.9	2.20±0.10	4.35±0.25
2.5	-	-	78.89±1.11	3.00±0.10	4.20±0.30
3	-	-	74.44±14.45	2.20±0.10	4.16±0.84
3.5	-	-	53.34±13.33	1.55±0.15	4.72±0.38
4	-	-	31.11±8.89	2.35±0.05	4.85±0.45
-	0.5	-	63.34±3.33	1.81±0.31	1.98±0.28
-	1	-	47.78±7.78	1.74±0.40	1.48±0.17
-	1.5	-	52.22±7.77	2.40±0.16	2.28±0.17
-	2	-	73.34±6.66	2.03±0.09	2.22±0.11
-	2.5	-	74.44±14.45	2.47±0.09	2.14±0.21
-	3	-	68.89±8.89	2.24±0.13	2.27±0.32
-	3.5	-	36.66±3.34	1.62±0.5	2.70±0.40
-	4	-	11.61±10.61	2.24±0.03	2.15±0.03
-	-	0.5	78.89±1.11	2.34±0.01	3.25±0.20
-	-	1	74.44±14.45	2.48±0.05	4.27±0.53
-	-	1.5	78.89±1.11	3.1±0.14	4. 35±0.38
-	-	2	84.44±4.45	2.28±0.17	3.32±0.33
-	-	2.5	68.89±8.89	2.69±0.19	2.48±0.19
-	-	3	78.89±1.11	1.63±0.38	2.50±0.27
-	-	3.5	73.34±1.11	3.02±0.10	2.76±0.22
-	-	4	58.89±18.89	2.05±0.06	2.94±0.06

both callusing and direct shoot induction after 21days of inoculation. Results show increase in percentage shoot induction with increased TDZ concentrations in MS media from 78.89 ± 1.11% (0.5 mgl^{-1}) to 84.44±4.45% (2 mgl^{-1}) (Figure 1c). A decrease in percentage shoot induction was observed when TDZ concentration was increased above 2 mgl^{-1}. The highest number of shoots per explants (3.1±0.14) with an average shoot length of 4.35±0.38 cm was observed when explants were cultured on MS media supplemented with TDZ (1.5 mgl^{-1}). A decrease in percentage shoot induction, average number of shoots per explant, average shoot length and shoot forming capacity was observed when TDZ concentrations was increased from 2.5 mgl^{-1} and 2 mgl^{-1} respectively (Figures 3-5). The regenerated shoots were thick as compared to the shoot regeneration observed in respective concentrations of BAP and Kn.

Out of the three cytokinins used, TDZ showed maximum results not only because of highest percentage shoot induction, average number of shoots per explants and average shoot length achieved with this cytokinin but also due to callus formation which is treated as another route of tissue culture regeneration of plants which is in contradiction with earlier report by (Anand and Nadha, 2006; Kaur et al., 2011; Haque and Ghosh, 2013), which showed best results on MS medium supplemented with BAP. TDZ has been reported to be the most active cytokinin for shoot induction in plant tissue culture (Siddique and Anis, 2007), and has been effective in terms of shoot regeneration in many recalcitrant species. It has been reported that TDZ-induced morphogenesis depends on the levels of endogenous growth regulators and TDZ modulates the endogenous auxin levels (Petric et al., 2011). In present study 84 % shoot induction was observed which is in contrary with earlier findings (Thomas and Philip, 2005) which showed 100% shoot induction on MS media rejuvenated with TDZ. The better concentration among the different concentrations of this cytokinin was TDZ (1.5 mgl^{-1}) for shoot regeneration from nodal explants of *Tylophora indica*. In case of BAP and Kn the better concentrations proved to be were 1.5 mgl^{-1} and 2.5mgl^{-1} respectively (Table 1).

Table 2. Effect of cytokinins (BAP, Kin and TDZ) and IAA on the induction of shoots from the nodal segments in *T. indica*

Conc. of harmone	Conc. of IAA (mg l^{-1})	Shoot induction (%)	Number of shoots/ explants (Mean±S.E)	Average Shoot length (cm)
BAP (1.5 mg l^{-1})	0.1	41.66±8.34	3.16±0.31	3.96±0.25
	0.25	50.00±16.67	2.50±0.37	4.06±0.07
	0. 5	58.34±8.33	2.02±0.21	3.81±0.32
Kin (2.5 mg l^{-1})	0.1	58.34±8.33	2.75±0.35	3.60±0.06
	0.25	58.34±8.33	2.57±0.45	3.62±0.39
	0. 5	50.00±16.67	1.44±0.21	3.65±0.67
TDZ (1.5 mg l^{-1})	0.1	66.66±16.67	4.38±0.40	3.93±0.71
	0.25	58.34±8.33	4.01±0.45	5.02±0.76
	0. 5	50.00±16.67	3.66±0.45	4.78±0.56

Optimum concentration of BAP (1.5 mgl^{-1}) was used in combinations with different concentrations of auxins (IAA and IBA) for in vitro shoot regeneration. A maximum of 58.34 ± 8.33 % explants showed direct shoot induction at BAP (1.5 mgl^{-1}) + IAA (0.5 mgl^{-1}) (Figure 2a). The highest average number of 3.16 ± 0.31 shoots per explant was seen in media suppplemeted with 1.5 mgl^{-1} BAP + 0.1 mgl^{-1} IAA and maximum average shoot length was at BAP (1.5 mgl^{-1}) + IAA (0.25 mgl^{-1}) (Table 2).

MS media supplemented with BAP (1.5 mgl^{-1}) in combination with different concentrations of IBA showed highest response (58.33 ± 8.33%) at BAP (1.5 mgl^{-1}) + IBA (0.1 and 0.5 mgl^{-1}) with an average number of 4.29 ± 0.76 shoots per explant at BAP (1.5 mgl^{-1}) + IBA (0.5 mgl^{-1}) only and maximum average shoot length at BAP (1.5 mgl^{-1}) + IBA (0.1 mgl^{-1}) (Figure 2 b). A decrease in percentage shoot induction was observed with addition of different concentrations of IBA to BAP (1.5 mgl^{-1}) supplemented to MS media. Apart from the direct shoot regeneration, callus formation was also perceived in this blend at BAP (1.5 mgl^{-1}) + IAA (0.25 mgl^{-1}) after culturing (Table 3).

Among different Kn concentrations, 2.5 mgl^{-1} Kn was revealed to be much effective over all other concentrations and was used in combinations with different concentrations of auxins (IAA and IBA) for in vitro shoot regeneration using nodal explants. A decrease in percentage shoot induction, average number of shoots per nodal explant was observed by addition of IAA (0.1, 0.25 and 0.5) mgl^{-1} to (Kn 2.5 mgl^{-1}) supplemented MS media (Figure 2c). A maximum of 58.34 ± 8.33% explants responded for shoot induction at Kn (2.5 mgl^{-1}) + IAA (0.1 $mgl^{-1}$and 0.25 mgl^{-1})(Table 2).Explants cultured on MS media divulged 58.34 ± 8.33 % shoot induction at Kn (2.5 mgl^{-1}) + IBA (0.5 mgl^{-1}) (Figure 2d). The highest 4.29 ± 0.76 average number of shoots per explant were observed on MS media supplemented with Kn (2.5 mgl^{-1}) + IBA (0.5 mgl^{-1}) (Table 3).

TDZ resulted to be over all better growth regulator among the three cytokinins used. Explants cultured on

Table 3. Effect of cytokinins (BAP, Kin and TDZ) in combination with IBA on the induction of shoots from the nodal segments in *T.indica*

Conc. of harmone	Conc. of IBA (mg l^{-1})	Shoot induction (%)	Number of shoots/ explants(Mean±S.E)	Average Shoot length (cm)	Callus formation
BAP (1.5 mg l^{-1})	0.1	58.33±8.33	4.00±0.87	3.81±0.19	_
	0.25	41.66±8.34	3.68±0.69	3.45±0.45	++
	0. 5	58.33±8.33	4.29±0.76	3.39±0.28	_
Kin (2.5 mg l^{-1})	0.1	50.00±16.67	3.05±0.19	3.47±0.19	-
	0.25	41.66±8.34	2.07±0.21	3.26±0.73	-
	0. 5	58.34±8.33	2.99±0.13	2.97±0.61	-
TDZ (1.5 mg l^{-1})	0.1	75.00±8.33	5.84±0.72	3.85±0.50	+
	0.25	58.34±8.33	4.19±0.44	2.30±1.54	+++
	0. 5	58.34±8.33	4.30±0.46	5.14±0.15	+++

Table 4. Effect of auxins on rooting from microshoots of *Tylophora indica* in half strength MS medium (after 30 days).

IBA (mg l^{-1})	NAA (mg l^{-1})	Root induction (%)	Mean number of roots/ explants	Average root length (cm)
0.5	-	90	3.25±0.25	3.87±0.37
1	-	75	1.75±0.25	2.25±0.14
1.5	-	40	1.5±0.28	1.5±0.28
-	0.5	85	3.4±0.4	3.7±0.2
-	1	60	2.6±0.2	2.8±0.25
-	1.5	30	2±0.54	1.8±0.25

MS media showed 66.66 ± 16.67 % shoot induction at TDZ (1.5 mgl^{-1}) + IAA (0.1 mgl^{-1}). The highest 4.38 ± 0.40 average number of shoots per explant were obtained on MS media supplemented with TDZ (1.5 mgl^{-1}) + IAA (0.1 mgl^{-1}). Maximum average shoot length was obtained at TDZ (1.5 mgl^{-1}) + IAA (0.25 mgl^{-1}) (Figure 2e). With increase in the concentration of IAA to TDZ (1.5 mgl^{-1}), increase in the percentage shoot induction and average number of shoots per explants was seen but the average shoot length at TDZ (1.5 mgl^{-1}) + IAA (0.25 mgl^{-1}) increases up to 5.02 ± 0.76 cm and then decreases at TDZ (1.5 mgl^{-1}) + IAA (0.25 mgl^{-1}) (Table 2). The explants exhibit both direct shoot induction and callusing after 21days but callus formation took place very late after 30 to 35 days from the date of inoculation. A maximum of 75.00 ± 8.33 % percentage shoot induction with 5.84 ± 0.72 average number of shoots per explant on media at TDZ (1.5 mgl^{-1}) + IBA (0.1 mgl^{-1}). The highest average shoot length was observed at TDZ (1.5 mgl^{-1}) + IBA (0.5 mgl^{-1}) (Figure 2f). Decrease in percentage shoot induction and average number of shoots per explant was observed with increase in the concentration of IBA to TDZ (1.5 mgl^{-1}). The average shoot length slightly decreased with increase in the concentration of IBA from 0.1 to 0.25 mgl^{-1} on MS media and increases largely at TDZ (1.5 mgl^{-1}) + IBA (0.1 mgl^{-1}). Large amount of callus formation was observed when media was added with TDZ (1.5 mgl^{-1}) + IBA (0.25 to 0.5 mgl^{-1}) (Table3).

In vitro shoot regeneration response from nodal explants was not significant when different concentrations of Auxins (IAA and IBA) were added to MS media fortified with combination of BAP (1.5mgl^{-1}) and Kn (2.5mgl^{-1}). The percentage shoot induction decreased with addition of these auxins but slight increase in the average number of shoots per explant and average shoot length was observed. Thus the micropropagation protocol for shoot induction developed for *Tylophora indica* can be successfully applied for large scale multiplication. For root induction, elongated shoots were subcultured for more than 5 weeks on medium containing TDZ. Both the auxins IBA and NAA supplemented to half strength MS medium initiated rooting. Maximum of 90% rooting was observed on medium with half strength basal salts containing 0.5 mgl^{-1} IBA with 90% rooting percentage, strong and good number of roots (Table 4).

Regenerated rooted plantlets were successfully transferred to pots containing soil, sand and manure in a 2:1:1 ratio. The pots containing plantlets were kept in growth room for 2 weeks and then transferred to green house. Healthy plantlets from pots were then transferred after 6-8 to field conditions for survival. Survival rate of 90% was observed. Plants were found to be similar to mother plants (Figure 2g).

The results of this study revealed that nodal explants had the highest regeneration rate in MS medium supplemented with various plant growth regulators. Furthermore, our results showed that TDZ was more effective among the cytokinins in shoot regeneration. TDZ in combination with IBA also showed good results with average number of shoots powered to 5-6 shoots per explants and at the same concentration best performance of callusing was witnessed. Half strength Basal medium with IBA was found most effective for root formation of *T. indica* when compared with NAA. Hence a protocol has been developed through for in vitro shoot regeneration and root regeneration under in vitro conditions to fulfil the natural reservoir of this plant and needs of pharmaceutical industries in future.

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Analysis of a novel comprehensive health system

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ABSTRACT

So far, different definitions of health systems have been provided by international health organizations in which it has been tried to show the capabilities of a health system. Of course a system that have all these capabilities has never been offered because these capabilities are associated with different health areas including telemedicine, and disease diagnosis, and there is no system that is capable of gathering all these features. In this study we attempt to define a system that meets the information needs a comprehensive health system at once; a system which covers most of the health areas, including medical, food and medicine. In this article we are going to discuss the most important capabilities that a comprehensive health system has. This application will bring economic efficiency for its users, because with its help, people can easily distinguish their body's needs based on specific circumstances and act accordingly. This will prevent many food caused diseases. Also, people using this application will not need to spend heavy expenses to prepare healthy food and they can prepare healthy foods with the help of healthy foodstuffs; as we know in many developed countries, to prevent food caused diseases, people have turned to diets such as vegetarian diet so they don't suffer such diseases due to inactivity. Users of this application can cook or prepare foods proposed by system with the items and healthy food and with the help of search program that is available in the system, and receive the most information about that food.

