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Continued Inside Cover

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Bioscience Biotechnology Research Communications
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Challenges and perspectives of health informatics and its management in developing Asian countries

Shaima S. Ali Miraj, PhD

Department of Public Health Management, College of Health Sciences, Saudi Electronic University Riyadh, Saudi Arabia

ABSTRACT

The health care industry has generated large amounts of complex and diverse data, driven by record keeping, compliance and regulatory requirements and patient care, however in absence of proper management and judicious use of health informatics and data generated through it, the vital information is not being used for the benefit of patients. Studieshave shown that costs can be dramatically reduced in health care by using health informatics and data collected over long periods of time, like patient's history, recurrence of pathologies, genetic trends, in heritance, and many other statistical tools, which can be used for huge benefits of patients. But all these require huge funds, proper training, judicious and honest use of such collected information, brilliant research - updated physicians, experts and managers can make wonders in health care delivery, benefitting both the government and the patients by synergistic use of health information system generated data. The literature indicates that these systems and their implementation is limited and at times spasmodic in developing as well as low-income countries, largely because of the financial and implementation challenges these countries face. These challenges are likely due to technological, organizational, financial or human resources barriers. With no signs of improvement, despite several governments claiming to soothe balms on the self-inflicted injuries, the health insurance sector, NGOs, WHO,IMF, World Bank and other benevolent rich nations will have to come together to fulfil the future vision of Health For All especially in poor and developing countries. To tackle the problems of corruption, malpractices in health care where health informatics data in such situations can be exploited for fraud and malpractices, better role of governments, sincere controlling and regulatory authorities in association with International Agencies is recommended to streamline these issues. Data analytics and applications in healthcare are at a nascent stage of development, but rapid advances in platforms and tools with proper management and control can accelerate their maturing process in developing nations as well.

KEY WORDS: DATA INFORMATICS, MANAGEMENT, SYNERGISTIC USE FOR PATIENT CARE

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**Corresponding Author: shaima.s.ali@gmail.com*

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INTRODUCTION

Healthcare industry has always been in a complex situation of handling or rather mishandling huge data of patients or in using health informatics, over a long period of time, as data collected but not judiciously used is as good as data wasted. The health careindustry has generated large amounts of complex and diverse data, driven by record keeping, compliance and regulatory requirements, and patient care, the latter seldom in priority. Looking to the recent computerisation of health care centres worldwide including the poor and developing nations, the current trend is toward rapid digitization of these large amounts of data.New data analytic tools to facilitate scalable, accessible and sustainable data infrastructure for effective management of large, multiscale, multimodal, distributed and heterogeneous data sets and convert data into knowledge for support cost-effective decision aids, disease management, and care delivery need to be developed, especially in health care centres of developing nations.

Driven by necessity, and the potential to improve the quality of healthcare delivery along with reducing the costs, these massive quantities of data (known as 'big data') hold the promise of supporting a wide range of medical and healthcare functions, including among other clinical decision support, disease surveillance, and population health management.They also include initiatives that enable use of data analytics in health systems for improved clinical decision making, enhanced efficiency of care provision, policy development and policy implementation, (Burghard 2012, Dembosky 2012, Feldman et al., 2012 Fernande et al., 2012 and Raghupati & Raghupati 2014 Andreau Perez et al 2015 and Sweeney 2017).

Reports demonstrate that data from the U.S. healthcare system alone reached, in 2011, 150 exabytes that's enough data to fill a stack of DVDs that would reach from Earth to Mars (NCBI, 2017). Remarkably, that volume continues to double every two years. At this rate of growth, data for U.S. healthcare will soon reach the zettabyte (10^{21} gigabytes) scale and, not long after, the yottabyte (10^{24} gigabytes), (IHTT (2013). By definition, big data in healthcare refers to electronic health data sets so large and complex that they are difficult (or impossible) to manage with traditional software and/or hardware; nor can they be easily managed with traditional or common data management tools and methods. Big data in healthcare is overwhelming not only because of its volume but also because of the diversity of data types and the speed at which it must be managed, (Frost and Sullivan 2016).

Developed nations which have huge amount to spend on health care both government and insurance based, can think of proper use of such technologies, infrastructure and latest developments in synergistic use of big data. Various studies in these countries have shown that costs can be dramatically reduced in health care by using health informatics and data collected over long periods of time, like patient's history, recurrence of pathologies, genetic trends, in heritance, and many other statistical tools, which can be used for huge benefits of patients. But all these require proper training, judicious and honest use of such collected information, brilliant research - updated physicians, experts and managers can make wonders in health care delivery, benefitting both the government and the patients by synergistic use of health information system generated data.

In one very famous example, California-based Kaiser Permanente associated clinical data with cost data to generate a key data set, the analytics of which led to the discovery of adverse drug effects and subsequent withdrawal of Vioxx from the market. Researchers at the Johns Hopkins School of Medicine discovered they could use data from Google Flu Trends to predict sudden increases in flu-related emergency room visits at least a week before warnings from the Centre for Disease Control. Likewise, the analysis of Twitter updates was as accurate as (and two weeks ahead of) official reports at tracking the spread of cholera in Haiti after the January 2010 earthquake, (IHTT 2013).

These electronic based health record systems like, Electronic Medical Record (EMR) Health Information System (HIS) are considered essential components of any healthcare organization, (Mitchell and Yaylacicegi 2013). Healthcare providers such as physicians and nurses spend long periods of time during their workday collecting information from patients, (Conrick, 2006). On the other hand, developing countries have tended to lag behind in the adoption and implementation of HIS and EMR systems, or even a basic health data system, (Sinha et al., 2013). The literature indicates that these system and their implementation is limited and at times spasmodic indeveloping as well as low-income countries, largely because of the financial and implementation challenges these countries face. These challenges are likely due to technological, organizational, financial or human resources barriers, (Luna et al., 2014, Naseem et al., 2014).

In developed countries like Saudi Arabia, initiatives for implementing Health Information System and other EMR systems have been occurring over the last four decades, (Altuwaijri 2008 and Hasanian et al., 2014). Over these past decades Saudi Arabia has spent billions of dollars to develop and improve the quality of health care. As well as funds to assist EMR implementation, the Saudi Ministry of Health (MOH) has made clear its intention to implement HIS nation-wide, (Altuwaijri 2008). Previous research showed that HIS implementation was low within Saudi public hospitals in its early stages, but Saudi Arabia is a developing country, it has made remarkable progress and achievements in health

care and its management, (Aldosari 2014, Alkhamis 2012, 2017). There are a number of major hospitals and healthcare organizations that have attained distinguished achievement in use of health informatics and other high technology gadget implementation in Saudi Arabia and many more well equipped public hospitals and centres have been developed, (Hasnain et al., 2014, 2015 and Alkahmis 2012, 2017).

Unfortunately, situation in poor and developing Asian countries like Bangla Desh, Afghanistan, Pakistan, Sri Lanka and India is in stark contrast and is seriously-alarming with reference to the use of health care informatics and data of patients collected for improving and reducing cost of healthcare. Leaving aside most of the state of the art private and few government hospitals in metro cities like Delhi, Mumbai, Bangalore, Chennai, Kolkata which are beyond access to general population, majority of hospitals serving thge public are in sorry state of affairs with regard to use of HIS, EMR and other state of the art technology based health informatics tools. Where it is extremely difficult to run heath care centres owing to severe shortage of funds, manpower, infrastructure, medicines and other bare necessities, how we can collect, generate and maintain health care data and keep it alive for better future use?

Health care data in such situations can be exploited for personal gains, or it can go waste for want of mismanagement, corruption, legal battles, fraud and malpractices in several sectors like the insurance, corporate health policy claims, government reimbursements and many others. Health, being one of the most essential and basic needs of an individual makes it a lucrative soft target for corruption worldwide especially in developing countries where poor or no control exists on health administration and management. Health having unique dimensions is susceptible to both economic and political influences and its corruption not only involves monetary incentives, but also involves corruption of knowledge, experience and other practices, thus use of advance technologies like health informatics, data collection and its maintenance in this sector warrant lot of sincerity, dedication, sacrifice and character, both from the government agencies and other parties.In this present scenario, where 50 % of world's population cannot afford essential health services, where each year about 100 million people including in India, are being pushed into poverty because they have to pay for health care out of their own pockets, (OOPs) we can only expect a miracle for better health for all in future by use of health informatics.

Tracking Universal Health Coverage: (2017) Global Monitoring recent Report by the World Bank and the WHO-TUHC (2017), it has been revealed that currently 800 million people spend at least 10 % of their earnings on health expenses for themselves, a sick child or other family member. For almost 100 million, these expenses are high enough to push them into extreme poverty, forcing them to survive on a meagre 1.90 US dollars or less a day. The report looks at catastrophic spending on health on the basis of out of pocket expenditures exceeding 10 % and 25 % of house hold total income or consumption. About one sixth of households in India (exceeding 10 % household income) and 3.9 % (exceeding 25% household income) bear such spending exceeding a household's ability to pay without reimbursement by a third party, WHO UHC (2017).

FUTURE PERSPECTIVES

With no signs of improvement, despite several governments claiming to soothe balms on the self-inflicted injuries, the health insurance sector, the Third Party Assurances, (TPAs), NGOs, WHO,IMF, World Bank and other benevolent rich nations along with the OOPs will have to come together to fulfil the future vision of Health For All especially in poor and developing countries. To tackle the problems of corruption, malpractices in health care where health care data in such situations can be exploited for fraud and malpractices, the role of sincere controlling and regulatory authorities in association with International Agencies can be recommended. Health, being one of the most essential and basic needs of an individual makes it a lucrative soft target for corruption worldwide especially in developing countries where poor or no control exists on health administration and management. Health having unique dimensions is susceptible to both economic and political influences and its corruption not only involves monetary incentives, but also involves corruption of knowledge, experience and other practices, thus use of advance technologies like health informatics, data collection and its maintenance in this sector warrant lot of sincerity, dedication, sacrifice and character, both from the government agencies and other parties.

However in the years to come we cannot keep our eyes closed and get into the ground to hide, let us start doing the innovations with a zeal enthusiasm and dedication amidst the challenges which are many and look like mountains. At minimum, health information analytics platform in healthcare we must support the key functions necessary for processing the health related data of patients. The criteria for platform evaluation may include availability, continuity, ease of use, scalability, ability to manipulate at different levels of granularity, privacy and security enablement, and quality assurance. In addition, while most platforms currently available are open source, the typical advantages and limitations of open source platforms apply.

To succeed, big data analytics in healthcare needs to be packaged, so it is menu-driven, user-friendly and transparent. Real-time big data analytics is a key requirement in healthcare. The lag between data collection and processing

has to be addressed. The dynamic availability of numerous analytics algorithms, models and methods in a pull-down type of menu is also necessary for large-scale adoption. The important managerial issues of ownership, governance and standards have to be considered. And woven through these issues are those of continuous data acquisition and data cleansing. Health care data is rarely standardized, often fragmented, or generated in legacy IT systems with incompatible formats. This great challenge needs to be addressed as well, (Raghupath and Raghupath 2014).

Health informatics pertaining to huge data analytics has the potential to transform the way healthcare providers use sophisticated technologies to gain insight from their clinical and other data repositories and make informed decisions. In the future we'll see the rapid, widespread implementation and use of big data analytics across the healthcare organization and the healthcare industry. To that end, the several challenges highlighted above, must be addressed. As big data analytics becomes more mainstream, issues such as guaranteeing privacy, safeguarding security, establishing standards and governance, and continually improving the tools and technologies will garner attention. Governments as participants and NGOs as watch dogs along with funding of helping hands like the IMF, WHO, UN and other philanthropic foundations like Bill Gates and others can make it possible with firm determination and will power to do it.

Big data analytics and applications in healthcare are at a nascent stage of development in Asian nations, but rapid advances in platforms and tools can accelerate their maturing process. Modern health sector has a questionable history, driven by hunger of profit through the inhibition and discouragement of less profitable therapies and treatments. There are many areas inhealth care system which on given occasions allow dubious practices including corruption to pierce in. It's important we have a check on these malpractices, which can disturb all the good work being carried out in this noble profession of serving humanity.