KEY WORDS: COMPREHENSIVE HEALTH SYSTEM, HEALTH CARE SYSTEM, DISEASE DIAGNOSIS SYSTEM

INTRODUCTION

Food is almost one of the basic human needs and according to its fundamental role in providing health, extensive research has been done about it. In this article we are going to discuss the most important capabilities that a comprehensive health system has. This application will bring economic efficiency for its users, because with its help, people can easily distinguish their body's needs based on specific circumstances and act accordingly. This will prevent many food caused diseases. Also, people using this application will not need to spend heavy expenses to prepare healthy food and they can prepare healthy foods with the help of healthy foodstuffs; as we know in many developed countries, to prevent food caused diseases, people have turned to

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diets such as vegetarian diet so they don't suffer such diseases due to inactivity. Users of this application can cook or prepare foods proposed by system with the items and healthy food and with the help of search program that is available in the system, and receive the most information about that food,(Health Systems, Goals, 22 January 2017).

The information system of this application is of database type and in the form of entity, (September 2005, http://whatis.techtarget.com/definition/entity), and relational or tabular. This product is a site to which the applicants join by paying a sum of money and use the site services and since a system with this characteristics and comprehensiveness does not exist and also it resolves the basic needs of the people, it most likely will be successful in the market and will have a good sale in the case of good and sufficient advertising, (Health systems, Goals, 22 January 2015).

Today, a high diversity of health systems are produced and each of them are somehow trying to solve medical and public health problems or provided facilities to fix the problems. According to the World Health Organization, health systems goals must be appropriately and equitably available to public, they also should have good, effective, and acceptable quality and evenly distributed for the general public, (health systems, World Health Organization definition, 22 January 2015,http://en.wikipedia.org/wiki/Health_system). These cases also known as 5C in the United States which include: Cost, Coverage, Consistency, Complexity, and Chronic Illness, (Health systems ,Goals, 22 January 2015).

SUMMARY OF MANAGEMENT TOPICS

The project is an application that is produced in the form of a website. The totality of project is as follows:

The product is an application relating to nutrition, health and wellness; a product, which provides comprehensive information about cooking, medicine and health and solves many problems that have been discussed below. Recently, people should not use food only for feeding. We argue that the proper use of this system will increase the people life to one hundred years. In fact, it is the system that decides what organic matter and how much energy and vitamins your body needs and therefore what foods you should consume. For this purpose, system receives complete information about your body and diseases that you likely suffer and the amount of your daily physical activity and sport and many other daily activities you were indifferent towards them so far and it also examines where you live in terms of weather and according to these information, teaches you the healthy lifestyle and how do stay healthy and warns you the necessary medical advices.

Some people do not want the system decides about their food and wish to choose their own food. This system has predicted this, too. The system has a complete list of a variety of dishes at once and allows you, for example, to prepare new and local dishes for your family according to the food in your cabinets and fridge. You can ask the system to analyze these foods for you and thus you will know that what food you have on table and eat and how much energy it gives to your body, or how much vitamins, fat, carbohydrates and other organic materials is in these foods that your body needs or is harmful to your body.

Even many people and disease physicians are eager to know what elements are in the food they eat every day. This also helps physicians in prescribing different regimens to their patients. For example, for a patient whose body needs iron in large amounts, the physician need to know what foods contain the highest amounts of iron. As we know, good food is the best medicine. Most people who care about their health should refer to a specialist for information about their obesity or overweight or malnutrition and pay a lot of time and money. The current information poverty has caused a lot of people to suffer from diseases and incur a lot of time and costs for curing them.

Most food caused diseases arise due to improper use of feedstuffs around us because people want to meet most of their body's needs by consuming carbohydrates (rice and wheat), despite the fact that most of these materials are converted into fat in body and other needs of the body remain unanswered. This system tells us in which food there are materials required for your body and what foods we should eat to resolve the issue.

BASIC DEFINITIONS

Health: As defined by World Health Organization, human health includes overall physical health, mental health and community,health, ,(http://en.wikipedia.org/wiki/Health_system).According to this definition, merely not being sick is not considered as being healthy. According to the World Health Organization definition, the main components of wellness in addition to health and lack of disease include social and economic environment, physical environment, individual's behavior and personality dimensions.

HEALTH CARE

Health Care includes activities such as diagnoses diseases and their complications, and preventing human diseases and mental disorders [health, 2 January 2015, http://en.wikipedia.org/wiki/Health].

In most cases, medical care is directly related to a country's economy and has allocated a budget of about 9.3 of GDP in OECD member countries.

Health care is divided to a variety of primary health care or the first category, secondary health care or second category, third category and fourth category.

- Primary health care or first category: related to professionals who advise their patients for the first time and make a connection with them, such as doctors or family physicians.
- Secondary health care or second category: includes service which is provided by those medical specialists who do not have the first contact with the client, such as cardiologist, urologist and dermatologist.
- Third category health care: is related to services that is often assigned to non-patients or people who are referred by primary or secondary medical care and provides facilities for specialized medical examinations and treatment. Some of these types of care include cancer management, neurosurgery, cardiac surgery, plastic surgery, treatment of severe burns, babies advanced services, and other complex medical and surgical interventions.

Fourth category of health care: the fourth category of medical care sometimes is used as a branch of the third category and in fact is considered as a part of the specialized advanced care which requires high levels of specializations which is not widely and easily available, such as some types of unusual methods of diagnosis or surgery. These services are limited in number in any area or in health care centers,(Health_care, 28 February 2015,http://en.wikipedia.org/wiki/Health_care).

HEALTH SYSTEMS

An organization or person is an institution or entity that will provide health care services to meet the health needs of a community(Health_care / Health care delivery, 28 February 2015).

HEALTH CARE SOFTWARE SYSTEMS

Health care software systems are mostly focused on physical health and diseases of individuals, than the social, emotional and intellectual aspects of people's health, but with increasing the smart software and information systems, it will also cover this area gradually and it is likely that these systems will cover all the areas of health care.

PREREQUISITES FOR HEALTH CARE SOFTWARE SYSTEMS

These systems should be web-based and provide online information and services, because health and community health systems must have powerful notification feature. This will causes the quickly release of data between the people of geographically dispersed regions and for example, if a particular disease spreads in one region or geographical area, without creating panic among the people of the whole country, the people of that area will particularly be notified and medical advice will be reminded to people in order to prevent the rapid spread of the disease; these systems' information must also be constantly updated.

On the other hand, since these systems deal with individual and community health, they should have the maximum accuracy of information, and their decision-making systems should have the least mistake; this category of systems are a part of information systems and the heart of every information system is its database, where information are categorized and can be searched and managed. These systems usually need to manage large volumes of information, but the database management systems should be used which are for heavy information management. On the other hand, the type of information used in these software systems are often linked together and sometimes system have to review extensive information for a simple decision.

Database management systems: Database management systems are software systems that are used to manage data and information. In follows, some of the most important of them are listed and analyzed.

SQL-based database management systems:These systems are also known as "Relational" systems and today, are mostly used in information systems. The advantage of these systems is that they communicate appropriately between dispersed and different stored information and in this respect, they are appropriate for the health care systems.

The problem with these systems is that they need a powerful and hardware stable for database management with heavy and high-volume information. Some of the most popular of these systems are Mysql, MS Sql, Oracle 11g, and Postgresql. The difference between these systems is that some of them, such as Mysql are designed to manage lighter and lower volume information and some of them, such as Oracle are designed to manage information with heavier volume. Also, some database management systems such as Postgresql are appropriate to manage medium-size databases.

SQL language has both structures of Data Definition Language (DDL) and Data Manipulation Language (DML).

NewSQL-based database management systems

NewSql systems are a modern class of relational systems of database management that while maintaining

the capabilities of relational systems are trying to have a significant speed when dealing with heavy data like Nosql systems and also have the ability of ACID (http://en.wikipedia.org/wiki/Health_care), (Health_system ,22 January 2015.http://en.wikipedia.org/wiki/Health_system). Some NewSql database management systems include Google Spanner, Clustrix, VoltDB, MemSQL, SAP HANA, Foundation DB, NuoDB, TransLattice, ActorDB [ACID, 20 February 2015,en.wikipedia.org/wiki/ACID).

Nosql-based database management systems

Nosql systems that are also interpreted as Not Only SQL systems provide a mechanism to store and retrieve information which is quite different from tabular method of relational systems. The advantage that has made these systems popular is that they are more easily designed and have more horizontal scalability as well as better control and greater access to data [new sql, 18 February 2015 ,http://en.wikipedia.org/wiki/NewSQL]. Data structure used in Nosql systems includes key-value, graph and document which is completely different from the data structure used in Sql systems and this leads to better and faster performance of nosql systems.

Some of the best Nosql systems

There are a variety of systems that use Nosql technology. Some of them are listed below: BaseX, Apache CouchDB, MongoDB, Oracle NoSQL Database, OrientDB, Big Table, Cassandra, Druid, HBase, Hypertable

The most appropriate data storage system for comprehensive health system

Given that health care systems or comprehensive health systems should set a lot of facts and items side by side to make decisions and announce the results and examine them, hence nosql systems will not work for these cases and will face difficulty. On the other hand, RDBMS systems, which are also called Traditional DBMS systems are used for programmers and even in universities and are more appreciated. Traditional systems are appropriate and easier for mid- and small-sized works, unless we offset these shortcomings and deficiencies using a more powerful hardware. Hence newsql systems are fit for the design and implementation of such systems and have better responsiveness capability.

	Old SQL	NoSQL	NewSQL
Relational	Yes	No	Yes
SQL	Yes	No	Yes
ACID transactions	Yes	No	Yes
Horizontal scalability	No	Yes	Yes
Performance / big volume	No	Yes	Yes
Schema-less	No	Yes	No

A comparison between database management technologies (VoltDB (in-memory database) : http://voltdb.com/)

Capabilities of the comprehensive health system

The capabilities that the comprehensive health system has in order to cover the maximum amount of information are set forth below:

Disease diagnosis

As it was seen in the definition of this system one of the features of this system is disease diagnosis. In medical science, disease diagnosis is based on two types of symptoms:

- Clinical symptoms: are also of two types:
  1. Outward symptoms, such as dizziness;
  2. Physical tests, such as touching the patient or measuring the fever.
- Paraclinical symptoms: include things such as:
  1. Routine tests;
  2. Specialized tests;
  3. Radiology;
  4. Bone marrow samples etc.

Important points in disease diagnosis

- One thing that should be considered when diagnosing a disease is that how often these symptoms of the disease happen.
- Sometimes a symptom indicates different diseases in different genders.
- The amount of symptoms in diseases may include some additional explanations. Example: abdominal pain, where? Does it increase with pressure or decrease?

- Sometimes during the process of diagnosis, the patient confirms some symptoms which are not related to their original condition, for example, symptoms of osteoporosis and yet declares symptoms related to "cyst" on some part of the body, in other words, a condition sometimes is hidden behind other disease or diseases.
- Sometimes a test should be repeated within a specific time interval.
- Sometimes some symptoms have a higher priority than others.
- In some cases, the symptoms do not indicate a disease; such as hematuria that can occur by eating beets.
- In those who have a history of alcohol or cigarettes, the dose and duration of their use are effective in diagnosing some diseases and prescribing.
- In the diagnosis process, if the system detects the type of disease, it should consider that some diseases shouldn't declare to the patient and should only be referred to a physician, such as cancer and multiple sclerosis.

Amounts of data in testing and during the process of diagnosis:

The system that must receive test data and disease symptoms and examine them should be designed to meet a variety of these experiments data.

Some of the most important of these data are:

- Numerical values: e.g. for glucose levels 200, or blood creatine 1340.
- Values of + and -: e.g. for pregnancy test, blood ph etc.
- Sentential values: e.g. for ultrasound and image.
- Values in the form of file: e.g. a picture or sound for heart examinations."

Offering medical advices

Health and medical care comprehensive system acts like a family physician and is always with people and recognizes the needs of individuals and declares important medical advice to them, when necessary. The system constantly receives people's health information, and people are connected with the system in different ways for hours, and monitor their past and present for the system and this is the system that receives all that are related to the patient's health such as allergies and examines these information constantly and evaluates them over and over again. Not like some doctors who examine all information of their patients for a few minutes and write a prescription for them and prescribe medications sometimes without evaluating them. Examples of these cases are described below:

- The first blood test should be done at age 30;
- If there is a history of diabetes in your family, do the glucose test at the age of 23;
- The breast exams should be done every 6 months after age 30.

Considering and evaluating people's consumption of organic and inorganic matter

One of the unique features of comprehensive health system is that it constantly examines the amount of organic and inorganic matters that people daily consume and if it is harmful for people's fitness as well as their diseases, the system notifies them and determines and recognizes the amount of material required for human body. There are some factors affecting the consumption of organic and inorganic matters, elements and daily calories such as age, weight, sex, addiction, alcohol, smoking, exercise and its type and amount during the day and also fitness. The cases in these topics that have been considered in this system include:

- Controlling food consumption: a lot of minerals and elements are essential for the body and should be consumed on a daily basis in a proportional amount (not less and not more);
- Some materials are not absorbed by the digestive system of some people and have to inject into the body, such as iron or B12 and B6 vitamins.

Comprehensive health system detects these and will not include high levels of these materials in their daily diet.

Medication prescription and its related issues

Of other features of the comprehensive health system is medicine prescription. The system helps physicians in this way and does a better medicine prescription and away from the errors. Some of the topics that have been studied in this system are as follows:

- Side effects of medicine use;
- Last date of medicine use;
- Side effects of a medicine for an age and weight class;
- Side effects of a medicine for some diseases;
- Allergenic side effects of some medicines;
- Sometimes by seeing a symptom on the patient's body taking the medicine should be discontinued;
- Amount of certain medicines use will vary in cases of using certain foods. For example :

A person with diabetes will take some sugar and sweets; the system determines how much insulin or alternative medicine such as NPH or Nevomix they should consume.

Study the amount of consuming organic matters and nutrients in people's food

A person, who has a certain diseases, should not daily consume for example, more than 100 grams of a food. This is another issue that comprehensive health system constantly examines. Some topics in this issue include:

- Grade diseases, such as diabetes: for each grade the quantities of medicine vary.
- The amount of different materials consumption in some diseases, if the person is physically active, according to the hours of activity or exercise is different.

Controlling the amount of physical activity

Another feature of comprehensive system health is controlling the amount of physical activity, including sports and jobs of people. Some types of exercise and daily activities or a job are disproportionate for some diseases.

DISEASE TREATMENT

Different methods to treat a disease

Variety of different methods to treat a disease and the difference in the duration of therapy in any treatment is another issue in examining the comprehensive health system. When prescribing, the comprehensive health system always examines that other than medicine or along with it, are there any other ways to treat a disease with fewer side effects or not? If the disease is treatable with different methods, we reach to this issue that with which of these methods the patient is more comfortable and which will lead to fewer complications and more definitive treatment. Some of these treatments include:

- Medicine
- Sport
- Food

Diseases that people are prone to them

Today, with advances in medical science we can determine people are prone to what diseases and use this information to prevent the development of those diseases. The system detects people who are prone to some diseases and will be able to offer them special cares. Some methods of detecting what people are prone to what diseases include:

- Such as the family history of people in one or more types of diseases.
- Diseases that affected an individual in the past and there is the possibility of recurrence.
- Calculating fitness on the basis of BMI formula.
- Considering some habits in diseases.
- Smoking, alcohol and alcoholic beverages, and drugs.

ENVIRONMENTAL FACTORS

Environmental factors affect the lives of healthy and sick people. These factors can be useful or harmful for a disease and if this adds to the system, it would help people to choose the best environment to live and work.

CONCLUSION

There are nearly 90 kinds of major disease that Health care systems prescribe for them. Today, these systems are mostly used as medical assistant but some of these systems, such as comprehensive health system have the ability to learn and become expert and it's not unlikely that one day they decide instead of physicians. Of course, the comprehensive health system will create a whole new world in the field of medical and health and nutrition for people. Now in the third millennium we see physicians making terrible mistakes in diagnosis, prescription and medical advices and yet the field of medicine and medical prescriptions is related to human factors, but what comprehensive health system brings for human fulfills a large part of the definition of World Health Organization and provides great facilities for the first time for public and without any discrimination.

People sit comfortably in their homes and communicate with the system. They declare their medical history, fitness status, feed rate and its type, their job and daily activities, their allergies and whatever is related to their health to the system and from this moment by the minute, the system consider every individual, and declares their needs and helps them to live a healthier life.

Who knows what toxins and how much of them are in a food? Who can tell how for many years people can live? The answer is comprehensive health system.

What can be done to improve people's lives? How much the environmental factors affect people's lives? And many other questions that their answers can be realized only with a scrutiny and long and costly trials which take many years; the comprehensive health system can tell all of this at an insignificant cost to people.

When computers came to assist the accountants for the first time and carried out many complex accounting matters only with a simple click, many people looked at these systems with skepticism and preferred to register and then analyze their daily accounts with accounting books and pens. If today someone is asked to compare between accounting office and computerized accounting systems and assess both of them even if the person lacks the expertise, computer systems will be recommend. Today this system may be criticized, but comprehensive health system will give you so many facilities that you will have no choice but to choose this system.

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Narmada river water quality assessment using benthic macro-invertebrates at Barwani, Rajghat Madhya Pradesh

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ABSTRACT

River Narmada is one of the 13 prominent rivers of India, which covers 98,797 sq km of total water-shed area. Narmada is considered to be the lifeline of Madhya Pradesh and is one of the most important west flowing rivers of India. The monitoring of water quality of Narmada river was carried out for one year August 2011 to July 2012. Barwani sampling stations were selected at downstream of Narmada River. The water samples collected were analyzed, as per standard methods parameters such as pH, Turbidity were measured in-situ. Raised values of physico-chemical parameters indicate the pollution of riverine ecosystem due to domestic wastes, municipal sewage, industrial effluent from Security Paper Mill (SPM) and agricultural run-off that influence the water quality directly or indirectly. Statistical analysis carried out through correlation method and also evaluates Average values (AV), Standard Deviation (SD), Standard Variance (SV),Standard Error (SE) and 95% Confidence Limit (CL) to assess the pollution load assessment. The results revealed that most of the water samples were below or out of limited; according to the WHO, BIS standards.

KEY WORDS: STATISTICAL ANALYSIS, NARMADA RIVER, WATER POLLUTION, RIVER WATER QUALITY, BARWANI, PHYSICO-CHEMICAL ANALYSIS

INTRODUCTION

Macro-invertebrates are most frequently used in bio-monitoring studies because the responses of macro-invertebrates to organic and inorganic pollution have been extensively documented (Thorne and Williams., 1997; Kazanci. and Dugal., 2000). They have sensitive life stages that respond to stress and integrate effects of both short-term and long-term environmental stressors (EPA., 1998) and they are important areas for maintaining biodiversity (Meyer et al., 2007 Richardson and Danehy 2007). The study of benthic macro-invertebrates provides a method to determine the water quality of a stream based on collection and identification of stream-bottom (benthic)

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macro-invertebrates. This study has been done to find out the diversity of benthic macro-invertebrates. Benthic study in Malwa region of Madhya Pradesh is scare except that of Varshney et al., 1976, Rao et al., 1985 , Sunny and Diwan., 1991 Sharma 2016, Sharma et al., 2007, Khichi et al., 2017).

MATERIAL AND METHODS

Description of Study Area The Narmada river is considered as the life line of Madhya Pradesh. The catchment area of the river exists in the States of Madhya Pradesh (86.18%), Gujarat (11.6%), Maharashtra (1.5%), and Chhattisgarh (0.72%). During its course, the river drops from an elevation of 1051 m to sea level, and flows through narrow gorges in the head reaches. The basin is bounded on the north by the Vindhya ranges, on the east by the Maikal range, on the south by the Satpura ranges and on the west by the Arabian Sea. Deep black soil covers the major portion of the basin. The river has 41 tributaries, of which 22 are on the left bank and 19 are on the right bank. The Barna, Tawa, Kolar, and Sukta dams have been constructed on the tributaries. The Bargi is constructed on the mainstream, while the Indirasagar, Omkareshwar, Maheshwar and Sardar Sarovar dams are under construction.

SAMPLING STATIONS BARWANI

Barwani, also known as Siddh Nagar, is an important city and a municipality in Barwani district in the state of Madhya Pradesh, India. The place is also famous for chool giri, Jain pilgrimage center of Bawangaja. The town is situated near the left bank of the Narmada river. Latitude (DMS) 22° 10', 60"N and Longitude (DMS) 74° 54', 0 " E.

PHYSICO CHEMICAL ANALYSIS OF WATER

The water samples were collected from the sampling station Barwani ,for the period of 12 months from August 2011 to July 2012. In the analysis of the Physico-chemical properties of water, standard methods prescribed in limnological literature were used. Parameters like Temperature, pH and Turbidity were determined at the site, while other parameters like Biochemical oxygen demand, Chloride, Alkalinity, total Solids were determined in the laboratory. The Physico- Chemical parameters of water were determined as per standard methods of APHA (2005), Welch (1998), Golterman (1991).

RESULTS AND DISCUSSION

WATER TEMPERATURE

The oxidation of organic matter is highly influenced by the temperature of water. Temperature of river water depends upon the season, climatic zone, where river is flowing, time of sampling and also upon the temperature of the effluents, which are being added in the river. Temperature fluctuation during August 2011 to July 2012 was as follows.

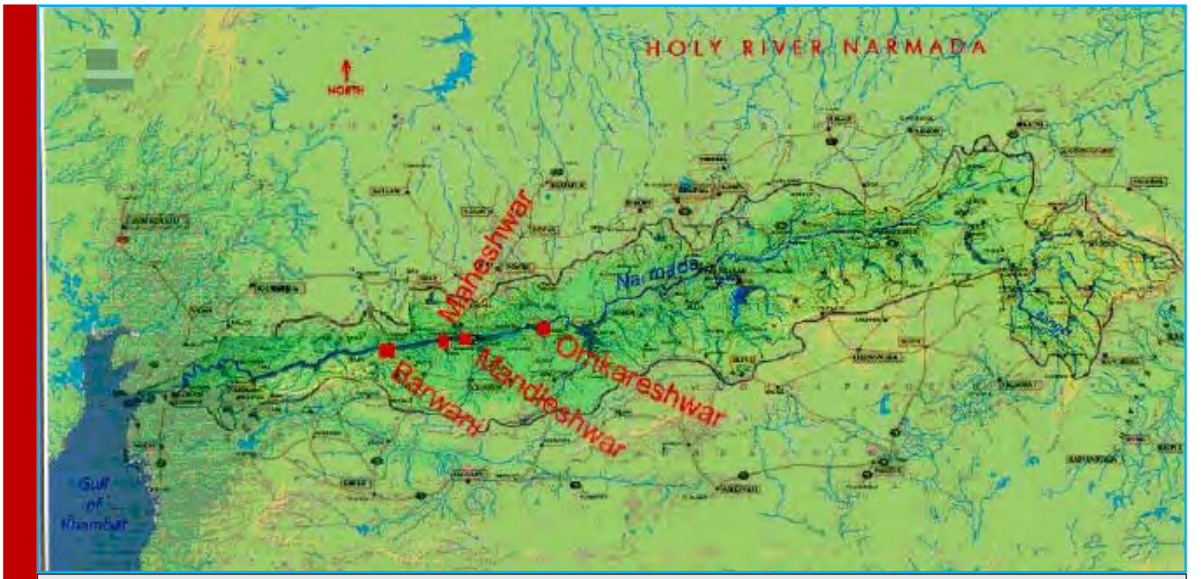
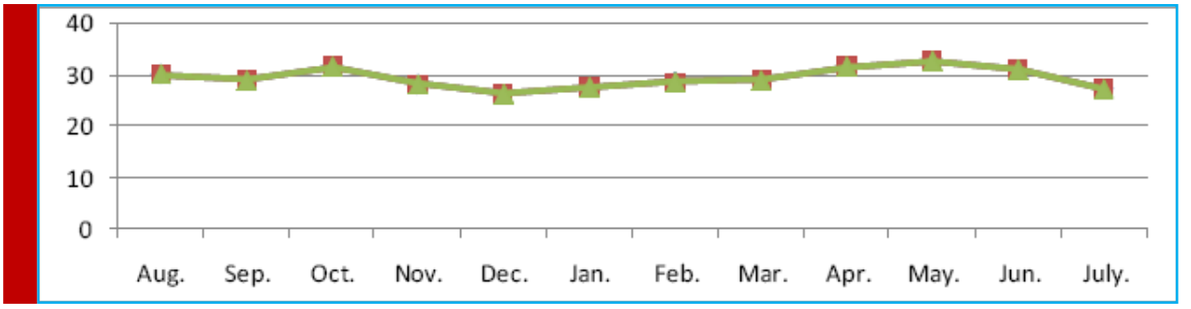


FIGURE 1. Map of Narmada river showing Sampling Stations

Table 1. Monthly fluctuation in water temperature (°C) of narmada river from August 2011 to July 2012 at barwani.

Aug.	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.	Jun.	Jul.	Avr.	Standered Ddeviation
30	28.8	31.5	28	26.2	27.5	28.5	28.8	31.5	32.6	30.9	27.10	27.85	1.91

The minimum water temperature was 26.2°C in December2012 and the maximum was reported 32.6°C in May 2012 at Barwani. The same observation were also reported by. Bhutaiani et al. (2016), Sharma et al (2011) and Shraddha et al (2008) in Narmada river, while studying the hydrological parameters of Narmada river at Hoshangabad recorded water temperature between 27.6°C to 38.4°C.



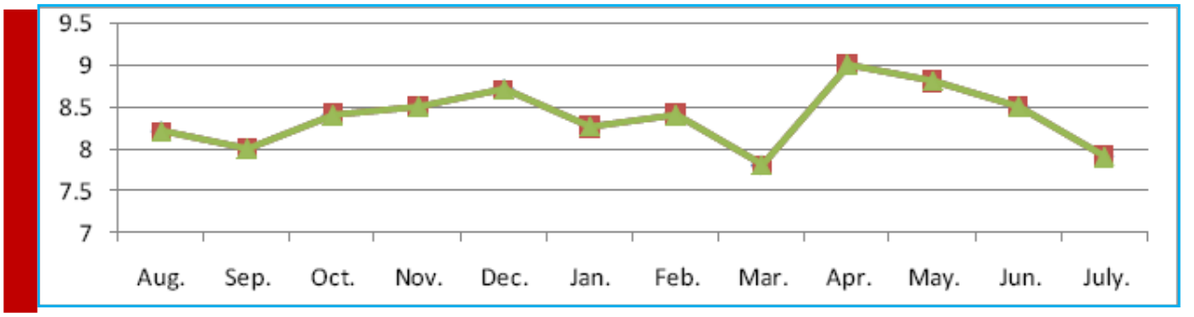
PH RANGE

pH is an important parameter which is important in evaluation the acid base balance of water. Natural waters generally have been found to range from 5.5 to 8.6 because of the presence of bicarbonates and carbonates of alkaline earth metals. Drinking water with a pH range from 6.5 to 8.3 has been necessary. During August 2011 to July 2012 the pH was fluctuated as follows.