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Evaluation of some biological properties of *Saussurea costus* crude root extract

Emad M. Abdallah,[1]* Kamal A. Qureshi,[2] Ahmed M. H. Ali[1,3] and Gamal O. Elhassan[2]

[1]*Department of Laboratory Sciences, College of Sciences and Arts at Al-Rass, Qassim University, Al-Rass, Saudi Arabia*
[2]*Department of Pharmaceutics, Unaizah College of Pharmacy, Qassim University, Saudi Arabia*
[3]*Department of Zoology and Entomology, Faculty of Science, Assiut University, Assiut, Egypt*

ABSTRACT

The current study aimed to evaluate some biological activities of *Saussurea costus* (*S. costus*) such as phytochemical constituents, antimicrobial activity and antifeedant potential on the larvae of *Spodoptera littoralis*. The results revealed that the methanol extract of roots of *S. costus* are rich in some bioactive phytochemical compounds such as alkaloids, phenols/polyphenols, flavonoids, terpenoids, tannins, coumarins, quinines, steroids, cardiac glycosides and resins. The antimicrobial screening revealed that, among 12 referenced microbial strains (10 bacteria and 2 fungi), 4 Gram-positive bacteria exhibited high susceptibility with the methanol and ethanol extracts of *S. costus*, namely *Bacillus cereus* ATCC 10876 (IZ 16.0±0.0, 15.5±0.5 mm, MIC100, 50 mg/ml, MBC 200, 100 mg/ml), *Staphylococcus saprophyticus* ATCC 43867 (IZ 14.5± 0.5, 15.5±0.5 mm, MIC50, 50 mg/ml, MBC100, 100 mg/ml), *Staphylococcus epidermidis* ATCC 12228 (IZ 13.5±0.5, 14.5±0.5mm, MIC50, 50 mg/ml, MBC 200, 100 mg/ml) and *Staphylococcus aureus* ATCC 29213 (IZ 11.0±0.0, 11.5±0.5 mm, MIC 100, 50 mg/ml, MBC 200, 100 mg/ml), respectively. Also, 1 fungal strain (*Aspergillus niger* ATCC 6275) revealed high susceptibility with the extracts (IZ 26.0±1.0 mm, MIC and MFC 50mg/ml). Other microorganisms recorded weak or no effect. Furthermore, the ethanolic extract of *S. costus* provided an anitfeedant effect toward *Spodoptera littoralis* larvae at different concentrations. In conclusion, the current findings provide evidence that roots of *Saussurea costus* is rich in bioactive phytochemical compounds and it might be a promising source of antimicrobial compounds as well as antifeedant activity against the larvae of *Spodoptera littoralis*.

KEY WORDS: ANTIBACTERIAL, ANTIFUNGAL, PHYTOCHEMICAL, *SAUSSUREA COSTUS*, ANTIFEEDANT, INSECTICIDAL, *SPODOPTERA LITTORALIS*, TRADITIONAL MEDICINE

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**Corresponding Author: emad100sdl@yahoo.com*
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INTRODUCTION

Medicinal plants are used for healing purposes throughout the human history; even in the current era, there are up to 80% of the world population most of them are living in the developing countries, rely on traditional herbal medicine on their primary health care systems, many of these herbal drugs prescribed in traditional medicine have inadequate knowledge and untested by scientific methods (Ekor 2013 Qazi and Molvi 2016). On the other side, Modern medicine stands helpless in the front of the growing phenomenon of antimicrobial resistance to antibiotics, which considered as a major health problem and required prompt attention. This crisis encouraged scholars and researchers to develop the current antibiotics, synthesize new antibiotics or to find new alternatives. The latter option is preferable because, in nature, plants arise as one of the largest pharmaceutical factories ever known and plants were the main source of drugs for humankind since antiquity. Many medicinal plants produce diverse groups of secondary metabolites known as phytochemical compounds, which may suppress the microbial growth by different modes of action such as interference with cellular metabolic processes, cellular membrane perturbations or by modulating the signal transduction or gene expression pathways (Omojate et al., 2014 Mohamed et al. 2017).

Saussurea costus (Falc.) Lipschitz, synonymous with *Saussurea lappa* C.B. Clarke, belongs to family Asteraceae, this family includes about 1000 genera and 30,000 species, widely distributed in different regions in the world; However, numerous species are found in India (Pandey et al., 2007). It is also distributed in Pakistan and some parts of Himalayas (Shah 2006). *Saussurea costus* (*S. costus*) is well known in Islamic medicine, which enlisted in the Holy Ahadith said by Prophet Muhammad (Peace be upon him) (Ahmad et al., 2009). It is known in Arab countries as "Al-Kost Al-Hindi" and used by traditional healers since the era of the Islamic civilization. For example but not limited to, *S. costus* is traditionally used as stimulant, antiseptic, carminative, sedative, bronchodilator and astringent agent (Wani et al., 2011). In the scientific literature, the biological activities of the roots of *S. costus* (synonymous with *S. lappa*) are widely investigated. Scientific investigations revealed that it has anti-trypanosomal activity (Julianti et al, 2011), it has "complement-inhibitor" substances helpful in the treatment of some diseases related to excessive activation of the complement system, like rheumatoid arthritis, respiratory distress and systemic lupus erythematosus (Fan et al., 2014). It was published that *S. costus* has a good anticancer activity on the tested cell lines (Robinson et al., 2008). The ethanol extract of *S. lappa* (synonymous *S. costus*) recorded a wide spectrum antimicrobial activity against some human pathogens (Hasson et al., 2013). In addition, many investigations reported other bioactive properties of *S. costus* roots such as anti-ulcer, anti-inflammatory, hepatoprotective, immunomodulator, hypoglycaemic, spasmolytic, anticonvulsant, antidiarrheal and antiviral activity (Zahra et al., 2014 Ghasham et al., 2017).

Egyptian cotton leafworm, *Spodoptera littoralis* (Lepidoptera: Noctuidae), is responsible for causing devastating damage for numerous vegetables and crops (Kandil et al., 2003; Adham, et al., 2009). This polyphagous pest is widely distributed in Africa and Middle Eastern countries (Abdel-Rahim and Azab 2008; Rizk et al. 2010; El-Zoghby et al. 2011). Several synthetic pesticides have been used to manage the agriculture crops from insect infestation. These synthetic insecticides cause serious hazard to the environment due to residual toxicity (White, 1995; El- Torkey 2008; Rizk et al. 2010). Therefore, scientists developed safe alternative insecticides with no residual activity. In this regard, various phytochemical plant extracts from several botanical sources against the specific pest have been evaluated (Kamaraj et al., 2010). The current study aimed to investigate some biological properties of the methanol extract of the roots of *S. costus*, including the phytochemical constituents, antimicrobial activity and antifeedant potential on the larvae of *Spodoptera littoralis*.

MATERIALS AND METHODS

The dry roots of *S. costus* (Figure 1) were purchased from a herbal market at Qassim region, Saudi Arabia. The herbal seller showed the trademark of the package and it has been confirmed that it was exported from India. The authentication of plant material was confirmed at the department of Laboratory Sciences, College of Sciences and Arts, Al Rass, Saudi Arabia.

12 standard pathogenic test organisms were used in this study; *Bacillus cereus* ATCC® 10876™, *Staphylococcus epidermidis* ATCC® 12228™, *Staphylococcus aureus* ATCC® 29213™, *Staphylococcus saprophyticus* ATCC®

FIGURE 1. The dried roots of *Saussurea costus*

43867™ and *Streptococcus pneumonia* ATCC® 49619™, *Escherichia coli* ATCC® 25922™, *Proteus vulgaris* ATCC® 6380™, *Klebsiella pneumonia* ATCC® 27736™, *Pseudomonas aeruginosa* ATCC® 9027™, *Shigella flexneri* ATCC® 12022™, *Candida albicans* ATCC®10231™ and *Aspergillus niger* ATCC®6275™.

The extraction of plant material was carried out by following the maceration method. The roots of the plant were crushed into small particles and then fine powder was obtained by using electrical mixer grinder. The maceration of plant material was carried out by mixing of fine powder into 70 % ethanol and 80 % methanol solvents separately. Then, all mixtures were incubated in a shaker incubator at 40°C temp., 50 r.p.m. shaking speed for up to 3 days in a well-tighten dark container. After incubation, the mixtures were centrifuged at 5000 r.p.m. for 15 minutes and then filtered through Whatman filter paper No.1. The filtrates were subjected to evaporate the solvents by using a rotary evaporator to get a semi-solid mushy crude extract, which was dried in hot air oven at 45°C for 48 hrs. The dried crude extracts were kept in a refrigerator until used (Ghasham et al., 2017). Methanol crude extract was analyzed for potential phytochemical molecules, methanol and ethanol extracts were used in the antimicrobial investigation, while ethanol crude extract was employed in the antifeedant examination against the larvae of *Spodoptera littoralis*.

In order to detect the various phytochemical constituents, the aqueous methanolic extract (100 mg/mL) was used. The colourimetric tests listed below were used as reported by (Ghasham et al. 2017, Sasidharan et al., 2011).

1 mL methanolic extract was mixed well with 1 mL of 1 % hydrochloric acid solution, followed by slight heating till the steaming. After that, 06 drops of Wagner's reagent were added into 1 mL of acidified extract. The formation of a brownish-red precipitate was observed for a positive test.

Carboxylic acid

2 mL of sodium bicarbonate solution was added to 1 mL of methanolic extract. The formation of effervescence was observed for a positive test.

Cardiac glycosides

1 mL methanolic extract was dissolved in 1 mL of chloroform, followed by addition of 2-3 drops of the sulphuric acid solution at the side of the test tube to form a layer. The formation of a brown ring at interphase was observed for a positive test.

Coumarins

1 mL of methanolic extract was mixed with 1 mL of 10 % sodium hydroxide solution. The formation of yellow colouration was observed for a positive test.

Emodins

1 mL of ammonia and 1.5 mL of benzene solutions were added to 1 mL of methanolic extract. The formation of red colouration was observed for a positive test.

Flavonoids

1 mL methanolic extract was added to 1 mL of 10 % lead acetate solution. The formation of a yellow coloured precipitate was observed for a positive test.

Leucoanthocyanins

1 mL of isoamyl alcohol was taken into a test tube, followed by slow addition of 1 mL of methanolic extract. The formation of red colouration at upper layer was observed for a positive test.

Lipids

0.5 mL of methanolic extract was mixed with 5 ml of ether. This mixture was allowed for evaporation on filter paper and dried the filter paper. The formation of an appearance of spot-on filter paper was observed for a positive test.

Phenols/Polyphenols

1 mL methanolic extract was added into 0.5 mL of 10 % ethanolic ferric chloride solution. The formation of blue-green to dark blue colouration was observed for a positive test.

Phlobatannins

1 mL methanolic extract was added to 1 mL of 1 % hydrochloric acid solution, followed by boiling the mixture. The formation of a red precipitate was observed for a positive test.

Quinones

1 mL of concentrated sulphuric acid was taken into a test tube, followed by addition of 1 mL of methanolic extract. The formation of red colouration was observed for a positive test.

Resins

Few drops of acetic anhydride solution were added to 1 mL of methanolic extract, followed by addition of 1 mL of concentrated sulphuric acid. The formation of orange to yellow colouration was observed for a positive test.

Saponins

5 mL of purified distilled water was taken into a test tube, followed by addition of 1 mL of methanolic extract and the whole mixture was well stirred. The formation of continuous effervescence was observed for a positive test.

Steroids

1 mL methanolic extract was mixed with 1 mL of chloroform, followed by addition of 2 mL of acetic anhydride and then few drops of concentrated sulphuric acid solution. The formation of dark green colouration was observed for a positive test.

Tannins

2-3 drops of 1 % lead acetate solution were added to 1 mL methanolic extract. The formation of dark blue or greenish grey colouration was observed for a positive test.

Terpenoids

2.5 mL of methanolic extract was mixed with 1 mL of chloroform and then 1.5 mL of concentrated sulphuric acid solution was added. The formation of reddish brown colour at the interface was observed for a positive test.

Volatile oil

0.5 mL of diluted sodium hydroxide and 0.5 mL of diluted hydrochloric acid were added to 2 mL of methanolic extract and mixed well. The formation of a white precipitate was observed for a positive test.

ANTIMICROBIAL TESTING

The antimicrobial potential of *S. costus* root extract was evaluated using agar-well diffusion method as described by (Abdallah, 2014) with some modifications. Before to the experimental phase, all identified microbial isolates were sub-cultured in a tighten bottles containing either Mueller-Hinton broth (18-24 hours, 35 °C) for bacteria or Sabouraud dextrose broth (48 hours, 25 °C) for fungi. After incubation, all turbid bottles- as a result of growth-were transferred and kept in the fridge (4 °C) to keep the microbial growth at the exponential phase until used. Autoclaved Bottles containing 20 ml of Mueller-Hinton agar or Sabouraud dextrose agar was poured hot on sterile Petri-dishes (90 mm in diameter) and left at room temperature until solidified. Working microbial strains were taken from the broth cultures (previously prepared) and adjusted as McFarland standard, then 100 µl from each microbial strain was put over Mueller-Hinton or Sabouraud dextrose agar plates (depending on the type of microorganism) and distributed above the agar using sterile cotton swabs. Wells were punched into the agar with a sterile cork borer (6 mm in diameter). Then, 100 µl from each extract (500 mg/ml) was dropped into the wells, extracts were previously reconstituted in 10% di-methyl-sulphoxide (DMSO) to make a concentration 500 mg/ml. 10 % DMSO did not show any inhibitory effect on microorganisms. Another well (in the centre) was loaded with 100 µl of 5 mg/ml Chloramphenicol for bacteria or 10 mg/ml clotrimazol for fungi. Plates were incubated at 35°C for 24 hours for bacteria or at 25°C for up to 48 hours for fungi. The antimicrobial activities of the tested extracts were determined by measuring the clear zone of inhibition in millimetre (mm) ± standard error of the mean.

MIC, MBC AND MFC ASSAY

Only microorganisms that showed high antimicrobial activity was tested for MIC, MBC and MFC. The minimum inhibitory concentration (MIC) was determined using microdilution method as described by Hassan et al. (2009) with slight modification. Briefly, in a set of sterile test tubes, serial two-fold dilutions were made to get 6 tubes containing 1 ml of 200, 100, 50, 25, 12.5 and 6.25 mg/ml of the extract, respectively. Additional two tubes were also used, one tube containing 1 ml of 10% DMSO to serve as negative control and the other tube containing 1ml of 5mg/ml chloramphenicol to serve as positive control. Then, 1 ml of sterile Mueller-Hinton broth and 100µl of the adjusted microbial strain were added to each tube (8 tubes). Tubes were gently shake and placed in the incubator at 35°C for 24 hours for bacteria or at 25oC for up to 48 hours for fungi. The lowest concentration (highest dilution) of the extract that showed no visible microbial growth (no turbidity) compared with the control tubes was considered as MIC. The minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) was evaluated by determined by sub-culturing the test dilution on to unseeded plates of Mueller-Hinton agar for bacteria or Sabouraud dextrose agar for fungi and incubated further for 18-24 h for bacteria or 48 h for fungi. The highest dilution that revealed no single bacterial colony on the plates was taken as MBC or MFC.

ANTIFEEDANT ASSAY AND STARVATION PERCENTAGE

A strain of *S. littoralis* was reared in the laboratory. Larvae were fed on fresh castor leaves, *Ricinus communius*. Adults were provided with 10% sugar solution. All the bioassays were conducted at 26± 2° C and 65±5 % R.H., with 8:16 L:D h photoperiod. The experiments were carried out on the 4th instar larvae. Serious of ascending crude concentrations were prepared (0.6 %, 1.25 %, 2.5 %, 5%, 10 % and 20%) by dilution in 70 % ethanol. Control discs were sprayed with the solvent alone. 400 larvae were starved overnight, then divided into 8 groups of 50 larvae each, six different concentrations of plant extract (*S. costus*), one group for the control and one group as starved larvae. Equal discs of castor bean leaves were rinsed in each treatment and in the control, the treated and untreated leaves were shad-dried. All

larvae control and treated leaves were weighted before and after treatment for 3 days. The dried leaves were placed individually in plastic Petri dishes. Ten larvae were transferred into each cup and allowed to feed on the treated and untreated leaves, the starved larvae were left without feeding for 24h. Five replicates for each treatment were carried out. The starvation percentages of tested larvae were calculated (Mostafa 1969 ; Abdel-Mageed et al. 1975).

$$\text{Starvation (\%)} = C - E/C - S \text{ X } 100$$

Where:
C = Mean weight gain of untreated larvae after 24 h;
E = Mean weight gain of treated larvae for each concentration after 24 h; and
S = Mean weight gain of starved untreated larvae after 24 h.

The antifeedant index (AFI) was calculated according to Sadek (2003).

$$\text{AFI (\%)} = [(C-T) / (C + T)] \text{ X } 100$$

Where:
C: the amount of food consumed (leaves) in the control; and
T: the amount of food consumed (leaves) in the treatment.

STATISTICAL ANALYSIS

Quantitative data were expressed as a mean ± standard error of means. One-way analysis of variance ANOVA was used and $P < 0.05$ was used in testing the statistical significance. Paired-Samples T-test was employed to determine any significant differences between methanol and ethanol extracts of the antimicrobial assay. The program used was SPSS-Statistical Package, version 11.

RESULTS AND DISCUSSION

PHYTOCHEMICAL SCREENING

The results of phytochemical screening suggest that, *Saussurea costus* roots are rich source of various bioactive constituents such as alkaloids, cardiac glycosides, coumarins, flavonoids, phenols, quinones, resins, steroids, tannins and terpenoids. These results are summarized in Table 1.

These phytochemical constituents are important for the use of health care. The findings of the present study agreed with previous studies; Chaudhary (2015) has reported that *S. lappa* (synonymous *S. costus*), is a rich source of alkaloids, steroids, flavonoids and resins. Moreover, Pandey et al., 2007 have reported that many bioactive molecules were identified and isolated from *S. costus*, such as sesquiterpene lactones, costunolide, isodehydrocostus, isozaluzanin-C, guiainolide, cynaropicrin, reynosin, santamarine and many more. Undoubtedly, the diverse biological activities of *S. costus* are attributed to its richness in phytochemical compounds. Accordingly, it is recommended that more studies may lead to the understanding which molecules are responsible for the antibacterial, antifungal and antifeedant activities against *Spodoptera littoralis* larvae.

Table 1. phytochemical analysis of *Saussurea costus* roots

Phytochemical constituents	Test results
Alkaloids	+
Cardiac glycosides	+
Coumarins	+
Flavonoids	+
Phenol/Polyphenols	+
Quinones	+
Resins	+
Steroids	+
Tannins	+
Terpenoids	+
Carboxylic acid	-
Leucoanthocyanins	-
Lipids	-
Emodins	-
Phlobatannins	-
Saponins	-
Volatile oil	-

+ = test positive, - = test negative

ANTIMICROBIL SCREENING

In the current study, 6 mm inhibition zone (IZ) means that there is no antimicrobial activity of the extract (the zone of the hole on the agar plate is 6 mm), above 6 mm to less than 10 mm means that there is a weak antimicrobial activity, from 10 mm to 12 mm means that there is a moderate antimicrobial activity (the double of the hole diameter), above 12 mm is noticeable or good antimicrobial activity. Philip et al. (2009) considered that IZ above 10 mm is good antimicrobial activity. Unlike the antibiotics, there is no standard criterion in explaining the IZ for the crude plant extracts. The results of the antimicrobial activity are demonstrated in (Table 2) and (Figures 2-6). The results of the antimicrobial efficacy of methanolic and ethanolic extracts of *S. costus* roots have shown that Gram-positive bacteria were more susceptible. *Bacillus cereus* ATCC 10876 has recorded the highest susceptibility (16.0±0.0, 15.5±0.5 mm), fol-

Table 2. The antimicrobial activity of the methanol and ethanol extracts of S. costus roots against different microorganisms

Tested Compound	Mean inhibition zones (IZ) in millimetre											
	Gram-positive bacteria					Gram-negative bacteria					Fungi	
	Sa	Se	Ss	Sp	Bc	Ec	Pa	Pv	Kp	Sf	As	Ca
MeOH of *S. costus* (500 mg/ml)	11.0 ±0.0	13.5 ±0.5	14.5 ±0.5	6. 5 ±0. 5	16.0 ±0. 0	8.5 ±0.5	9.5 ±0.5	9.0 ±0.0	6.0 ±0.0	6.0 ±0.0	26.0 ±1.0	6.0 ±0.0
EtOH of *S. costus* (500 mg/ml)	11.5 ±0.5	14.5 ±0.5	15.5 ±0.5	6. 5 ±0. 5	15.5 ±0. 5	8.5 ±0.5	7.5 ±0.5	9.0 ±0.0	6.0 ±0.0	6.0 ±0.0	27.5 ±0.5	7.5 ±0.5
Chloramphenicol (5 mg/ml)	34.0 ±0.5	38.0 ±3.0	36.5 ±1.5	20.5 ±1.0	34.0 ±2.0	34.0 ±1.0	19.5 ±0.5	33.0 ±1.0	33.5 ±0.5	34.5 ±0.5	-	-
Clotrimazole (10 mg/ml)	-	-	-	-	-	-	-	-	-	-	40.5 ±1.5	36.0 ±2.0
10% DMSO	6.0 ±0.0	6.0 ±0.0	6.0 ±0.0	6.0 ±0.0	6.0 ±0.0	6.0 ±0.0	6.0 ±0.0	6.0 ±0.0	6.0 ±0.0	6.0 ±0.0	6.0 ±0.0	6.0 ±0.0

*MeOH= methanol extract, EtOH=Ethanol extract, Sa= *Staphylococcus aureus* ATCC 29213, Se=*Staphylococcus epidermidis* ATCC 12228, Ss=*Staphylococcus saprophyticus* ATCC 43867, Sp=*Streptococcus pneumonia* ATCC 49619, Bc= *Bacillus cereus* ATCC 10876, Ec=*Escherichia coli* ATCC 25922, Pa=*Pseudomonas aeruginosa* ATCC 9027 Pv=*Proteus vulgaris* ATCC 6380, Kp=*Klebsiella pneumonia* ATCC 27736, Sf=*Shigella flexsneri* ATCC 12022, As= *Aspergillus niger* ATCC 6275, Ca= *Candida albicans* ATCC 10231.

lowed by *Staphylococcus saprophyticus* ATCC 43867 (14.5±0.0, 15.5±0.5 mm), *Staphylococcus epidermidis* ATCC 12228 (13.5±0.5, 15.5±0.5 mm) and *Staphylococcus aureus* ATCC 29213 (11.0±0.0, 11.5±0.5 mm), respectively. While, *Streptococcus pneumonia* ATCC 49619 has shown very weak susceptibility, which was 6.5±0.5 mm for methanolic and ethanolic extracts. On the other side, the Gram-negative bacteria exhibited weak or no susceptibility at all. Weak susceptibility was found with *Pseudomonas aeruginosa* ATCC 9027 (9.5±0.5, 7.5±0.5 mm), *Proteus vulgaris* ATCC 6380 (9.0±0.0, 9.0±0.0 mm) and *Escherichia coli* ATCC 25922 (8.5±0.5, 8.5±0.5 mm) for methanolic and ethanolic extracts, respectively.