Table 2. Monthly fluctuation in water pH of narmada river from August 2011 to July 2012 at barwani.

aug.	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.	Jun.	Jul.	Avr.	Standered Ddeviation
8.2	8	8.4	8.5	8.7	8.26	8.4	7.8	9	8.8	8.5	7.9	8.37	0.34

The minimum in March 2012 and the maximum in May 2012 at Barwani. Sharma et al (2011) observed pH fluctuation between 7.6 to 9.9 in Hoshangabad area of Narmada river. Prasanna and Ranjan (2010) observed pH value between 7.5 to 8.5 in Dharma estuary.



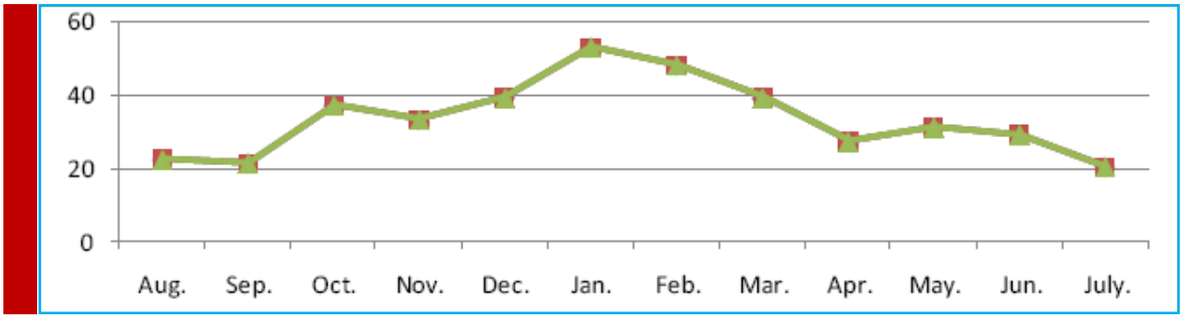
TRANSPARENCY

Transparency has been long known to hinder disinfection by shielding microbes, some of them perhaps pathogens. This is most important significance of transparency monitoring and therefore it has been an indication of effectiveness of filtration of water supplies (Hauser 2001). During August 2011 to July 2012 transparency was fluctuated as follows.

Table 3. Monthly fluctuation in water transparency mg/l of narmada river from August 2011 to July 2012 at barwani.

Aug.	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.	Jun.	Jul.	Avr.	Standered Ddeviation
22	21	37	33	39	53	48	39	27	31	29	20	33.16	10.56

The minimum in July 2012 and maximum in January 2012 at Barwani. These observations were also supported by Prasanna and Panda (2010), Shraddha et al (2008) Tiwari, M and Dwivedi, A (2016) and Trivedi et al (2009).



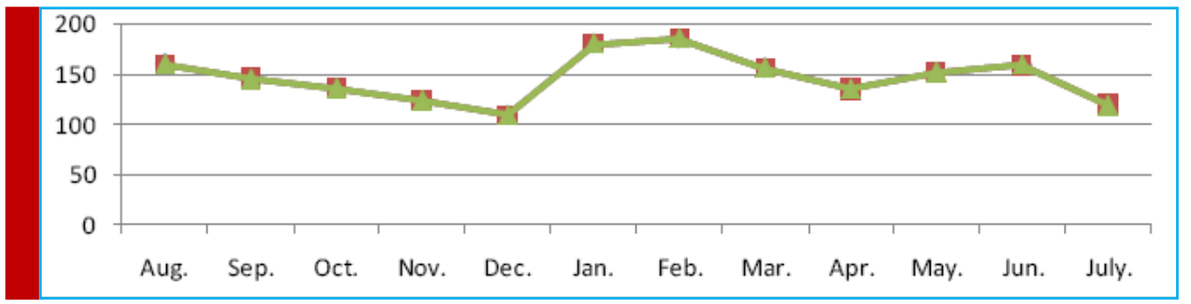
TOTAL DISSOLVED SOLIDS

The total solids are the total amount of chemical substance present in the water. The total dissolved solids and total suspended solids together make the total solids in the water. The presence of solids in water vary greatly at different times and affect the density of water and there by the quality of aquatic environment. During August 2011 to July 2012 the value of total solids varied as follows.

Table 4. Monthly fluctuation in water total dissolved solid mg/l of narmada river from August 2011 to July 2012 at barwani.

Aug.	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.	Jun.	Jul.	Avr.	Standered Ddeviation
160	145	136	124	109	180	185	156	135	152	159	119	146.6	22.31

The minimum value was recorded in December 2011 and maximum in February 2012 at Barwani. Nduka et al (2008) also recorded total solids between 100 to 220 mg/l in Niger delta of Nigeria and Khanna, D.R, and Rawat, S., Bhutiani, R., (2014).



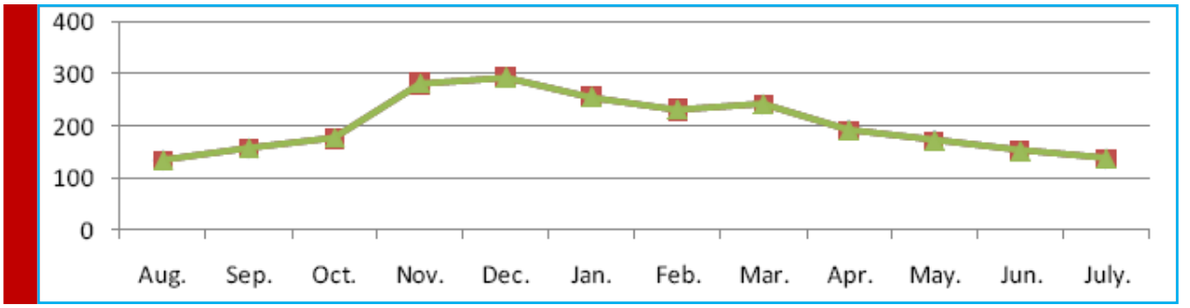
ALKALINITY

Alkalinity measures the buffering capacity of water and content of CO2 in its various forms are involved in this carbonate-bicarbonate carbonic acid buffering system. In the present study the value of Alkalinity varied as follows.

Table 5. Monthly fluctuation in water alkalinity of narmada river from August 2011 to July 2012 at barwani.

aug.	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.	Jun.	Jul.	Avr.	Standered Ddeviation
132	155	175	280	293	255	230	240	190	170	150	135	146.6	22.31

The minimum value in Auguest 2011 and maximum in December 2011 atBarwani. Trivedi et al (2009) also observed the same value in Ganga river India.



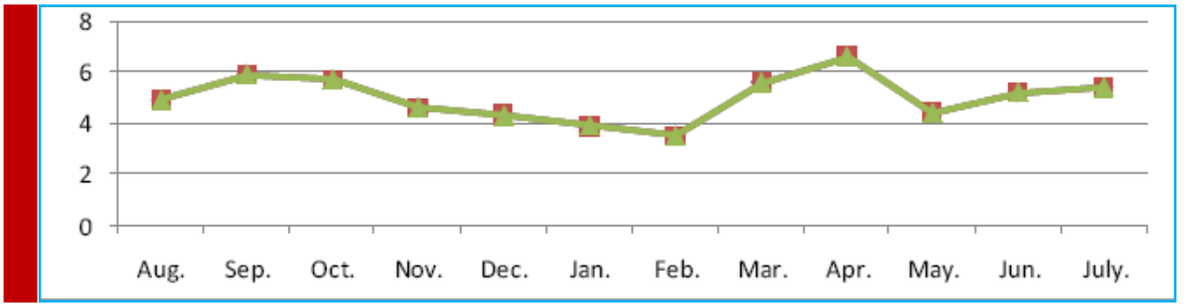
BIOCHEMICAL OXYGEN DEMAND

Biochemical oxygen demand is the amount of oxygen utilized by microorganism in stabilizing the organic matter in aerobic condition. DO measurement forms the basis of BOD analysis. It gives an indication of load of biodegradable organic material present in the water body. During the present study the BOD was fluctuated as follows.

Table 6. Monthly fluctuation in water biochemical oxygen demand mg/l of narmada river from August 2011 to July 2012 at barwani.

Aug.	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.	Jun.	Jul.	Avr.	Standered Ddeviation
4.9	5.9	5.7	4.6	4.3	3.9	3.5	5.6	6.6	4.4	5.2	5.4	5	0.86

The minimum BOD was recorded February 2012 and maximum in April 2012 at Barwani. Same observations were also recorded by Nnaji et al (2010) Katakwar, M (2014) and Mary et al (2008).



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Biofuel energy: Resources, production and its impact on environment

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ABSTRACT

In order to fulfil the energy demand of world, there is a need to adopt a new energy system which is based on use of renewable energies resources. Emphasis should be on integration of the various forms of renewable energy like solar energy, geothermal energy, wind energy and biofuels energy. These energy resources have potential to replace the dependency of world on fossil fuels if proper policies for utilization of these resources have to be adopt. Change in climate and independency of energy are master stroke for bioenergy development. Many researcher have focused on the biofuels performance in reducing carbon emissions, with questions marks on their potential in this respect. Governments of many countries are setting targets to enhance the proportion of biofuels in their energy mix for the purposes of fulfill their energy security and rural development. There are lot of benefits of biofuels energies like job creation and diversification of the economy in rural areas, reliable market for agricultural produce especially for energy crops especially in developing countries. In present review different biofuels sources with their impact on environment and amount of production biomass in world has been discussed

KEY WORDS: BIOFUELS, ENERGY, BIODIVERSITY, GREEN ENERGY, BIOMASS

INTRODUCTION

In last few decade world has obtained a period of intense economic growth. With economic growth the requirements of energy also increases securing future energy supplies in a shrinking energy market is vital if this is to continue. After several year when oil reserves have shown a pronounced drop, then it is highly necessary to find new sources. In last three decades the world is moving towards renewable energy resources such as solar energy, wind energy, geothermal energy and biofuels energy. The main concern of all these is that they are green sources of energy that is they have negligible harmful effect on environments

The term biofuels is being appropriated to refer just to fuels like ethanol and biodiesel, it should ideally imply

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fuels from plant-based sources, which can be produced, processed and consumed in diverse forms. Bioenergy can be defined as energy produced from any source of biomass; i.e. plants, animals, and organic waste. Biofuels account for only a small proportion of energy use, currently providing approximately 1% of the global energy supply and approximately 4% of the global liquid transport fuel use. Despite its small current contribution to transport energy, the production of liquid biofuels has increased rapidly in recent years. This increase can be attributed in part to government targets and subsidies example EUs renewable energy target, which have been established to promote the use of biofuels for reasons of energy security, climate change mitigation, and rural development

SOURCES OF BIOFUEL ENERGY

The supply of biomass can be classified into two sections traditional biomass and modern biomass. Traditional biomass implies the use of sources like wood, crop residues, animal dung, and charcoal used for heating and cooking at the household level. Traditional biomass is an informal activity as it is generally collected by women and children from public lands or privately owned lands. Liquid biofuels for transportation like ethanol and biodiesel are one of the fastest-growing sources of alternative energy in the world today which comes under modern sources of biofuels. Sources of biomass energy can be categorized in the following three category : Agriculture, Forestry and Waste.

Conversion of agricultural residue to bioethanol by enzymatic treatments is done in many crops, (**Prasad et.al 2007**). India is the largest producer and consumer of coriander in the world. A coriander crop residue in India is estimated to produce 188.3 kT/Yr biomass having 22 MW power potential and 5.2 Mcal/sec. calorific potential, **(Anil et. al 2015)**. This calorific value can be converted in bio-ethanol. Coriander is cultivated both for leaf as well as for seed; biomass in form of leaf of young plant is consumed, whereas the crop residue of the seed purpose coriander is left as an agricultural waste. The general practice adopted by farmers for management of that residual waste is using it for composting, or it is being burnt in the fields as it is not preferred by animals for feeding. Conversion of this biomass to bio-ethanol can be an opportunity for making biofuel. Biomass is coriander is highly variable as per the variety sown, production technology adopted, nutrient application, and environmental condition

Sugar and starch-based crops: Crops rich in sugar and starch like sugarcane and corn (maize), respectively, supply almost all the ethanol that is produced today. Other major crops being used include, wheat, sorghum, sugar beet, and cassava **(**Moonmoon **et al. 2014)**. Technologies for conversion of sugar and starch are also the most technologically and commercially mature today

Most bioenergy systems can be explained using the schematic shown in figure 1

FIGURE 1. Sugarcane (Inhouse)

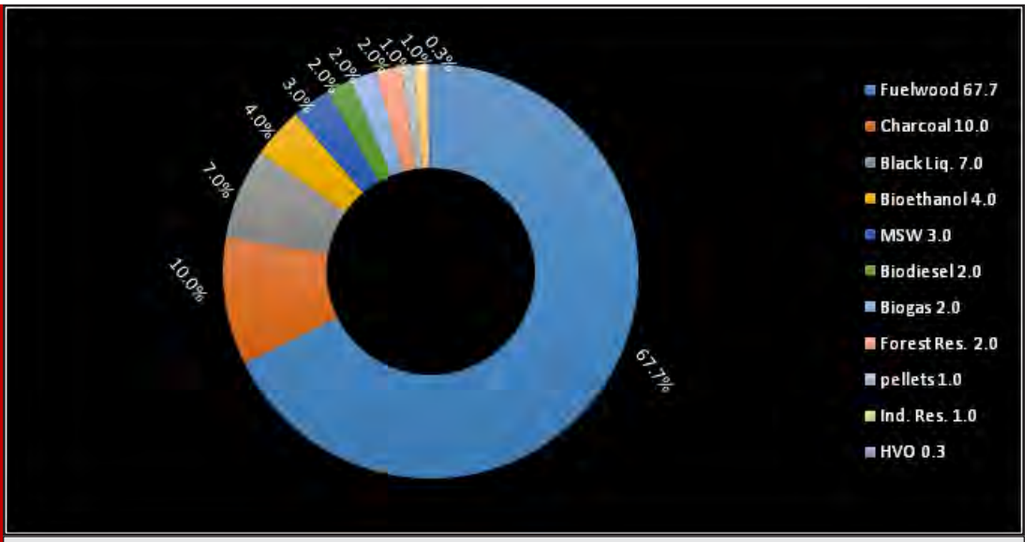

FIGURE 2. Primary energy supply of biomass resources globally in 2013 (WBA global bioenergy statistics 2016)

Palm (*Arecaceae*): Palm oil can be used to create biodiesel. It can be used as a simply processed.