While, *Klebsiella pneumonia* ATCC 27736 and *Shigella flexneri* ATCC 12022 revealed no susceptibility against the tested extract, which agrees with the results of Mohamed et al. (2017) who stated that, methanolic extract of *S. costus* roots has significant level of antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* (Gram-positive) and showed no effect against *Escherichia coli* and *Pseudomonas aeruginosa* (Gram-negative). Interestingly, these results are in

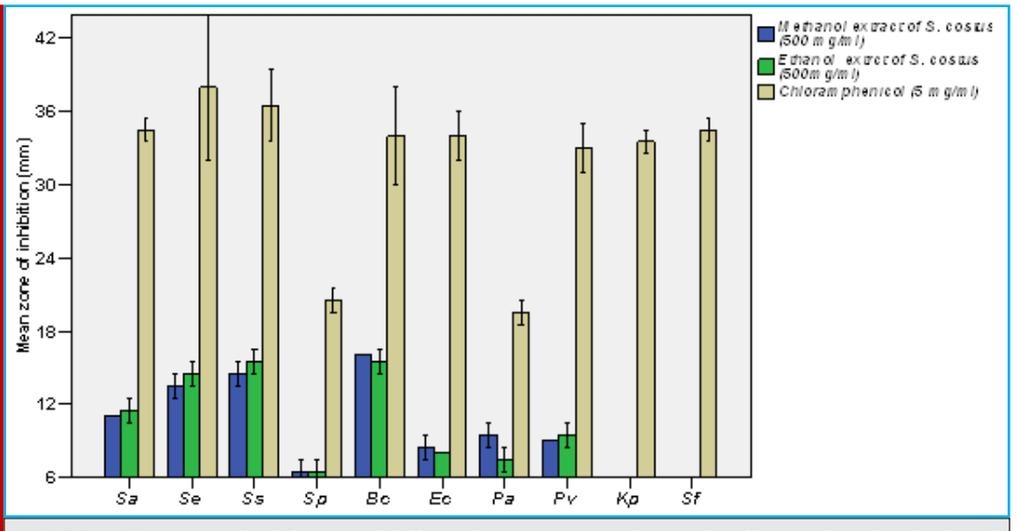

FIGURE 2. Mean zone of inhibitions of different bacterial strains due to the effect methanol and ethanol extracts of *S. costus* compared with chloramphenicol*
*Abbreviations of the names of microorganisms are detiled under (Table2).

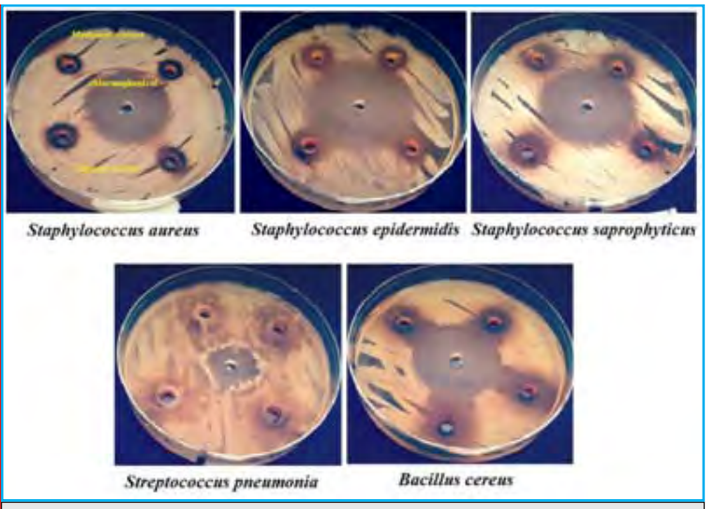

FIGURE 3. Susceptibility of gram-positive bacteria to methanol and ethanol extracts of *S. costus* roots compared with chloramphenicol

agreement in-partial with the findings of Hasson et al. (2013), in their study they have reported that *S. lappa* (synonymous *S.costus*) has exhibited significant level of antibacterial activity against different Gram-positive and Gram-negative pathogenic bacteria, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumanii*, *Escherichia coli* and *Klebsiella pneumonia*.

After comparing the results of our findings with the results of Hasson et al., 2013, we have concluded that, the former study has used 99.9 % ethanol as a solvent to extract the crude and in our study, we used 70 % Ethanol and used 80 % methanol. It is well known that absolute ethanol can collect non-polar constituents better than 70 % ethanol. Whereas, methanol can collect some non-polar and polar constituents from the plant materials. The statistical analysis (Paired-Samples T test) showed that, there was no significant difference between antibacterial activity of 70 % ethanolic and 80 % methanolic extracts, which means that the effective antibacterial compounds are present in the non-polar fraction. This consumption is supported with the findings of Pandey et al. (2008), mentioned that, the essential oil of *S. costus* roots has exhibited better antibacterial effects as compared with the methanolic extract. In addition, *S. costus* has showed high significant level of antifungal activity

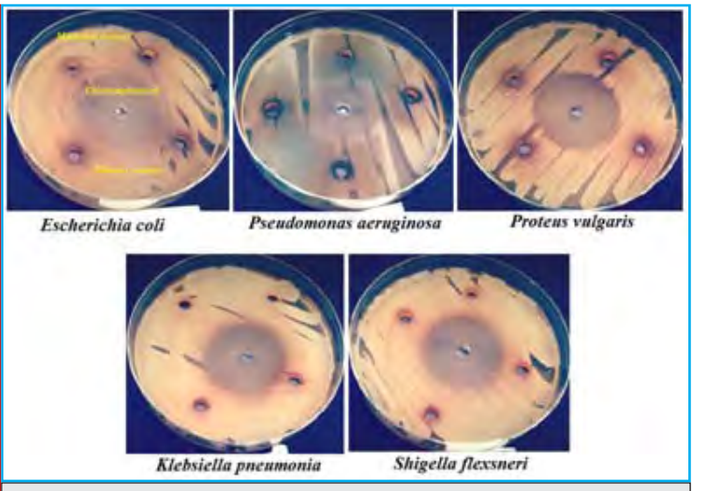

FIGURE 4. Susceptibility of gram-negative bacteria to methanol and ethanol extracts of *S. costus* roots compared with chloramphenicol

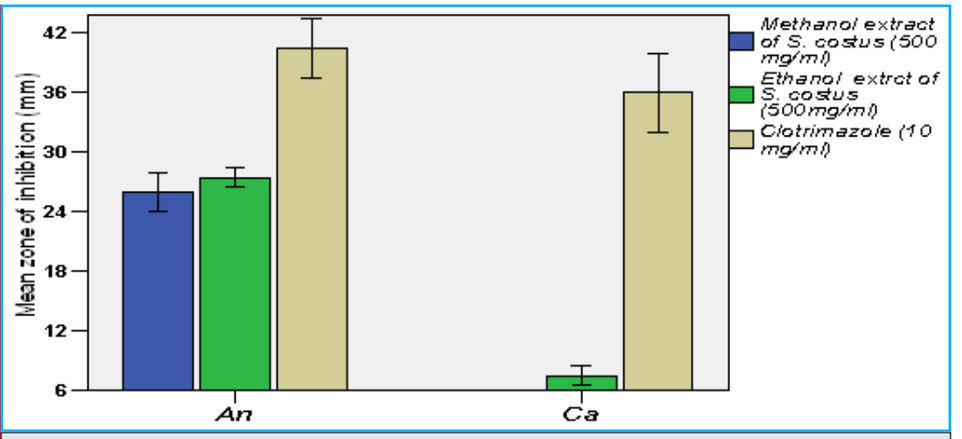

FIGURE 5. Mean zone of inhibitions of different fungal strains due to the effect methanol and ethanol extracts of *S. costus* compared with clotrimazole*
**An=Aspergillus niger, Ca= Candida albicans*

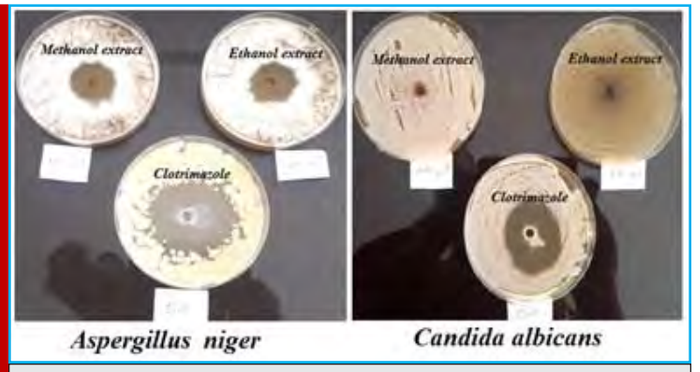

FIGURE 6. Susceptibility of fungalstrains to methanol and ethanol extracts of *S. costus* roots compared with clotrimazole

against *Aspergillus niger* ATCC 6275 and weak effect against *Candida albicans* ATCC 10231.

It disagrees with the findings of Mohamed et al. (2017) who reported good antifungal activity of *S. lappa* against *Candida albicans*. This contradiction is related to the solvent used in extraction, as reported by Patil et al. (2009) that, diethyl ether fraction has showed prominent fungicidal activity against *Candida albicans*. However, the antimicrobial activity resulted from the current investigation was not competitor to chloramphenicol or clotrimazole. These referenced antibiotics are present in a pure form (single compound), while the extracts are investigated as a crude. Therefore, the antimicrobial efficacy of *S. costus* roots could be competitor to antibiot-

Table 3. MIC and MBC of the methanolic and ethanolic extracts of the *S. costus* roots

Bacterial strain	MIC mg/ml		MBC mg/ml		MBC/MIC	
	MeOH	EtOH	MeOH	EtOH	MeOH	EtOH
Staphylococcus saprophyticus ATCC 43867	50	50	100	100	2	2
Staphylococcus epidermidis ATCC 12228	50	50	200	100	4	2
Bacillus cereus ATCC 10876	100	50	200	100	2	2
Staphylococcus aureus ATCC 29213	100	50	200	100	2	2

*MeOH= methanol extract, EtOH=Ethanol extract.

Table 4. MIC and MFC of the methanolic and ethanolic extracts of the *S. costus* roots

Fungal strain	MIC mg/ml		MFC mg/ml		MFC/MIC	
	MeOH	EtOH	MeOH	EtOH	MeOH	EtOH
Aspergillus niger ATCC 6275	50	50	50	50	1	1

*MeOH= methanol extract, EtOH=Ethanol extract.

Table 5. Antifeedant activity of ethanolic extract of *S. costus* against 4th instar larvae of *S. littoralis*.

Concentration	Antifeedant index (%) ±SE		Mean*
	Days post-treatment		
	1st	2nd	
0.6%	28.39± 4.22e	26.01± 1.64d	27.20 %
1.25%	39.54± 3.27cd	39.75± 5.50c	39.64%
2.5%	35.16± 3.36de	38.34± 3.21c	36.75%
5%	46.58± 1.96c	43.03± 2.52bc	44.80%
10%	58.34± 4.75b	50.90± 2.67b	54.60%
20%	85.36± 2.24a	70.16± 3.23a	77.76%

*Data are expressed as mean ± SE (n=5), total mean of each treatment at different time intervals, values were analyzed by one-way ANOVA, where means within each column followed by different letters are significantly different (P< 0.05 by LSD).

ics if the bioactive compound (s) isolated and studied in a future studies. This hypothesis is boosted by the results of MIC, MBC and MFC as shown in (Tables 3 and 4), which revealed that, the methanolic extract was bacteriostatic to *Staphylococcus saprophyticus*, *Staphylococcus epidermidis*, *Bacillus cereus* and *Staphylococcus aureus* at 50, 50, 100 and 100 mg/ml, respectively; and the bactericidal activity was at 100, 200, 200, 200 mg/ml, respectively. As well, the ethanolic extract was bacteriostatic to *Staphylococcus saprophyticus*, *Staphylococcus epidermidis*, *Bacillus cereus* and *Staphylococcus aureus* at 50, 50, 50 and 50 mg/ml, respectively; and the bactericidal activity was detected at 100, 100, 100, 100 mg/ml, respectively. In addition, the methanolic and ethanolic extracts were bacteriostatic and bactericidal to *Aspergillus niger* at 50 mg/ml. The values of MBC/MIC were ranging between 2-4 for bacterial strains and 1 for fungal strain. Djeussi (2013) has stated that, the

Table 6. Starvation percentage (%) of the 4th instar larvae of *S. littoralis* treated with the ethanolic extract of *S. costus*

Treatments	Time	Average weight (mg/larva)	Difference* (mg/larva)	Starvation (%)	Average
0.6	0 min 24h 48h	63.13 74.30 93.62	-------- +11.17 +30.49	------- 25.09 24.18	24.63%
1.25	0 min 24h 48h	69.10 76.24 84.12	-------- +7.14 +15.02	------- 42.38 52.05	47.21%
2.50	0 min 24h 48h	60.11 70.00 70.73	-------- +9.89 +10.62	------- 30.58 59.98	45.28%
5	0 min 24h 48h	60.83 64.20 72.31	-------- +3.37 +11.48	------- 58.55 58.43	58.49%
10	0 min 24h 48h	68.23 64.75 77.80	-------- -3.48 +9.57	------- 87.94 61.87	74.90%
20	0 min 24h 48h	68.00 63.25 65.78	-------- -4.75 -2.22	------- 93.39 83.11	88.25%
Control	0 min 24h 48h	68.10 85.12 112.01	-------- +17.02 +43.91	------- ------- -------	------- ------- -------
Starved larvae	0 min 24h 48h	61.68 55.39 50.09	-------- -6.29 -11.59	------- ------- -------	------- ------- -------

plant extract is a bactericidal when the ratio of MBC/MIC equals 4 and bacteriostatic when MBC/MIC ratio is >4. Accordingly, *S. costus* roots may possess new natural antimicrobial agents that require isolation of these novel and natural bioactive molecules.

ANTIFEEDANT PROPERTIES

The antifeedant potential results of the ethanolic extract of *S. costus* on the larvae of *Spodoptera littoralis* are presented in (Table 5), the crude methanol extract exhibited antifeedant effect on the 4th instar larvae of *S. littoralis*. The antifeedant activity are varying from 27.2%, 39.64%, 36.75%, 44.8%, 54.6 to 77.76% at 0.6, 1.25, 2.5, 5, 10 and 20% concentrations, respectively. It was noticed that the antifeedant activity on the larvae increased by days in all concentrations after treatment. Data in (Table 6) shows the starvation percentage of the 4th instar larvae of *S. littoralis* treated with the ethanolic extract of *S. costus*. The starvation percentage same as antifeedant activity which increased with the increasing of the concentration during 48 hours. The root of *S. lappa* which have the essential oil and the alkaloid considered as insect repellent (Kapoor, 2001). The Costunolide that isolated from root extract of *S. lappa* showed 80% antifeedant activity to citrus pest *Papilio demoleus* (Vattikonda et al., 2014). The plant extract of of *S. costus* may be useful for effective control of *S. littoralis* at larval stages.

CONCLUSION

Roots of *Saussurea costus* (*S. costus*) are widely used in the traditional medicine; it is frequently mentioned in the Islamic medicine as well as ancient Indian and Chinese medicine. the current investigation revealed the presence of many bioactive phytochemical molecules, antimicrobial activity, and antifeedant effect against *Spodoptera littoralis* larvae, which offers a scientific basis for traditional uses of *S. costus* roots as antimicrobial and insect repellent. We recommend further future studies using different solvents and extraction systems as we assume that there are perhaps more bioactive compounds in the non-polar or aromatic fraction. Moreover, it is worthy to separate and identify these bioactive compounds from the roots of *S. costus* in order to get new natural and effective drugs.

SOURCE OF SUPPORT

Nil

CONFLICT OF INTEREST

None declared

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Production and partial characterization of extracellular polysaccharide from endophytic *Bacillus cereus* RCR 08

Ananya Mukherjee[1], Rituparna Das[1], Anju Sharma[1], Arundhati Pal[2] and A. K. Paul[1]*

[1]*Microbiology Laboratory, Department of Botany, University of Calcutta, 35, Ballygunge Circular Road, Kolkata, India*

[2]*Post Graduate Department of Botany, Serampore College, 9, William Carey Road, Serampore, Hooghly, India*

ABSTRACT

The present study focuses attention on the production of extracellular polysaccharide (EPS) by bacterial endophytes of *Ricinus communis* L. Among the 28 endophytic bacterial isolates screened for EPS production, a potent isolate identified as *Bacillus cereus* RCR 08 (GenBank accession number MF159112) produced significant amount of EPS in mineral salts medium under batch culture. In single factor system of analysis, glucose and ammonium chloride were most suitable carbon and nitrogen sources respectively for EPS production. Maximum growth (7.1 g/L) and EPS yield (10.24 g/L) was attained when glucose and ammonium chloride were used in the ratio of 25:1. The isolated polymer contained carbohydrate (88.8%), protein (3.18%), RNA (6.0%) as well as DNA (3.2%) and showed characteristic FTIR absorption spectrum with peaks at 3404, 2,933, 1,655, and 1,042 cm^{-1}. The emulsifying activity of the EPS was more or less comparable with Tween 80. Though the EPS failed to show any antibacterial activity, it exhibited moderate DPPH radical scavenging activity and displayed a dose-dependent cytotoxic activity against hepato cellular carcinoma (Huh 7.5) cell line in MTT assay. A detailed physico-chemical analysis is, therefore, essential to assess the significance and potential importance of this endophytic EPS in biotechnology.

KEY WORDS: *BACILLUS CEREUS*, ENDOPHYTIC BACTERIA, EXTRACELLULAR POLYSACCHARIDE, FTIR ANALYSIS, *RICINUS COMMUNIS* L.

INTRODUCTION

Endophytes are microorganisms which colonize living internal tissues of plants without causing any apparent negative impact on the host plant. They occur ubiquitously in almost all plants and are benefitted from the host by deriving organic nutrients, shelter as well as transmission to the next host generation. On the other hand endophytes favour the infected host plants by fixation of atmospheric nitrogen, production of growth

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**Corresponding Author: amalk_paul@yahoo.co.in*
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promoting substances, imparting tolerance to stress and toxicity to herbivores, nematodes and pathogens (Borges et al., 2009). Recently, attention has been paid to the endophytes for the production of biopolymers including extracellular polysaccharides (EPS) and their utilization for potential industrial applications (Donot et al., 2012; Kusari et al., 2014).

Microbial extracellular polymeric substances, the heterogenous matrix of polymers comprising of polysaccharides, proteins, nucleic acids, uronic acids, humic substances, lipids etc. (Wingender et al., 1999) are biosynthesized by bacteria and fungi via intracellular or extracellular processes (Freitas et al., 2011). In recent years, a variety of structurally different EPSs with bioactive potentials have been reported from endophytes (Guo et al., 2014; Mahapatra and Banerjee, 2016; Liu et al., 2017).

Production of such endophytic EPS in culture depends on media components such as carbon and nitrogen sources, minerals, surfactants and cultivation conditions including incubation temperature, pH and aeration (Liu et al., 2009). The EPS so produced in their natural habitat play a key role in plant-endophyte interactions and are essential for the survival in the host plant (Wingender et al., 1999). Owing to their interesting physico-chemical and biological activities, the endophytic EPS has been considered as potential candidate for nutraceuticals, bioleaching, bioremediation, waste water treatment and pharmaceutical industries. Special attention has also been paid for the use of EPS as a hydrophilic matrix for controlled release of drugs (Gandhi et al., 1997), anti HIV agent (Yamada et al., 1997), enhancement of nonspecific immunity (Sutherland, 1998), antimicrobial (Orsod et al., 2012), antioxidant (Liu et al., 2009) and antitumour activities (Chen et al., 2013). Furthermore, they have been found to be extremely susceptible for biodegradation in nature and thus are environment friendly.

Ricinus communis L. (Euphorbiaceae), a perennial flowering shrub, is an indigenous oil-yielding plant of India having medicinal as well as agrochemical importance. Its oil and seeds have been used in folk medicine for disorders like severe constipation, worm infestation, rheumatism, intestinal inflammation and also for birth control. Castor oil is an effective motor lubricant and also used as a component of flavour and ingredient for preparing protective coatings for tablets. Apart from these, a range of biologically active compounds have been isolated especially from rhizosphere and endosphere associated fungi of *R. communis* L. with probable industrial applications (Rajkumar and Freitas, 2008; Jain and Sharma, 2015).

The increasing demand for natural polymers with industrial applications has thus led to an interest in EPS production by microorganisms which have high yield and better quality than plant or animal derived polysaccharides (Moscovici, 2015). In this article we report the screening of bacteria endophytic to *R. communis* L. for production of EPS, determine the influence of nutritional and environmental conditions for EPS production by a selected potent strain under batch culture and characterization of the partially purified polymer.

MATERIAL AND METHODS

A total of 28 endophytic bacterial isolates of *Ricinus communis* L. used in the present study were isolated in the Microbiology Laboratory, Department of Botany, University of Calcutta. Pure cultures of endophytic bacterial isolates were maintained on slopes of nutrient agar by repeated sub-culturing at an interval of 30 days.

The selected potent EPS producing isolate was characterized following standard morphological and physiobiochemical tests (Gerhardt et al., 1994). Antibiotic sensitivity of the bacterial isolate was detected following the Kirby Bauer disc-diffusion assay (Bauer et al., 1966) using antibiotic impregnated discs (Himedia, India, 6 mm dia).

The 16S rRNA gene sequence of the isolate was determined by direct sequencing of PCR amplified 16S rDNA. The genomic DNA was isolated from the overnight grown culture and purified according to the modified method of Marmur (1961). The 16S rDNA was amplified using the universal primers 27F (5'AGAGTTTGATCCTGGCTCAG3') and 1492R (5'GGTTACCTTGTTACGACTT3') and the amplified product was purified using QIAquick gel extraction kit (Qiagen, Netherlands). The sequencing reaction was performed with ABI PRISM Dye Terminator cycle-sequencing ready reaction kit (Applied Biosystems) and products were purified and electrophoresed on polyacrylamide sequencing gel using an ABI 377 automated DNA sequencer. Sequencing data were analyzed by ABI version 3.0.1 b3 software and compared with reference sequences using the NCBI BLASTN programme. Multiple sequence alignments were carried out by using BLOSUM 62 matrix with the program package Clustal-W employing the neighbour-joining algorithm method (Saitou and Nei, 1987) with MEGA version 6.0.

Mineral salts medium was inoculated with freshly prepared inoculum (2% v/v) of the endophytic bacteria and incubated at 32 °C on rotary shaker (120 rpm). Growth was determined by measuring dry weight of the washed cell mass harvested by centrifugation (10,000×g, 20 °C for 10 min). Cell pellet was transferred to pre-weighed aluminium cup and dried to constant weight at 80 °C. The EPS of the cell-free culture filtrate was precipitated with double volume of chilled acetone, kept overnight at 4 °C and recovered by centrifugation (12,000×g, 4 °C for 20 min). Cell-bound EPS was extracted with EDTA (0.05M), precipitated with chilled acetone and recovered

by centrifugation. The soluble and cell-bound EPS fractions were pooled and dissolved in known volume of distilled water prior to quantification.

The EPS was quantified following the phenol sulphuric acid method of Dubois et al. (1956). To 1 mL of EPS solution, 1 mL of 5% (w/v) phenol solution was added and mixed thoroughly. To the reaction mixture, 5 mL of concentrated H_2SO_4 was purged in and the optical density was measured at 490 nm using Systronics colorimeter. The amount of EPS was determined from the calibration curve using glucose as the standard.