Palm oil mixed with petro diesel, or can be processed through trans esterification to create a palm oil methyl ester blend **(Senthil et.al 2015),** Byproduct of trans esterification is glycerin. The real process used to produce biodiesel all over the world is different in different countries and as per the requirements of different markets. The next-generation biofuel production processes are being tested in relatively small trial quantities.

Oilseed crops: Biodiesel is produced from oilseed crops like rapeseed, soybean and oil palm but like sugar and starch crops, oilseed crops are also characterized by low yield and high use of inputs.

Wood: Wood is mainly used for cooking and heating at the household level and for producing electricity at a small scale. When used directly at the household level, it is generally collected from forests or other lands. Commercial plantations of woody trees like poplar in temperate zones and eucalyptus and acacia exist today albeit on a small scale. Today the predominant use of commercial plantations is for the supply of wood to paper and pulp industries. Future cellulosic technologies, which permit the conversion of wood to ethanol **(Mohan et al 2006),** may compete with general uses of wood.

FIGURE 3. Palm tree (**Arecaceae**) [Source book on REN. Energy ISBN NO: 978-93-81191-09-9]

FIGURE 4. Jatropha (*Jatropha curcas)* (Source book REN. Energy 2017)

Pongamia pinnata: Pierre is a fast-growing leguminous plant with the potential for high oil seed production and it has the ability to grow on marginal land. All these properties support the suitability of this plant for large-scale vegetable oil production required by a biodiesel industry. The future success of *P. pinnata* as a source of feedstock for the biofuels industry is dependent on an extensive knowledge of the genetics, propagation and physiology of this legume. Moreover *P. pinnata* has recently been recognized as a viable source of oil for the burgeoning biofuel industry.

Wastes and residues: The global annual potentional bioethanol from crop residues and waste crops is 491 GL [IEA 2017]. After the processing of one ton of the main product, 1.5 tons of crop residues are generated [**Neetu et. al** 2015]According to a report there are about 73.9 million tonnes of dry wasted crops and about 1.5 billion tonnes of dry ligno-cellulosic biomass from seven crops namely, oats, barley, maize, rice, sorghum, wheat, and sugarcane. These could yield around 490 billion liters of ethanol or around 30% of global gasoline use today (**Kim et al 2008**). 2.12 EJ of waste was converted to energy globally – large part of it was from municipal waste from households and industries also the waste to energy sector has increased at an annual rate of 4% during 2000 – 2014.55% of all waste to energy conversion occurs in Europe while the rest is in Asia and Americas, (**WBA report 2017**).

Jojoba (*Simmondsia chinensis)*: Jojoba oil is the liquid wax produced in the seed of the jojoba (*Simmondsia chinensis*) plant, a shrub native to southern California, southern Arizona, and northwestern Mexico. The oil makes up about 50% of the jojoba seed by weight. Unrefined jojoba oil looks like a clear golden liquid at room temperature with a slightly fatty odor. Refined jojoba oil is odorless and colorless. The melting point of jojoba oil is approximately 10°C and the iodine value is approximately 80, **(Lou et al 2017)** Jojoba oil is comparatively shelf-stable when compared with other vegetable oils because it does not contain triglycerides, unlike most other vegetable oils such as grape seed oil and coconut oil. It has an Oxidative Stability Index of about 60, which means that it is more shelf-stable than oils of canola oil, safflower oil, almond oil or squalene but less than coconut oil and castor oil.

Jatropha (*Jatropha curcas)*: Jatropha is a genus of flowering plants in the spurge family, Euphorbiaceous, (Divakara et. al 2010). Most of these plants are native to the Americas, with 66 species found in the Old World. Mature plants produce male and female flowers separately. In 2007 Goldman Sachs acknowledged *Jatropha curcas* as one of the best candidates for future biodiesel production. Healthy seeds (Bold) showed higher oil content (29.5 % to 36.8%) as compared to the Shrunken seeds (3.5% to 8.8% (Aruna et. al 2017).

On crushing the jatropha seeds, the resulting jatropha oil can be processed to produce a high-quality biodiesel **(Kazi et al 2010)** that can be used further in a standard diesel car, while the left over residue (press cake) can also be processed and used as biomass feedstock to power electricity plants or used as fertilizer. However, despite their abundance and use as oil, none of the Jatropha species have been properly domesticated, hence their productivity is variable, and the long-term impact of their use on soil quality and the environment is unknown. Estimates of Jatropha seed yield vary widely, due to the lack of research data, genetic diversity of the crop, the range of environments in which it is grown, and Jatropha's perennial life cycle. Seed yields can range from 1,500 to 2,000 kilograms per hectare, corresponding to extractable oil yields of 540 to 680 liters per hectare (**Bhupender et al 2012**) or 58 to 73 US gallons per acre. Time magazine 2014 acknowledged the potential for as much as 1,600 gallons of diesel fuel per acre per year.

IMPACTS ON ENVIRONMENT AND BIODIVERSITY

The biodiversity impact of biofuels will depend on the biofuel crop and the previous land use. Biofuels can be beneficial to biodiversity when appropriate crops are grown in suitable areas. Furthermore, if they contribute to climate change mitigation, they have the potential to be indirectly beneficial to biodiversity as a whole. However, biofuels have already been shown to negatively impact biodiversity when direct conversion of natural ecosystems or indirect land conversion of non-degraded land occurs. The expansion of biofuel production in the tropics has resulted in the loss of tropical forest and wetlands, and in temperate regions biofuel production has encroached into set-aside lands. Biofuel feedstock plantations (particularly oil palm and maize plantations), have been shown to support far lower levels if biodiversity than natural ecosystems, and can cause soil erosion and the pollution of watercourses. How a feedstock plantation is managed influences the level of biodiversity impacts. Well managed plantations can in some instances prove beneficial to biodiversity especially if these are on degraded or marginal lands. Biofuel targets have largely been set as part of renewable energy policies in the context of climate change mitigation. Biofuels can undoubtedly contribute to climate change mitigation when grown in appropriate areas. For example, recent studies have suggested that when sugarcane is used to produce ethanol, 80-100% greenhouse

gas savings could be achieved **(**Howarth et al 2009), and that oilseed rape production for biodiesel can similarly achieve emissions savings of 20-85% **(**Howarth et al 2009).Where biofuels achieve real emissions reductions, this would have biodiversity benefits through reducing climate change impacts, (UN-Environment report 2017).

This is an important trade-off to keep in mind when considering some of the potential negative impacts on biodiversity resulting from the cultivation of feedstocks. Moreover, the time frame, over which the impacts on biodiversity resulting from biofuel production are examined, also needs to be considered. However, recent research has suggested that the production of energy crops may do little to mitigate climate change where they replace natural ecosystems; even increasing emissions by as much as 17-420 times compared to that of fossil fuels **(Fargione et al 2008**). One recent study has estimated that land conversion for biofuels could result in emissions of 753-1825 Mt CO2 per year, compared to the 840 Mt CO2 that is emitted from 10% petrol consumption **(Ravindernath et al 2009).** Furthermore, although the use of palm oil can achieve large greenhouse gas emissions savings, the conversion of rainforest and peat soils can actually result in 800-2000% higher emissions than equivalent fossil fuels.

Net carbon reduction: This refers to the net reduction in carbon emissions resulting from the consumption of a unit of biofuel. Biofuels are generally expected to result in lower net addition of carbon to the atmosphere because the carbon emitted on combustion is eventually sequestered during re cultivation, whereas carbon emitted during combustion of fossil fuels is more **(Kevin et al 2011).**

Net petroleum offset: This refers to the reduction in petroleum consumption that can be achieved by using biofuel. One way to measure the net petroleum offset is to calculate the number of gallons of gasoline displaced by one gallon of ethanol. This indicator can be useful for studying the implications of biofuel for oil depletion, oil imports, etc.

Reducing land conversion: biodiversity impact will largely depend upon the previous land use. Although planting second generation biofuels on degraded land could reduce natural land conversion whilst providing soil stability, nutrients, and increasing water retention. It has been estimated that yields of grassland perennials are 50% higher if grown on fertile land, **(**Ji X and Long et al 2016). Plantations on degraded land would therefore have to be properly incentivized, and it has been suggested that regulations would be required to stop crops such as switchgrass being grown like traditional biofuels. Land compatible with switchgrass is the same as that currently set-aside under the USDA Conservation Reserve Programmed, and it is likely that biodiverse 'prairies', pasture land and cropland would be required to meet biofuel targets. Similarly, incentives for short rotation coppice (SRC) could result in the conversion of forest to poplar and willow plantations.

Conversion of degraded or marginal lands: When appropriate crops are planted in suitable areas, they can actually benefit biodiversity. This is particularly true where biofuels are grown on marginal and degraded lands. They can increase soil productivity, reduce soil erosion, reduce pressure on natural ecosystems, and create habitats. Jatropha is one biofuel crop that can be grown on degraded land and is receiving increasing attention, particularly in Africa and India. It has been suggested that this crop can be grown by traditional pastoralists under traditional systems that maintain biodiversity and it is not a crop used for food production. However, its land and water requirements and suitability for large scale production are yet to be fully determined, and reports suggest that it will grow better on more productive land meaning that production on degraded land would need to be incentivized. On the other hand, some countries currently have biofuel crops on no sensitive lands or still have large expanses of such land available for energy crop production (UNCTAD 2016).

Temperate ecosystems: The European Environment Agency is concerned that the profitability of biofuels will outweigh the incentives for farmers to participate in agro-environmental schemes (House of Commons Environmental Audit Committee 2016). This is a significant issue for biodiversity, particularly for bird species, as it has been estimated that set-aside lands in the US have bird nesting rates that are ten times higher than those on cropland. In Europe, encroachment into set-aside land by oilseed rape and sugar beet threatens semi-natural steppes and long fallow dry cereal systems, which are among Europe's most biodiverse habitats. This has been linked to a decline in key habitats for endangered species (WWF Report 2016) and to declines in farmland birds (Brown et al 2008). A recent study of the potential biodiversity impacts of biofuel policy in Europe has suggested that although impacts will vary spatially and depend upon crop choice, they will be negative across all taxa and particularly so for mammals.

Post land conversion impacts: the true environmental impacts of biofuels are often overlooked. Life cycle analyses (LCAs) provide a mechanism for these impacts to be assessed. Along with greenhouse gas emission, LCA provides a mechanism for investigating biofuels' potential for acidification, eutrophication, toxicity, photochemi-

cal ozone, and ozone and resource depletion. However, few LCAs provide a comprehensive coverage of potential environmental impacts and impacts on biodiversity are not covered due to a lack of indicators Although overlooked, the pollution from fertilizers and pesticides is likely to be another major negative biodiversity impact associated with biofuels; particularly for aquatic ecosystems (Sala et al. 2009).

One study on the environmental impact of sugarcane plantations has shown that soil erosion is high in plantations in comparison to forest and pasture, and that the resulting sediments are deposited into wetlands, rivers, and streams. The same study noted that watersheds in major sugarcane areas had only 13-18% of the original riparian vegetation, which has led to decreasing small mammal species richness (Johnston *et al*. 2009). However, the cultivation of appropriate native species could result in less pollution, water stress and provide wildlife habitats .The environmental impacts of corn-based ethanol (a fertilizer and pesticide intensive crop) are thought to be the highest of any agricultural crops in the US. Again, many of these impacts are felt downstream, making it difficult to quantify. For example, ethanol production in the Mississippi River system has been linked to hypoxia in the Gulf of Mexico. In addition, biofuel crops can cause water stress. Many of the crops require heavy irrigation, which can involve the drainage of wetlands and use of water from rivers and lakes, with consequent implications for biodiversity (AEA Technology 2008).

Despite this, in-depth studies into the water footprints of different biofuel crops are only just becoming available. There is also significant concern that some biofuel crops will be invasive in some areas. For example, a study in Hawaii has suggested that 70% of regionally suitable biofuel crops have a high risk of becoming invasive, compared to only one quarter of the non-biofuel plant species assessed (**Carolina et**.al 2011). Biofuels that do not require high-energy inputs and can be grown in polycultures of native species will be more biodiversity friendly than monocultures. This does not apply to most of the first generation biofuels, but it has been suggested that the development of second-generation biofuels such as grassland perennials could potentially lead to more 'biodiversity friendly' biofuel production monocultures. Choice of crop and management regime is therefore an important element in determining whether impacts on biodiversity are positive or negative. The 'off farm' impacts of biofuel production will be similar to those observed from conventional agriculture, a full analysis of which is beyond the scope of this report. These impacts have been reviewed and related to biofuels in a report by AEA Technology for the UK government (AEA Technology 2008).