For isolation and purification of the EPS, the selected isolate RCR 08 was grown in mineral salts medium under continuous shaking for 64 h. The cell-bound EPS was extracted following washing of cell mass with 0.05 M EDTA and separated by centrifugation (12,000 × g for 20 min). The EPS was recovered from the supernatant by precipitation with chilled acetone. The soluble EPS from the cell-free culture filtrate was obtained by the same acetone precipitation method. The soluble and bound EPS were pooled, dissolved in distilled water and subjected to dialysis in sterile water for 24 h at 4 °C with regular change of dialysate. On completion of dialysis, the EPS was further treated with chilled acetone at 4 °C and the precipitate was collected by centrifugation (12,000×g, 4 °C, 20 min) as partially purified EPS. To remove protein and nucleic acid, trichloroacetic acid (20%) was added to the partially purified EPS solution and incubated in ice for 30 minutes prior to centrifugation (15,000 × g, 4 °C, 30 min) (Bales et al., 2013). The supernatant was treated with double volume of chilled ethanol at 4 °C and the precipitate was collected by centrifugation (12,000 × g, 4 °C, 20 min).

The partially purified EPS was analyzed for its carbohydrate, protein and nucleic acid contents. While the carbohydrate content was estimated following the phenol-sulphuric acid method of Dubois et al. (1956), protein content was estimated by folin-phenol reagent using bovine serum albumin as standard (Lowry et al., 1951). DNA and RNA contents of the EPS were estimated by diphenylamine (Soni et al., 2011) and orcinol methods (Almog and Shirley, 1978) respectively.

The absorbance of the crude and purified EPS in distilled water was recorded in the range of 200 to 300 nm using UV-VIS spectrophotometer (Jenway, Model 6505).

The Fourier transform infrared (FTIR) spectra of the purified EPS were recorded in a Perkin Elmer RX-1 FTIR spectrometer. The dried sample was grinded with potassium bromide (KBr) and pressed into pellet for spectrophotometric scanning in the frequency of 400 to 4000 cm^{-1}.

The emulsification assay was carried out following the method as described by Cooper and Goldenberg (1987). The purified EPS solution (2.5 mL, 0.5% w/v) was mixed with 2.5 mL hydrocarbons, vortexed to homogenity and left to stand for 24 h at 4 °C. The emulsifying activity was expressed as the percentage of the total height occupied by the emulsion. The hydrocarbon substrates used were benzene, palm oil, olive oil, soybean oil, kerosene, petrol, octane, hexane, tetradecane and hexadecane.

The aqueous solution of purified EPS was filter sterilized and screened for antibacterial activity following agar-cup assay method using four test organisms like *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas cepacia*. Modified method of Liu et al. (2010) was used for the DPPH radical scavenging activity of the EPS. The reaction mixture containing 0.5 mL of purified EPS, 0.2 mL of DPPH solution (0.4 mM DPPH in methanol) and 2.5 mL distilled water was shaken vigorously, incubated for 30 min at room temperature and the optical density was measured at 517 nm. Vitamin C (ascorbic acid) was used as the positive control. The percentage of scavenging of free radical was calculated according to the following formula:

$$\% \text{ scavenging activity} = \{1- (A_1-A_2/A_0)\} \times 100$$

Where,

A_1 = O.D. of reaction mixture
A_2 = O.D. of reaction mixture without DPPH
A_0 = O.D. of reaction mixture with DPPH but without sample

Proliferation of Huh 7.5 cells in response to EPS produced by *B. cereus* RCR 08 was measured by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Slater et al. (1963). Huh 7.5 cells in DMEM medium were incubated overnight in 96 microtiter plate. The cells were treated with filter sterilized EPS of different concentrations and incubated for 48 h following an additional incubation of 4 h with 20 µL of MTT (5 mg/mL). The MTT-transformed crystals were dissolved in MTT solvent [4 mM HCl, 0.1% Nondet P-40 (NP40) in isopropanol] and the absorbance was measured at 595 nm with a reference filter of 620 nm by using a microplate reader (Molecular Devices, Sunnyvale, USA). The relative cell viability was expressed as the mean percentage of viable cells relative to the respective control.

All experiments were carried out in triplicates and results represent mean ± standard deviation.

RESULTS AND DISCUSSIONS

Endophytic microorganisms, the bacteria in particular have long been recognized as important bioresources for production of structurally and functionally diverse extracellular polymeric substances. All 28 endophytic

Table 1. Screening of bacterial endophytes of *Ricinus communis* L. for production of extracellular polysaccharide

Category of producer	Production of EPS, g/L	% EPS producer*
Good	>1.0	3.57
Moderate	0.31-1.0	85.71
Poor	0.14-0.3	10.71

**Expressed out of total 28 isolates*

bacteria isolated from *Ricinus communis* L. were screened for EPS production during growth under batch cultivation in glucose containing mineral salts medium. The EPS content (bound and free) of each isolate was quantified in terms of their carbohydrate content (Dubois et al., 1956) and almost all the endophytic isolates of *R. communis* L. were capable of producing EPS (Table 1). Majority of the isolates were poor to moderate producers with the exception of isolate RCR 08, which produced good amount of EPS (1.5 g/L) and was selected for further studies. Liu et al. (2017) in a recent review have summarized the EPS-producing endophytic bacteria and their host plants which include rice, sorghum, sugarcane, *Artimisia annua*, *Ophiopogon japonicas*, etc.

Morphological and physiological analysis revealed that the endophytic isolate RCR 08 endophytic to root tissues of *R. communis* L. is a rod-shaped, Gram-positive, motile and endospore forming bacterium which form white smooth colonies on mineral salts agar (Figure 1). The isolate could tolerate wide range of pH (3.5-8.0) and temperature (30-40 °C) and produced a number of hydrolytic enzymes such as catalase, amylase, protease, pectinase, lipase, gelatinase and inulinase. It produced acid from glucose, fructose, sucrose, maltose and galactose and was resistant to antibiotics ampicillin, bacitracin, penicillin and methicillin. Based on these characteristics, the endophytic isolate RCR 08 was tentatively identified as a member of the genus *Bacillus*. Sequence analysis of 16S rDNA of the isolate *Bacillus* RCR 08 showed 99% similarity with *Bacillus cereus* strain ATCC 14579, reasonably high score and e-value being zero. The evolutionary relationship of the endophytic isolate RCR 08 as depicted from the dendrogram showed clear rooted evolution (Figure 2). The 16S rDNA sequence of the isolate RCR 08 has been deposited to the GenBank under the accession number MF159112 and the isolate has been designated as *Bacillus cereus* RCR 08. Similar to *B. cereus* RCR 08, production of EPS by *B. cereus* SZ1 endophytic to *Artimisia annua* L. is not uncommon (Zheng et al., 2016). Likewise endophytic *B. amyloliquefaciens* (Chen et al., 2013) and *B. licheniformis* (Singh et al., 2011) isolated from *Ophiopogon japonicas* and *Gracilaria dura* respectively are well recognized as EPS producers.

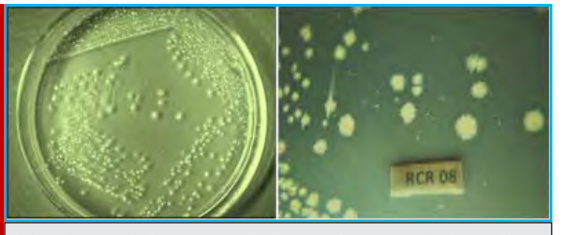

FIGURE 1. Colony morphology of potent EPS producing bacterial isolate *Bacillus cereus* RCR 08 endophytic to root tissues of *Ricinus communis* L. in mineral salts agar plate

The production of EPS by bacteria in culture depends on phases of growth, media components, nutritional status and the environmental conditions. Media components including carbon and nitrogen sources, mineral elements, etc. on EPS production have been tested using the single factor method. Out of eight different media tested, *B. cereus* RCR 08 showed maximum EPS production (7.65 g/L) in ammonium chloride containing mineral salts medium (Table 2). Yeast extract medium supported significant biomass formation but not the EPS production. Tryptic soy and Luria Bertani media failed to support both biomass as well as EPS production by *B. cereus* RCR 08.

During growth under shake flask condition in mineral salts medium, the extracellular polymer accumulation by the endophytic isolate *B. cereus* RCR 08 was found to be more or less parallel with growth and continued to increase till late stationary phase of growth. The highest EPS production (9.48 g/L) was obtained after 64 h of incubation (Figure 3). This supports the earlier observations of Decho (1990) and Manca et al. (1996). EPS synthesis was accompanied by increasing cell mass formation until glucose, the sole source of carbon, was consumed. In addition, production of EPS was accompanied with decline of pH of the medium (data not shown).

Ability to utilize nine different carbon sources for growth and EPS production by *B. cereus* RCR 08 was tested and EPS production was highest (9.48 g/L) in glucose followed by mannitol (6.93 g/L) and maltose (5.07 g/L) (Figure 4). Though, the isolate *B. cereus* RCR 08 preferred maltose for growth, it failed to utilize galactose. Different carbon sources have been utilized for EPS production by endophytes (Liu et al., 2009; Bragadeeswaran et al., 2011) and glucose and sucrose are reported to be the most suitable ones.

Recently, Li et al. (2016) and Mahapatra and Banerjee (2016) have showed that organic nitrogenous compounds supported higher biomass and EPS yield than the inorganic ones. Supplementation of both inorganic and organic nitrogen in the growth medium at 0.1% (w/v)

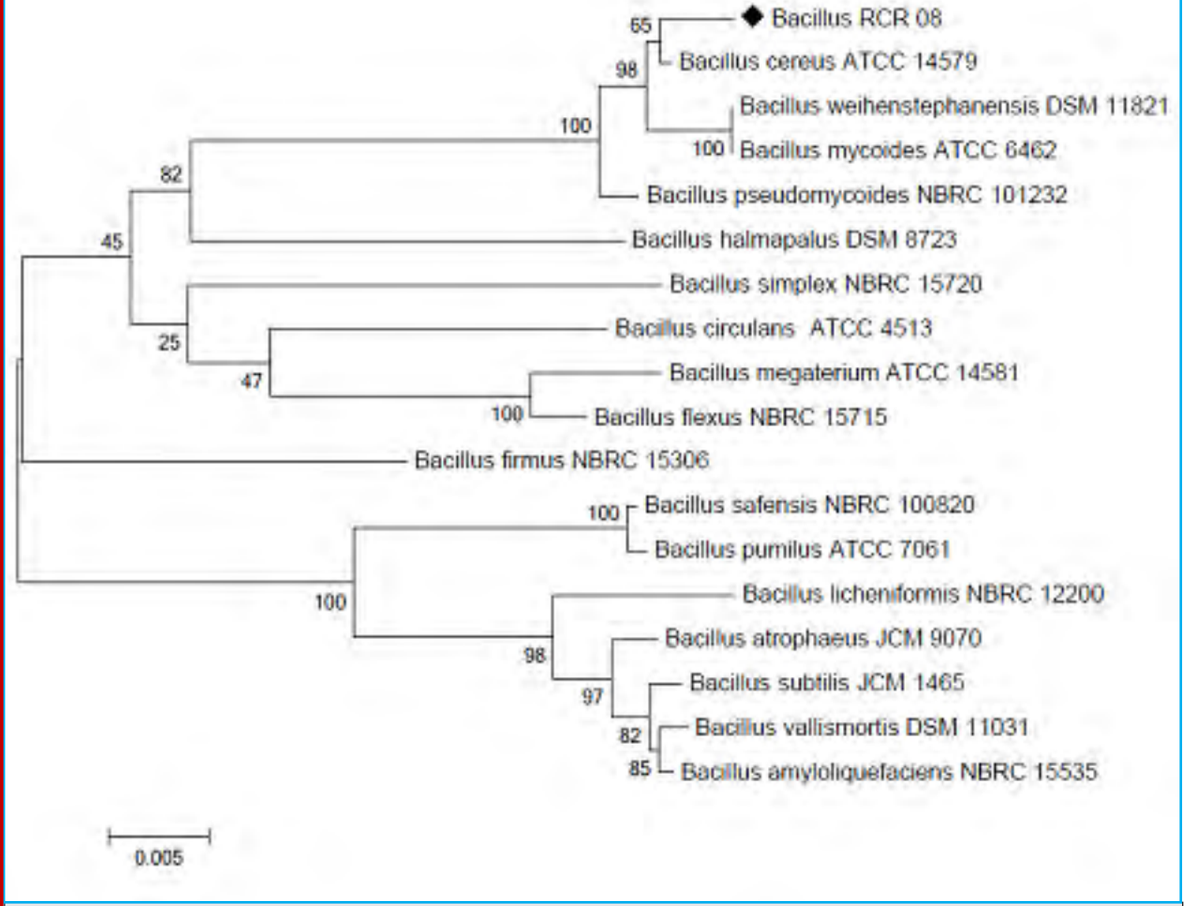

FIGURE 2. Phylogenetic relationship of *Bacillus* RCR 08 endophytic to *Ricinus communis* L. with closely allied *Bacillus* spp. based on 16S rDNA sequence analysis.

Table 2. Effect of different media on growth and EPS production by the endophytic bacterial isolate *B. cereus* RCR 08

Medium	Growth, CDW, g/L		EPS, g/L	
	48 h	72 h	48 h	72 h
Davis and Mingioli's medium	1.4 ± 0.02	2.4 ± 0.02	2.09 ± 0.04	1.81 ± 0.03
Mineral salts medium	1.6 ± 0.01	1.9 ± 0.04	1.05 ± 0.03	1.38 ± 0.04
Mineral salts medium with NH4Cl	6.7 ± 0.04	6.4 ± 0.03	6.07 ± 0.05	7.65 ± 0.07
Glutamate-mannitol medium	4.0 ± 0.02	4.0 ± 0.03	1.36 ± 0.04	2.13 ± 0.04
Tris-Glucose medium	1.8 ± 0.01	2.0 ± 0.02	1.65 ± 0.02	1.90 ± 0.02
Yeast extract medium	4.7 ± 0.03	4.5 ± 0.01	2.94 ± 0.03	3.75 ± 0.02
Tryptic soy medium	4.6 ± 0.03	4.5 ± 0.03	1.06 ± 0.03	1.41 ± 0.04
Luria Bertani medium	3.4 ± 0.01	2.8 ± 0.04	1.73 ± 0.02	1.19 ± 0.02

Values represent mean of triplicate readings ± S.D.

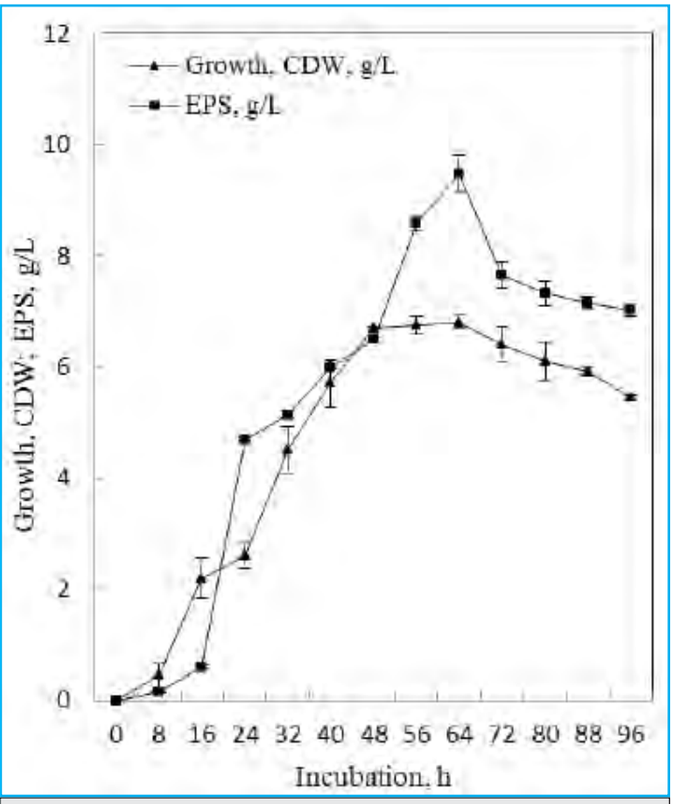

FIGURE 3. Time course of growth and EPS production by the endophytic bacterial isolate *B. cereus* RCR 08 in batch culture

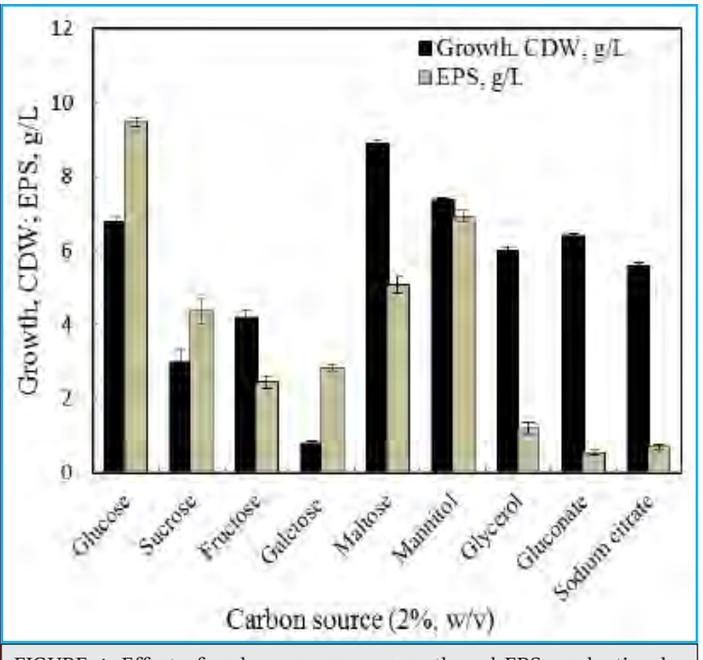

FIGURE 4. Effect of carbon source on growth and EPS production by endophytic bacterial isolate *B. cereus* RCR 08

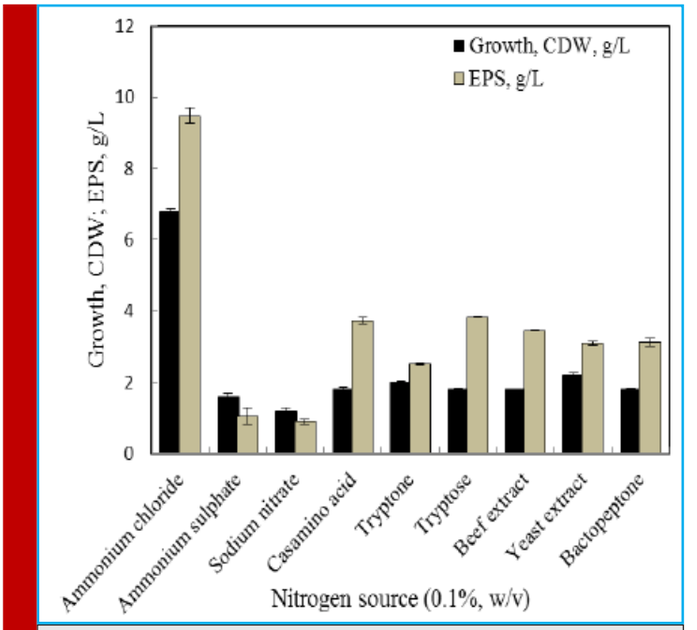

FIGURE 5. Effect of nitrogen source on growth and EPS production by the endophytic bacterial isolate *B. cereus* RCR 08

level showed discrete variation in the growth and polymer production by the isolate *B. cereus* RCR 08, however maximum EPS production (9.48 g/L) was recorded in presence of ammonium chloride and was followed by organic nitrogenous compounds such as tryptone, casamino acid and beef extract (Figure 5). When glucose and ammonium chloride in the medium were maintained at a ratio of 25:1 maximum growth (7.1 g/L) and EPS production (10.24 g/L) were recorded (Figure 6).

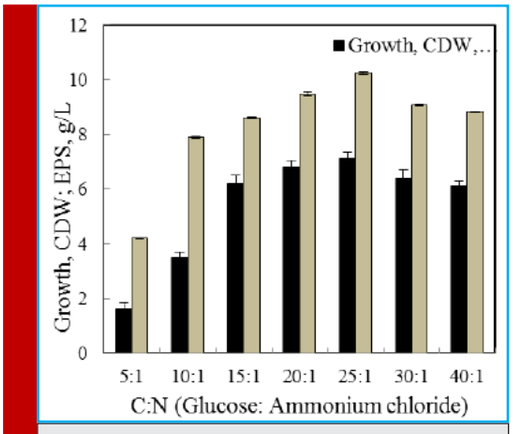

FIGURE 6. Effect of C:N (glucose: ammonium chloride) ratio on growth and EPS production by the endophytic bacterial isolate *B. cereus* RCR 08

Miqueleto et al. (2010) studied the influence of different carbon sources and the C/N ratio on the production of EPS by immobilized bacterial biomass and found that high C/N ratio favored the biopolymer production.

Compositional analysis of the partially purified EPS of *B. cereus* RCR 08 revealed that it was composed of 88.8% carbohydrate, 3.18% protein, 6.0% RNA as well as 3.2% DNA. The partially purified EPS showed characteristic peaks of protein and nucleic acids at 260 and 280 nm, respectively (Figure 7). Following TCA treatment, the EPS, however showed characteristic spectrum similar to those of authentic polysaccharides such as galactan and dextrin (Figure 7).

FTIR spectrum of purified EPS showed characteristic absorption peaks at 3404, 2,933, 1,655, and 1,042 cm^{-1} (Figure 8). The strong band at 3404 cm^{-1} was assigned to the hydroxyl stretching vibration of the polysaccharide, while the band at 2933 cm^{-1} was due to C-H stretching vibration. The bands in the region of 1500 and 1200 cm^{-1} were assigned to C-H deformation vibration and the bands between 1100 and 1075 cm^{-1} corresponded to C-O-C and C-O-H stretching vibration. A characteristic absorption at 928 cm^{-1} was possibly due to the stretching vibration of pyran ring (Liu et al., 2010). A similar spectrum was also observed by Sonawdekar and Gupte (2016) for EPS from *B. cereus*.

The emulsifying activity of extracellular polysacharides as tested by the method of Cooper and Goldenberg (1987) revealed that all the hydrocarbons (except petrol and hex-

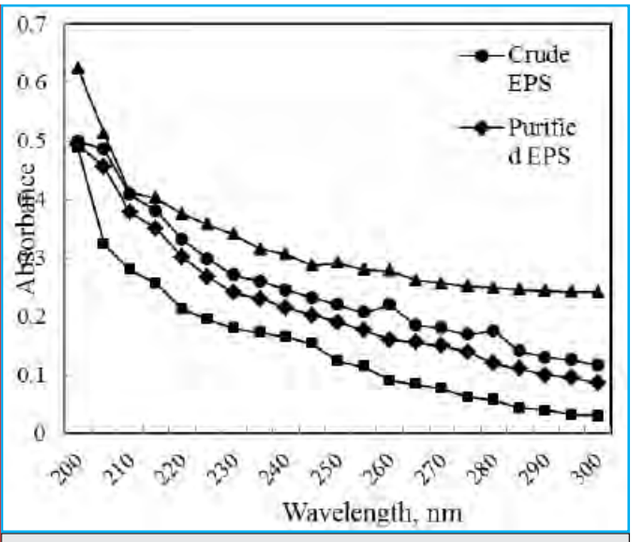

FIGURE 7. Comparision of UV absorption spectra of the purified exopolysaccharide obtained from the endophytic bacterial isolate *B. cereus* RCR 08 with other authentic polysaccharides

adecane) showed effective emulsification (Table 3). The highest emulsifying activity of the EPS was obtained with benzene (76.37%) followed by tetradecane (70%) and hexane (66.66%). However, tween 80 showed higher emulsifying activity for kerosene (73.07%) than the EPS of *B.cereus* RCR 08. Chowdhury et al. (2011) reported high emulsifying activity of *B. megaterium* RB-05 EPS in coconut oil, mustard oil and xylene while *B. cereus* isolated by Sonawdekar and Gupte (2016) showed 53% emulsification.