PRODUCTION

With the recent famous climate agreement COP21 in Paris, will be the driver of the investments in bioenergy. The agreement will provide highly needed impetus in shifting from fossil fuels to renewables. Greenhouse gas (GHG) mitigation is also a major driver for development of bioenergy markets, in recent years. Earlier it was often noticed that the security of supply and high import costs of fossil fuels was the main argument. Now, the substitution of fossil fuels, and the reduction of fos-

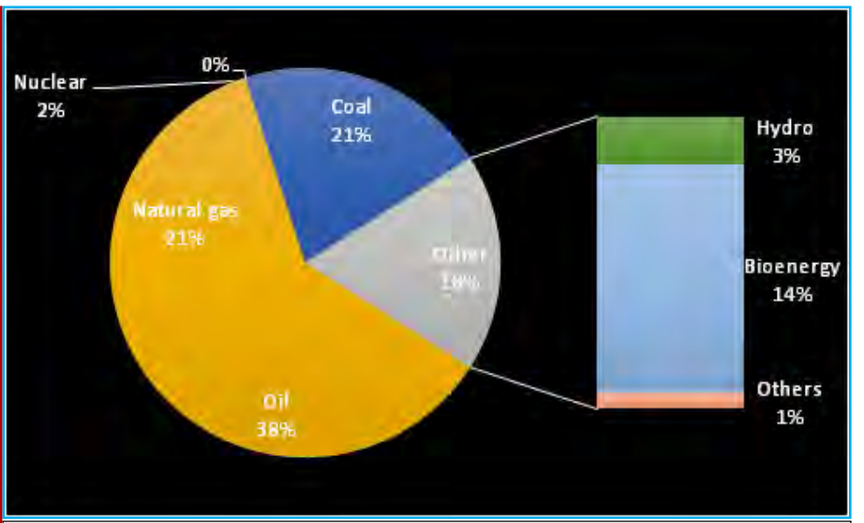

FIGURE 5. Global final energy consumption in 2013 (WBA global bioenergy statistics 2016)

Table 1. Ten countries for ethanol and biodiesel mandatory mix (WER Bioenergy report (2016) by WEC)

Sr. No	Country	Ethanol blend percentage	Biodiesel blend percentage
1	Brazil	27.5%	7%
2	Paraguay	25%	1%
3	India	5%	-
4	Jamaica	10%	-
5	Philippines	10%	10%
6	Argentina	10%	5%
7	Costa Rica	7%	20%
8	EU	10% (food crop biofuels limited to 7%)	-
9	Canada	National: 5%	National: 2%
10	China	10% (in nine provinces)	-

sil carbon emissions are major arguments and benefits for bioenergy. Another major driver is the carbon taxes, a key instrument for energy transition. Carbon tax is a simple and effective tool to reduce fossil fuel use and increase energy efficiency. It can be tax neutral as other taxes like income tax can be reduced. This will also lead to a more sustainable lifestyle and investment for the future. To put the Paris agreement into concrete action, there has been a global call for introduction of carbon pricing. It was one aspect in which everyone was in agreement including companies, NGOs, governments etc. Efforts are underway for initiating a global carbon price. Many countries, for example Sweden, have successfully implemented the carbon tax leading to an increase use of bioenergy.

In Europe and North America the use of biomass for electricity is prominent. Highly produced from forestry products and residues. Cogeneration plants enable the use of biomass with increased efficiency, so much so that the combined efficiency of producing heat and electricity more 80%. The Europe and Americas continent contribute more than 70% of all consumption of biomass for electricity. In 2013, 462 TWh of electricity was produced globally from biomass (**WBA Report 2016**). In the past few years, biomass is work more effective in developing countries in Asia and Africa where popula-

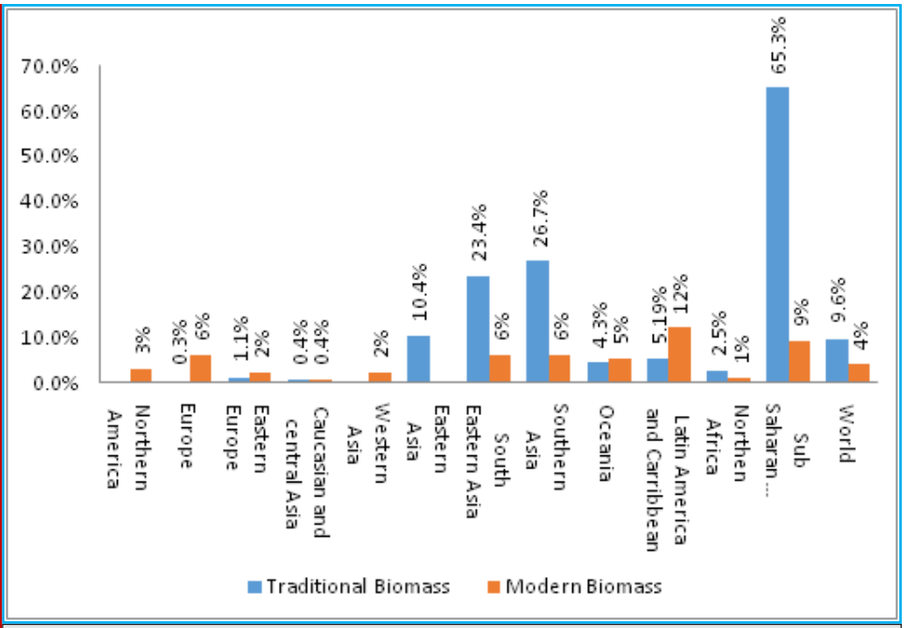

FIGURE 6. Share of biomass in final energy consumption in 2010 (in %) (WBA global bioenergy statistics 2015)

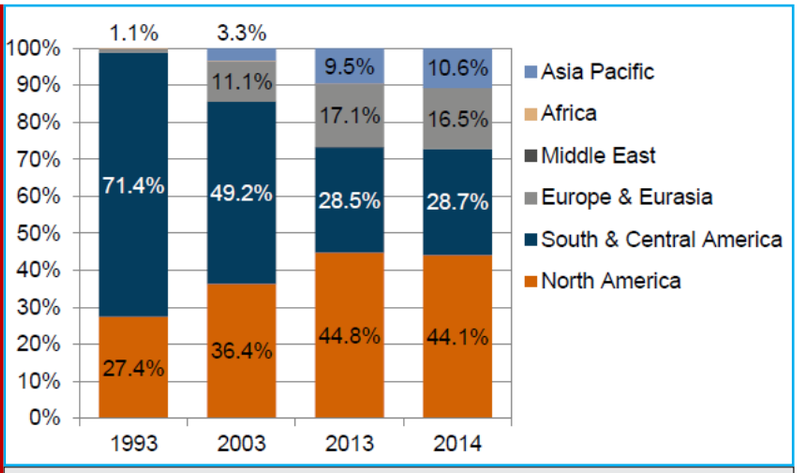

FIGURE 7. share of biofuels production by region(Source: BP (2015) Statistical Review of World Energy)

Table 2. Biomass production and gross electricity generation from biomass by different countries (source WEC 2016)

Year	Country	Biomass production in tonne of oil equivalent	Gross electricity generation from biomass in tonne of oil equivalent
2013	Argentina	2261919	212038
2013	Austria	4376995	295529
2013	Belarus	1505136	7395
2014	Belgium	1104090	226226
2013	Benin Republic	2230917	86
2013	Bolivia	999403	4816
2013	Brazil	67195353	3438264
2014	Bulgaria	1086510	11866
2013	Cambodia	3999929	946
2013	Cameroon	4778184	5847
2013	Canada	11468425	373517
2013	Chile	10235383	491918
2013	China	200962120	3293208
2013	Colombia	3929947	170164
2013	Costa Rica	674501	15736
2013	Cote d'Ivoire	9640896	5847
2013	Cuba	1235049	58470
2014	Czech Republic	2300994	171282
2014	Denmark	1304505	254343
2013	Ecuador	554481	25452
2013	El Salvador	779450	34136
2013	Estonia	1122099	62855
2014	Finland	8066400	943079
2014	France	9073899	140843
2013	Gabon	1262850	860
2014	Germany	11424692	1020465
2013	Greece	869208	18573
2013	Guatemala	7561432	154515
2013	Honduras	2258073	62425
2013	Hungary	1402790	146346
2013	India	398969	179544
2013	Indonesia	54240686	19605
2013	Iran	498448	1978
2013	Ireland	210400	22528
2013	Israel	20350	7223
2014	Italy	6539410	328719
2013	Jamaica	493838	16681
2013	Japan	8461069	2760706
2013	Kenya	15392	15392
2014	Luxembourg	65803	1806
2013	Malaysia	3505040	95271
2013	Mauritius	209015	40671
2013	Mexico	8867632	87619
2014	Netherlands	1290103	180568
2013	New Zealand	1081471	33964
2014	Norway	840499	1032
2013	Philippines	7793375	13070
2013	Poland	6179493	787705
2013	Republic of Korea	952542	42993
2014	Romania	3645696	39037
2013	Slovakia	759507	78762

2013	South Africa	15554123	25624
2014	Sweden	8958107	780482
2013	Switzerland	1002437	26140
2013	Tanzania	20049036	1806
2013	Thailand	22577936	528031
2013	Turkey	3152193	3010
2013	Ukraine	1880769	8685
2014	United Kingdom	3047794	1281858
2013	United States of America	51375036	3915306

tion is dense and access to electricity is low. There is a cost competition in Biogas and decentralized bioenergy systems. It is found that cogeneration plants using agricultural residues like Bagasse in India, Mauritius, Kenya and Ethiopia are successful.

According to REN21 2015 global status report USA National: The Renewable Fuels Standard 2 (RFS2) requires 136 billion liters of renewable fuel to be blended yearly with transport fuel by 2022. Also Table 1 show Ten countries where ethanol and biodiesel mandatory mix

CONCLUSION

Bioenergy reduced dependency of countries on fossil fuels and enhance in a gradual decarburization of the energy system. In present date countries like Finland and Sweden have successful utilization of bioenergy. Their sustainable use forest products or forestry make them to be world leaders in renewables. Data show Brazil has maximum blending of biofuels in the transportation sector and becoming independent of oil. This is possible only because of effective policies such as carbon taxes, blending mandates and high investments in research and development. These policies have been driven by strong support from the technical institution, associations and companies. Also Countries in Asia and sub-Saharan Africa has led to an increasing dependence on biomass. It has provided much energy to rural areas which is highly needed and is the source of livelihood in many countries

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Description of a new species of a Cestode parasite, *Circumoncobothrium devidasensis*, from a teleost fish *Mastacembelus armatus*

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ABSTRACT

The genus *Circumoncobothrium* was erected by G.B.Shinde in 1968, as a type species *Circumoncobothrium ophiocephali* from the intestine of fresh water fish *Ophiocephalus leucopunctatus*. The present form deals with the description of a new species *Circumoncobothrium devidasensis,* a Cestodian parasite from a teleost fish, *Mastacembelus armatus*. The worm under discussion is having scolex, long bluntly rounded rostellum with rostellar hooks and contains two bothria. The neck is short, mature segment medium in size, testes and ovary also medium in size. The species shows moderate differences in its morphology and reproductive organs, when compared with the other known species. The above noted characters are valid enough to erect a new species and hence the name *Circumoncobothrium devidasensis* for this new species is proposed in the honor of authors father.

KEY WORDS: *CIRCUMONCOBOTHRIUM DEVIDASENSIS, MASTACEMBELUS ARMATUS*, MANJRA DAM

As the genus *Circumoncobothrium* was erected by G.B. Shinde, in 1968. Latron in 1976 Chincholikar added two new species of the genus *C. shindei* from fresh water fish *Mastacembelus armatus* and *C. bangariusi* from *Bangarius species*. Shinde in 1977, added a new species *C. khami*. Jadhav and Shinde in 1980 added two new species *C. aurangabadensis* and *C. raoi*. Patil et al., in 1998, Shinde and Kalse in 1999, Tat and Jadhav in 2004, Pardeshi and Kalse in 2007. Supugade et. al, (2005) added *C. vitellaiensis* from *M. armatus*. Kharade et al., (2007) added *C. cirrihinae* from *Cirrihina mrigala*. Shelke et al., (2007) added *C. mehdii* from *M. armatus*. Jawalikar et al. (2008) added *C. yogeshwari* from *M. armatus*. Borde and Jawale, (2008) added *C. purnae* from *M. armatus*. Shah, (2010) added *C. paithenensis* from *M. armatus*. Menkudle and Jawale, (2010) added *C.*

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thapari from *Ophiocephalus stratus*. Pardeshi and Hiware, 2011 added *C. Jadhavae* from *M. armatus*. Dhole and Kadam, (2011) added *C. clariase* from *Clarias batrachus*. Recently, Fartade and Chati, (2017) have added *C. govindii* from *Channa marulius*.

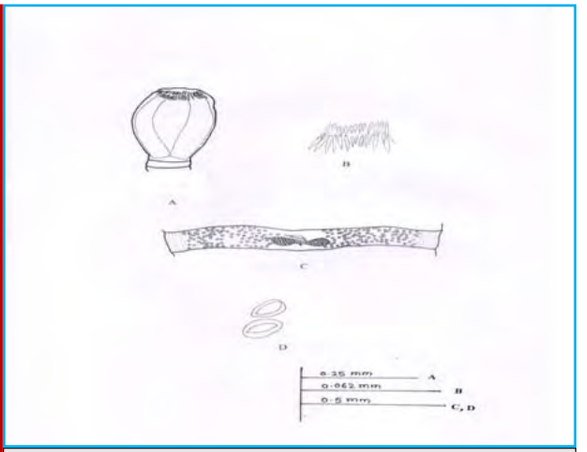
In the present study, the six cestode parasites were collected from the intestine of fresh water fish *Mastacembelus armatus*. The collected parasites were flattened, preserved in 4 % formalin, stained with Harris haemotoxylin, passed through various alcoholic grades, cleared in xylol and mounted in D.P.X. The whole mount slides were prepared for anatomical studies. The drawings are made with the aid of Camera Lucida.

The scolex was found to be long, bluntly rounded tip, broader in the middle, narrow posteriorly and measured 1.208-1.235 in length and 0.302-0.625 in breadth. The rostellum is medium, transversely elongated, oval and measures 0.038-0.061 in length and 0.112-0.120 in breadth. The rostellar hooks are 48 in number, variable in size, straight, rod like and measures 0.026-0.078in length and 0.003-0.010 in breadth. The bothria are two, extends from anterior to posterior extremity of the scolex, slightly overlapping on each other and measures 0.846-0.897 in length and 0.061-0.072 in breadth. The neck is short and measures 0.030-0.054 in length and 0.276-0.282 in breadth. The mature segments are medium, broader than long and measures 2.012-2.223 in length and 0.324-0.421 in breadth. The testes are 200-210, medium, round, in two groups on lateral sides of ovary, unequally distributed and measures 0.106-0.113 in diameter. The cirrus pouch is medium, oval, obliquely placed in the middle of the segment and measures 0.162-0.181 in length and 0.020-0.088 in breadth.

The cirrus is thin, slightly curved and measures 0.148-0.157 in length and 0.007 in breadth. The vas deferens is short, thin, slightly curved and measures 0.072-0.079 in length and 0.007 in breadth. The vagina is short, thin tube, runs obliquely and measures 0.114-0.140 in length and 0.007 in breadth. The genital pore is small, round, preovarian anterior to isthmus is located in the centre of the segment and measures 0.117-0.126 in diameter. The ovary is medium, bilobed, roughly dumbbell shaped lobes of unequal size, is connected by isthmus and measures 0.535-0.710 in length and 0.024-0.161 in breadth. The isthmus is thick, long and measures 0.146-0.170 in length and 0.043-0.050 in breadth. The ootype is small, rounded, placed between the ovarian lobes and measures 0.086-0.095 in diameter. The vitellaria are granular, 8-10 rows in the corticular region of the segment.

The present communication deals with the description of *Circumoncobothrium devidasensis* new species. After going through the literature the worm is found to differ from all known species of *Circumoncobothrium*.

1) The worm under discussion differs from *C.ophiocephali*, Shinde (1968) in having scolex distinct, rostellar hooks 80, rod shaped, neck present, testes 70-80, ovary single, conical mass to irregular shapes, lobes 2-3, vitellaria in 14-15 rows.2) The present worm differs from *C.aurangabadensis*, Jadhav and Shinde, (1976) in having the scolex broader in the middle, narrow at both the ends, rostellar hooks 42 in number, rod shaped, neck present, testes 135-145, round, ovary bilobed with 3-4 acini, vitellaria granular, near the lateral margins.3) The present form differs from *C.raoi* , Shinde and Jadhav, (1976),in having scolex broad in the middle, narrow at both the ends, rostellar hooks 46 in number, rod shaped, testes 210-215, round, in two fields, ovary almost to the posterior margin of the segment, vitellaria granular in the lateral sides of the segment.4) The present worm differs from *C.shindei*, Shinde and Chincholikar,(1976)in having rostellar hooks 49,rod shaped, neck present, tes-



Circumoncobothrium devidasensis a new species

tes 260-275 (273), round, ovary dumbbell shaped, lobes rounded, situated in the centre of the segment, vitellaria granular. 5) The present cestode differs from *C.khami*, Shinde,(1976) in having the scolex cylindrical, rostellar hooks 48 in number, mature segment squarish, testes 190-200 (194), rounded, Ovary bilobed, vitellaria follicular, rounded in a single layer, near the lateral margins.

6) The worm under discussion, differs from *C. bagariusi*, Chincholikar and Shinde, (1976)in having rostellar hooks 55 in numbers, rod shaped, testes 275-285 (278) in number in two fields, ovarian lobes with 5-6 globular acini, in the middle 1/3 of proglottids, vitelline follicles with irregular shape in 4-5 rows on each side.7) The present form differs from *C.gachaui*, Jadhav and Shinde, (1976)which is having scolex pear shaped in appearance, rostellar hooks 46 in number, neck present, testes 375-400 in number, rounded, densely placed in two fields, ovarian lobes with 5-6 short, blunt acini and vitellaria corticular in position with 1-2 rows on each side.8) The worm under discussion differs from *C.yamaguti*, (Jadhav and Ghavane (1991) which is having rostellar hooks 56 in number, neck present, testes 130-150, rounded, ovary bilobed, centrally placed near posterior margin of proglottids, vitellaria granular, corticular, distributed along lateral margin of proglottids.9) The present worm differs from *C.alii*, Shinde et.al.(1994) which is having rostellar hooks 34 in number, neck present, testes 230-240 in number in two lateral fields, ovary bilobed, centrally placed compact, vitellaria granular on the lateral fields.

10) The present form differs from *C.vadgaonensis*, Patil et.al.(1998) which is having scolex large in size, rostellar hooks 56 in number, arranged in four quadrants, neck absent, testes 490-510 in number, oval in shape, ovary distinctly bilobed, each lobe compact in the posterior half of the proglottids, vitellaria follicular, in two rows on each side.11) The present cestode differs from *C.baimaii* Wongsawad and Jadhav, (1998) in having the scolex pear shaped, hooks 48 in numbers, neck present,testes 88-100 in numbers, ovary compact and reported from *Mastacembelus armatus* in Chang Mai.12) The present worm differs from *C. punctatusi* Kalse and Shinde,(1999) in having scolex rectangular, hooks 40-50 in numbers, neck present, mature proglottids squarish, testes 140-150 in numbers, vitellaria follicular, arranged in 3-6 rows and reported from *Ophiocephalus punctatus*, in India.

13) The present worm differs from *C. armatusae* Shinde et. al., (1999) in having scolex triangular, hooks 58 in numbers, neck present, testes 90-100 in numbers, ovary compact and vitellaria follicular, arranged in 3-4 rows on lateral side of the segments.14) The present parasite differs from *C. mastacembelusae* Shinde et. al., (2002) in having scolex pear shaped hooks 30 in numbers, testes130-140 in numbers, ovary compact and vitellaria follicular, arranged in 2-3 rows on each lateral side.15) The present cestode differs from *C.armatusae (minor)* Pawar et. al., (2002) in having scolex triangular, hooks 58 in numbers, testes 190-200 in numbers and vitellaria follicular.16) The present form differs from *C. manjari* Tat andJadhav,(2004) in having the scolex triangular, hooks 48in numbers, in single circle, neck present, testes 128-145 in numbers, vitellaria follicular and reported from *Ophiocephalus gachua,* in India.17) The present parasite differs from *C. vitellariensis* Supugade et. al., (2005) in having scolex large, triangular, hook 48 in numbers, testes 250-260 in numbers and vitellaria follicular, arranged in 3-4 rows.

18) The present parasite differs from *C. cirrhinae* Kharade et al., (2007) in having scolex large, cylindrical, barrel shaped, rostellar hooks 56 , neck short, testes 300-305, medium,oval, ovary dumbbell shaped, medium. 19) The present parasitediffers from *C. mehdii* Shelke et al. (2007) in havinghooks 56 arranged in single circle, neck short, squarish, mature segment medium, squarish, testes 280-290 medium, ovary large, distinctly bilobed, vitellaria follicular, 3-4 rows. 20) The present cestode differs from *C.ambajogaiensis* Pardeshi et al., (2007) in having hooks18-20 in numbers, neck absent, mature segment ten times broader than long, testes 250-300 in numbers, ovary bilobed, dumbbell shaped, vitellaria follicular. 21) The present worm differs from *C.yogeshwari* Jawalikar et al., (2008) in having hooks 53 in numbers, neck very short, testes 95-98 in numbers, vitellaria follicular, arranged in two rows.

22) The present worm differs from *C. purnae,*Borde and Jawale, (2008) in having hooks 52 in numbers,neck absent, mature segment squarish, slightly broader than long, testes 230-235 in numbers, ovary bilobed and vitellaria follicular, arranged in 3-4 rows. 23) The present parasite differs from *C. naidui* Kalse et al., (2009) in having scolex cylindrical, hooks 40 in numbers, neck absent, testes 200-210 in numbers, medium rounded, ovary oval, single mass, compact, transversely elongated with acini. 24) The present cestode differs from *C. paithenensis* Shah, (2010) in having scolex triangular, cylindrical, hooks 58, single circle in four quadrant, neck very short, mature segment two time broader than long, testes 70-80, oval, and vitellaria follicular in tworows. 25) The present form differs from *C. thapari* Menkudale and Jawale (2010) in having host *Ophiocephalus* stratus, hooks 52 in numbers, neck absent, testes 95 in numbers, medium, oval, ovary medium, lobed, vitellaria follicular 2-3 rows.

26) The present parasite differs from *C. jadhavae* Pardeshi and Hiware, (2011) in having scolex triangular, dome shaped, hook 35-45 in numbers, neck present, mature segment broader than long, testes 95-105 oval to round, ovary bilobed, vitellaria follicular, arranged in two rows. 27) The present worm differs from *C.clariasi* K.N.Kadam and Jaywant Dhole (2011), having scolex triangular,

hooks 48 in numbers, testes oval in shape 249-259 in numbers, vitellaria follicular arranged 2-3 rows. Reported from *Clarias batrachus* in India. 28) The worm under discussion differs from *C.govindii* Fartade and Chati (2017), having scolex conical ,rostellar hooks 58-60 in number, neck present, testes oval to round 50-59 in number, ovary irregular bilobed, vitellaria granular in two rows and reported from *Channa marulius*.

The above noted characters are valid enough to erect a new species and hence the name *Circumoncobothrium devidasensis* a new species is proposed in the honor of author's father name from the host *Mastacembelus armatus* from Manjra dam, Tq.Kaij, Dist.Beed, M.S.India.

Type species	*Circumoncobothrium devidasensis*, a new species.
Date of collection	4th December, 2012.
Host	*Mastacembelus armatus*
Habitat	Intestine
Locality	Manjra Dam, Taluq Kaij, District. Beed, M.S. India.

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Effect of ginger, *Zingiber officinale* on sex hormones and certain biochemical parameters of male Wistar rats

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ABSTRACT

It is well demonstrated that phytoestrogens play an important role in the male reproductive system. So, the aim of the current study was to determine effect of ginger on sex hormones and blood biochemical levels in rat. A 40 male Wistar rats (200–250 g) kept as folk and fed basal chew pellet for a week, then randomly divided into 4 experimental groups. Group 1 was kept as control and fed basal diet (commercial chew pellet) and orally gavage with distilled water. Groups 2-4, provided basal diet and orally gavage of the 100, 200 and 300 mg ginger powder in distilled water, respectively for 4 weeks. At the end of the study, Serum glucose, cholesterol, triglyceride, LDL, HDL, albumin, total protein, urea as well as LH, FSH and testosterone determined. Then animals sacrificed and sperm was collected from epididymis and prepared for spermatozoa characteristics, semen testosterone, LH and FSH levels. According to the results, dose dependent increase observed on spermatozoa forward movement (P=0.0001). A dose dependent increase observed on sperm viability (P=0.0001). Orally administration of the 100, 200 and 300 mg of the ginger significantly increased serum total protein (P=0.0008) and decreased glucose (P=0.014) compared to the control group. Administration of the ginger in a dose dependent manner increased serum triglyceride (P=0.01) and HDL (P=0.0006) levels in rat compared to the control group. Orally gavage of the ginger significantly increased semen testosterone levels in comparison to the control group (P=0.009) while had no significant effect on LH and FSH levels (P>0.05). No significant effect observed on serum LH, FSH and testosterone levels compared to the control group (P>0.05). These results suggested ginger improves spermatozoa characteristics and semen hormone level.

KEY WORDS: GINGER, LH, FSH, TESTIS, BLOOD BIOCHEMICAL, RAT

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INTRODUCTION

Infertility is one of the major health problems in the world (Ghalehkandi, 2014). Infertility is a multi-parametric phenomenon which more than 30 % of infertilities are related to a male factor (Vincent et al. 2012). Several factors affect spermatogenesis and sperm quality such as drug treatment, chemotherapy, toxins (Adeeyo et al. 2011), air pollutions and insufficient vitamins intake (Barkhordari et al. 2013). It is reported administration of 100 mg/kg of ginger increased sperm percentage, motility viability, and serum testosterones in rat (Khaki et al. 2009). Based on the literature has a long history in order to fertility regulation (Kooti et al. 2015). Also, there are increasing interests on plant-derived chemicals on the endocrine system and the activity of sexual organs (Kooti et al. 2015).

Ginger (*Zingiber officinale R.*) is a medicinal plant which is gaining popularity amongst modern physicians (Sakr and Badawy, 2011). The major isolated bioactive ingredients of the ginger are gingerdiol, zingibrene, gingerols, protodioscin, saponins and shogaols (Sakr and Badawy, 2011). Ginger has many therapeutic effects such as antioxidant, antiemetic, antithrombotic, anti-hepatotoxic, anti-inflammatory and cholagogue (El-Morsy Ibrahim and Al-Shathly, 2015). Ginger relieves nausea and vomiting associated with motion sickness, pregnancy and surgery (Gilani and Rahman, 2005). Ginger has protective role in reproductive toxicities such as cyclophosphamide, cisplatin, malathion and diabetes (Riaz et al. 2017).

It is reported that aqueous extract of ginger (24 mg/ml) has a positive effect on metiram-inhibited spermatogenesis and induced apoptosis in mice (Sakr and Badawy, 2011).Ginger decreases body weight, serum glucose, cholesterol and serum alkaline phosphatase in adult male rats (Bhandari et al. 2005). Many positive effects of ginger on male reproductive system have been reported. It is reported that co administration of ginger (0.5-1 g/kg) with lead resumed the plasma testosterone level to near normal levels (Riaz et al. 2011). Since years ago, there are increasing interests focus on verification of pharmacological and physiological actions in ginger as a therapeutic agent (James et al. 2015).

The protodioscin and saponins of ginger increase testosterone and luteinizing hormone (LH) hormone levels as well as libido which can be used in traditional medicine to treat sexual dysfunctions (Morakinyo et al. 2008). Tribestan (patented extract of ginger) increases libido, infertility and menopausal disorders (Imani and Ainehchi, 2014). Also, ginger increases estrogen, pregnenolone and testosterone levels and sexual potency in men (Sabik et al. 2009). Despite researches have been done on effects of the ginger on male reproductive system, its role on sex hormones in blood and testicular levels is not fully elicited. So, the aim of the current study was to determine effect of the ginger on blood biochemical follicle stimulating hormone (FSH), LH, testosterone levels as well as semen FSH, LH, testosterone hormones in rat.

MATERIAL AND METHODS

Animals: A 40 male Wister rats (200–250 g) kept as folk and fed basal chew pellet for a week, then randomly divided into 4 experimental groups (n=10). Animals were kept in groups of 8–10 per cage (45 cm × 30 cm × 15 cm) at a controlled room temperature (23 ± 1 °C), relative humidity of 55–65% and were maintained on a light-controlled regime (12-h light cycle, lights on at 07:00 h) according to European Union recommendations for laboratory animals. During the study, all animals had *ad libitum* access to fresh water. Animals were acclimatized to laboratory conditions for one week prior to experiments. All experimental procedures were carried out during the light phase (10:00–17:00 h) and executed in accordance with the Guide for the Care and Use of Laboratory Animals to Investigate Experimental Pain in Animals (Zimmermann 1983). Animal handling and experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (USA) and the current laws of the Iranian government.

Study design: All animals had free access to basal diet (commercial chew pellet) for a week prior the study. Then animals randomly allocated into 5 experimental groups. Group 1 kept as control and fed basal diet (commercial chew pellet) and orally gavage with distilled water. Groups 2-4, provided basal diet and orally gavage of the 100, 200 and 300 mg ginger powder in distilled water, respectively. All animals provided *ad libitum* access to the experimental diets based on their groups for 4 weeks. At the end of the sixth week, animals were food deprived for 12, blood samples were taken, centrifuged at 4°C for 10 minutes at 250×g and the serum stored at -20°C until assayed. Serum glucose, cholesterol, triglyceride, LDL, HDL, albumin, total protein, and urea were obtained using colorimetric assay using commercial kit (Pars Azmoon Co., Tehran, Iran). Serum concentration of LH and FSH were determined in duplicated samples using radioimmunoassay. Rat FSH and LH kits obtained from Biocode Company-Belgium, according to the protocol provided with each kit. The sensitivities of hormone detected per assay tube were 0.2ng/ml and 0.14ng/ml for FSH and LH respectively (Khaki et al. 2009). Serum concentration of total testosterone was measured by using a double antibody radioimmunoassay kit

Table 1. Effect of the ginger on spermatozoa characteristics in rat

Groups	Forward movement	Motility without movement	Without movement	Dead sperm (%)	Viability (%)
Control	21[b]	22.56[a]	60.54[a]	70.50[a]	29.50[c]
Ginger (100 mg)	20.70[b]	20.80[b]	58.30[a]	51.95[b]	48.05[b]
Ginger (200 mg)	20.20[b]	22.20[b]	57.60[a]	44.90[c]	55.10[a]
Ginger (300 mg)	37.45[a]	31.45[a]	30.80[b]	44.80[c]	55.20[a]
P value	0.0001	0.0001	0.0024	0.0001	0.0001
SEM	2.99	1.99	4.30	2.15	2.15

SEM standard error of mean. Different letters (a, b and c) indicate significant differences between treatments at each time ($p< 0.05$).

(immunotech Beckman Coulter Company-USA). The sensitivities of hormone detected per assay tube were 0.025ng/ml (Huang et al. 1995).

Surgical procedure: At the end of the study, rats fasted overnight and were intraperitoneally (i.p) injected with pentobarbital (40 mg/kg). Peritoneum on each animal was opened by an incision and testes were taken out. Semen samples were collected from the Cauda epididymis and homogenized in 10% (W/V) ice-cold buffer (0.1 M phosphate buffer, pH 7.4 + 150 mM KCl). The homogenate was centrifuged at 9000 rpm for 20 min to obtain a supernatant which was used to determine semen testosterone, LH and FSH levels using radioimmunoassay kits (Biocode Company-Belgium) and (immunotech Beckman Coulter Company-USA) (Huang et al. 1995).

Spermatozoa characteristics: At the end of the study, semen samples were collected from the Cauda epididymis carefully separated from the testis and placed in a Petri dish containing Ham's F10. Epididymal caudal was minced with scissors to release sperm and then was placed in the incubator for 15min (Padmanabhan et al. 2008). Approximately, 10µL of the diluted sperm suspension was transferred to each counting chamber of the hemocytometer and allowed to stand for 5 min (Wyrobek et al. 1983). The cells which settled during this time were counted by a light microscope at 200X magnification (Seed et al. 1996).

Statistical analysis: Data were prepared in excel, analysed with analysis of variance (ANOVA) using SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL, USA) followed by Tukey's post-hoc tests and presented as mean ± SEM. $P < 0.05$ was considered to denote significant differences between groups.

RESULTS AND DISCUSSION

The results of the ginger on sex hormones and blood biochemical levels in rat are presented in tables 1-5. As seen in table 1, a dose dependent increase observed on spermatozoa forward movement (P=0.0001). Also, spermatozoa motility without movement significantly increased by administration of the ginger (100, 200 and 300 mg) compared to the control group (P=0.0001). Also, spermatozoa without movement significantly decreased in rats treated with ginger (100, 200 and 300 mg) compared to the control group (P=0.0024). A dose dependent increase observed on spermatozoa viability (P=0.0001) and decreased dead sperm (P=0.0001).

Effect of the ginger on blood biochemical levels in rat is presented in table 2. According to the results, orally

Table 2. Effect of the ginger on blood biochemical levels in rat

Groups	Total protein (g/dl)	Albumin (g/dl)	Glucose (mg/dl)	Urea (mg/dl)
Control	7.68[c]	5.2	105.85[a]	29.53
Ginger (100 mg)	8.24[b]	5.3	113.62[a]	41.2
Ginger (200 mg)	8.82[a]	5.46	110.62[a]	36.95
Ginger (300 mg)	8.22[b]	5.46	72.98[b]	36.95
P value	0.0008	0.65	0.014	0.4
SEM	0.17	0.16	9.53	2.05

SEM standard error of mean. Different letters (a, b and c) indicate significant differences between treatments at each time ($p< 0.05$).

Table 3. Effect of the ginger on blood lipid profile in rat

Groups	Cholesterol (mg/dl)	Triglyceride (g/dl)	HDL (mg/dl)	LDL (mg/dl)
Control	253.7	51.65^{b}	46.55^{b}	23.61
Ginger (100 mg)	215.5	71.73^{a}	55.65^{a}	27.65
Ginger (200 mg)	214.80	55.66^{b}	45.78^{b}	26.74
Ginger (300 mg)	218.50	62.51^{ab}	48.78^{b}	25.98
P value	0.15	0.01	0.0006	0.60
SEM	13.7	4.22	1.66	2.18

SEM standard error of mean. Different letters (a and b) indicate significant differences between treatments at each time ($p< 0.05$). HDL: high density lipoprotein; LDL: low density lipoprotein.

administration of the 100, 200 and 300 mg of the ginger significantly increased serum total protein levels compared to the control group (P=0.0008). Serum glucose significantly decreased in rat received ginger (100, 200 and 300 mg) compared to the control group (P=0.014). No significant difference observed on serum albumin and urea levels in rat treated with ginger in comparison to the control group (P>0.05).

Administration of the ginger in a dose dependent manner increased serum triglyceride (P=0.01) and HDL (P=0.0006) levels in rat compared to the control group. No significant difference observed on serum cholesterol and LDL in animals received ginger than control group (P>0.05) (table 3).

As seen in table 4, orally gavage of the ginger significantly increased semen testosterone levels in comparison to the control group (P=0.009) while had no significant effect on LH and FSH levels (P>0.05).

According to the table 5, ginger (100, 200 and 300 mg) had no significant effect on serum LH, FSH and testosterone levels compared to the control group (P>0.05).

Infertility is one of the major problems which the both male and female related factors are not yet clearly understood (Nassiri et al. 2009). Better understanding of underlying mechanisms in fertility is important to improve diagnosis and treatment (Nassiri et al. 2009). Male factor is involved in 40-50% of infertility and numerous conditions include in spermatogenesis and sperm quality (Mazaheri et al. 2014). Despite many achievements in modern medicine, side effects of synthetic chemical drugs are still the main problem (Lim, 2016). Hence, there are growing interests to use of herbal medicine due to it possessing lower side effects (Lim, 2016). Ginger is a famous medical plant by anti-oxidant, anti-serotonergic and anti-inflammatory properties (Lim, 2016).

According to the results, dose dependent increase observed on spermatozoa forward movement. Dose dependent increase was observed on sperm viability. In this regard, Khaki et al. (2009) reported administration of the ginger rhizome powder (50 and 100mg/kg) for 20 consequence day increased sperm viability and motility in rat. Orally administration of the *Zingiber officinale* (1000mg/kg for 28 days) increased epididymal sperm count and motility (Morakinyo et al. 2008).which our results were similar to their findings. Orally administration of the 100, 200 and 300 mg of the ginger significantly increased serum total protein and decreased glucose levels. In this regard, it is reported ginger has anti hyper glycaemic effect by decrease glucose levels in rats (Al-Amin et al. 2006). Despite direct mechanism of action ginger on blood glucose level is not fully elicited, Khan et al. (2003) reported ginger increase

Table 4. Effect of the ginger on semen sex hormone in rat

Groups	LH (Iu/l)	FSH (Iu/l)	Testosterone (ng/ml)
Control	2.54	0.24	0.75^{c}
Ginger (100 mg)	1.84	0.08	1.03^{bc}
Ginger (200 mg)	2.13	0.09	1.2^{ab}
Ginger (300 mg)	1.34	0.11	1.48^{b}
P value	0.30	0.47	0.009
SEM	0.44	0.04	0.14

SEM standard error of mean. Different letters (a, b and c) indicate significant differences between treatments at each time ($p< 0.05$). LH: luteinizing hormone; FSH: follicle stimulating hormone.

Table 5. Effect of the ginger on serum sex hormone in rat

Groups	LH (Iu/l)	FSH (Iu/l)	Testosterone (ng/ml)
Control	4.9	0.59	1.02
Ginger (100 mg)	2.52	0.34	1.44
Ginger (200 mg)	2.96	0.34	1.59
Ginger (300 mg)	2.025	0.15	2.02
P value	0.31	0.45	0.007
SEM	0.69	0.10	0.19

SEM standard error of mean. Different letters (a and b) indicate significant differences between treatments at each time ($p< 0.05$). LH: luteinizing hormone; FSH: follicle stimulating hormone.

glucose uptake and glycogen synthesis and phosphorylation of the insulin receptor.

Based on the findings of the current study, administration of the ginger in a dose dependent manner increased serum triglyceride and HDL levels in rat. Orally gavage of the ginger significantly increased semen testosterone levels while had no significant effect on LH and FSH levels. In contrast it is reported administration of the 50 and 100mg/kg ginger rhizome powder increased testosterones without effects on LH and FSH hormones (Khaki et al. 2009). According to our findings, no effect observed on serum LH, FSH and testosterone levels in ginger-treated rat.

However, Riaz et al. (2017) reported orall administration of the ginger (1.5gm/kg) significantly decreased plasma testosterone and LH levels in male rats after lead induced toxicity. Imani and Ainehchi, (2014), reported ginger (20 and 40 mg/kg) increased serum LH while only 20 mg/kg increased serum FSH levels in rats. In the current study we used 100, 200 and 300 mg of the ginger powder in water. So, perhaps only low levels of the ginger increase the FSH levels which needs further investigations to determine direct cellular and molecular of actions. Ginger extract has androgenic activity which elevates semen testosterone and accumulation of sperm in the seminiferous tubules in rat (Amr and Hamza et al. 2006; Rekha et al. 2010).

As observed in our study, ginger had no effect on serum and semen LH and FSH levels while increased sperm viability and motility. Ginger extract has antioxidant effect by antioxidant enzymes including super oxide dismutase, glutathione peroxides and catalase in rats (Khaki et al. 2009). It seems, ginger increase sperm motility via protective effect (Amr and Hamza et al. 2006). However, because of the limitations of the current study, we were not able to measure antioxidant enzyme levels in serum and testis tissue of the ginger-treated rat. Ginger roots induce antidiabetic activity and enhance male fertility in diabetic rats (Hafez 2010). As observed in this study, ginger decreased serum glucose level and increased sperm viability and movement. Observed effects of the ginger are attributed to its major ingredients including Zingerone, gingerdiol, Zingiberene, gingerols and shogoals (Morakinyo et al. 2008). In conclusion, these results suggested ginger improves spermatozoa characteristics and semen hormone level.

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