Though there are several reports of EPS with antimicrobial activities (Orsod et al., 2012), the EPS produced by the endophytic isolate RCR 08 failed to show any antibacterial activity when tested against *E. coli, B. subtilis, S. aureus* and *P. cepacia* by agar-cup assay. Similarly, antioxidant properties of EPS (Liu et al., 2009) are also not rare. The DPPH radical scavenging activity of the EPS isolated from *B. cereus* RCR 08 increased with increasing concentrations and a scavenging activity of 16% was recorded at a concentration of 10 mg/mL but was much lower as compared to vitamin C (Figure 9). The scavenging activity exhibited by the EPS might be attributed due to their hydrogen donating abilities. The effect of EPS extracted from *B. cereus* RCR 08 on the viability of Huh 7.5 cells was determined by MTT assay.

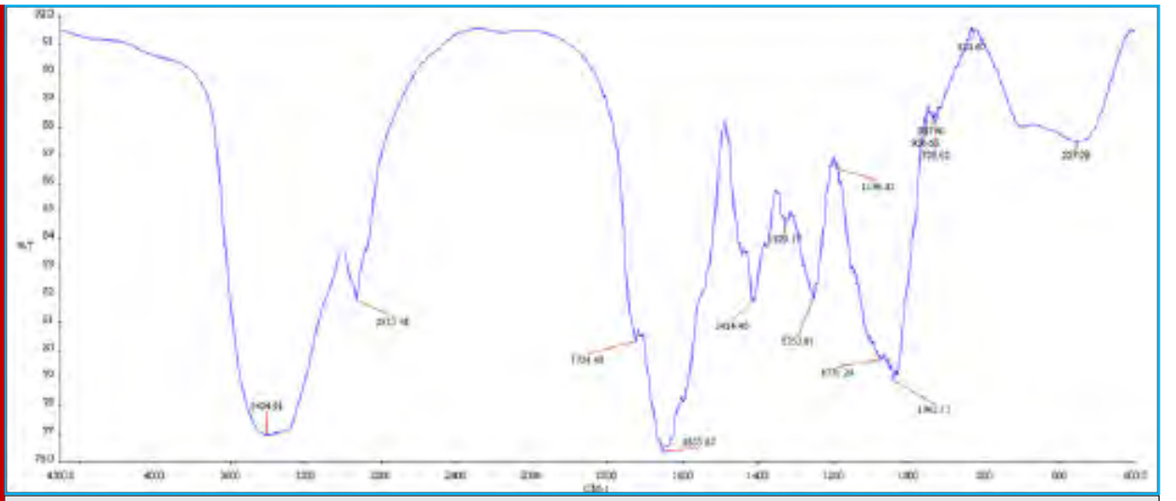

FIGURE 8. FTIR spectra of the purified EPS produced by the endophytic bacterial isolate *B. cereus* RCR 08

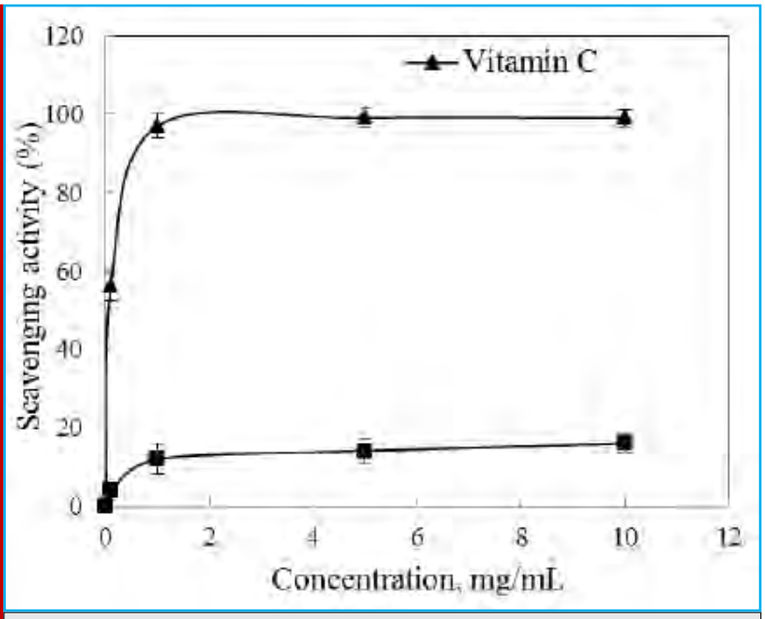

FIGURE 9. DPPH radical scavenging activity of the EPS produced by the endophytic bacterial isolate *B. cereus* RCR 08

The EPS displayed a dose-dependent cytotoxic activity against Huh 7.5 cell line in culture. The antiproliferative activity of the EPS gradually increased with increasing concentration. The EPS exhibited 60.8% viability of the Huh 7.5 cells at a concentration of 2000 ng/mL (Figure 10). Li and Shah (2016) also reported strong antiproliferative activity of EPS isolated from *Streptococcus thermophilus* ASCC 1275 on Caco-2 and HepG2 cells.

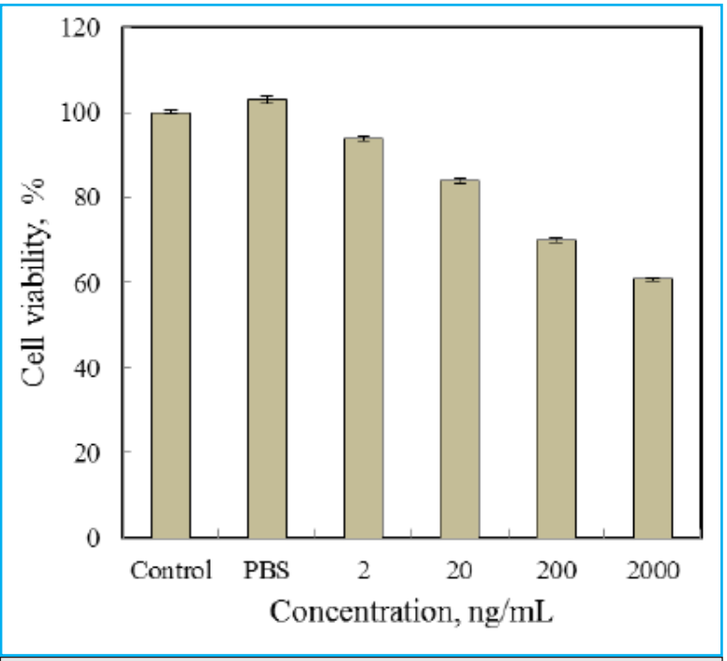

FIGURE 10. Effect of EPS produced by the endophytic bacterial isolate *B. cereus* RCR 08 on the viability of Huh 7.5 cell line

Table 3. Emulsifying activity of EPS produced by *B. cereus* RCR 08 and commercial emulsifier Tween 80

Hydrocarbons	Emulsification, %	
	EPS of B. cereus RCR 08	Tween 80*
Benzene	76.37 ± 2.50	68.00 ± 1.20
Palm oil	47.61 ± 1.22	48.00 ± 1.22
Olive oil	60.00 ± 1.25	68.18 ± 0.09
Soybean oil	63.15 ± 1.23	61.53 ± 0.12
Kerosene	62.50 ± 1.26	73.07 ± 0.08
Petrol	-	62.50 ± 0.01
Octane	60.00 ± 1.23	68.00 ± 0.00
Hexane	66.66 ± 1.27	64.00 ± 0.15
Tetradecane	70.00 ± 1.26	61.23 ± 0.12
Hexadecane	-	29.62 ± 1.25

**Expressed as the percentage of the total height occupied by the oil water emulsion after 24 h; each value represents the average of three measurements ± S.D.*

CONCLUSION

Endophytes have been recognized as important sources of structurally and functionally novel extracellular polysaccharides which could find applications in medical, pharmaceuticals, chemical and other industries. The present study demonstrates that *Bacillus cereus* RCR 08, endophytic to *Ricinus communis* L., is capable of producing a substantial amount of extracellular polymeric substance employing a suitable carbon and nitrogen source in a definite ratio. Results so obtained appear to be beneficial for further assessment of enhancing the production of *B. cereus* RCR 08 EPS in large scale. The significant oil emulsifying activity along with antioxidant and antiproliferative activity against Huh 7.5 cell line deserve special attention. Thorough chemical analysis of these carbohydrate polymers is required to exploit them in pharmacy in adjunction to cancer trials.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

All authors have declared no conflicts of interest in this communication.

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Resveratrol nano-capsule as an efficient tool for blood pressure regulation: A study on metabolic syndrome induced mice

Alemeh Shahraki,[1]* Saeed Bahadorikhalili,[2] Mahmoud Hashemzaei,[3] Mohammadreza Hajinezhad,[4] Ali Afsharimoghaddam,[5] Farugh Sarani[6] and Omid Tajrobekar[7]

[1]*Medicinal Plants Research Center, University of Medical Sciences of Zabol, Zabol, Sistan and Baloochestan, Iran*
[2]*Department of chemistry, College of science, University of Tehran, Tehran, Iran*
[3]*Department of Pharmacology and Toxicology, University of Medical Sciences of Zabol, Zabol, Sistan and Baloochestan, Iran*
[4,5,6]*Department of Basic Sciences, Faculty of Veterinary Medicine, University of Zabol, Zabol, Sistan and Balochestan, Iran*
[7]*Department of Microbiology and Parasitology, University of Medical Sciences of Zabol, Zabol, Sistan and Baloochestan, Iran*

ABSTRACT

Insulin resistance and overweight have been associated with major risk factors such as blood pressure (BP) for cardiovascular disease. In this study the effect of Nano-capsules of resveratrol (RV-NC) on BP control is evaluated. RV-NC nanoparticles were analyzed by SEM, Zeta sizer, Potentiometer and HPLC. The analysis resulted from RV-NC synthesis showed that the Nano capsules have characteristics such as size of 207 nm, zeta potential of -7.15 and loading efficiency of 99.54% ± 1.02. BP reduction was associated with reduction of weight and enhance of QUICKI index which represents insulin resistance. RV-NC were prepared by interfacial deposition and then its applicability was evaluated in metabolic syndrome induces mice. The effect of RV-NC was studied on fourteen mice. Induction of syndrome by high fat diet and high BP was observed. The collected data were analyzed by ANOVA and Turkey criteria were used to compare the distinction between the groups. Finally, the results indicated that RV-NC-treated mice have regulated in systolic and diastolic blood pressure (compare to the other group ($p < 0.05$). The effective formulation of nano-capsules for resveratrol delivery not only can be helpful in increasing the in vivo stability, but also in regulation of the patient's blood pressure with at least cost of therapy.

KEY WORDS: RESVERATROL, NANO-CAPSULATION, CARDIOVASCULAR DISEASE, DRUG DELIVERY

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INTRODUCTION

Hypertension (BP: 140/90 mmHg), as one of the main symptoms of metabolic syndrome, can be caused by fatty and high-calorie diet associated with obesity and insulin resistance. This problem has been introduced as a serious warning sign in patients with heart disease. (Danaei et al, 2013; Jahandideh et al, 2016; Nonogaki et al, 2016). Global Research has been found that, approximately % 45 and 51% of deaths are resulted by coronary stroke and artery disease, respectively (Brook, 2013; Movahed et al, 2016). The clinical studies have shown that prescription of anti-hypertensive drugs for the hypertensive patients might cause a number of side effects. Therefore, medical researchers are interested in using natural sources instead of chemicals for the production antihypertensive drugs (Aluko et al, 2015; BC Guidelines, 2016).

Recently, plant polyphenols such as resveratrol have been successfully applied in improving the symptoms of insulin resistance and obesity in metabolic syndrome, and therefore it has opened a special place in global trade as a medicinal compound in the regulation of blood pressure in patients with heart problems, diabetes and other diseases (Raj et al, 2013; Liu et al, 2015; Movahed et al, 2016). However, the natural polyphenols suffer from a number of disadvantages such as low biological half-life, high volatility and rapid removal, which limits the in vivo applicability of these compounds (Cottart et al, 2015; Khaled et al, 2016)

Therefore, new studies have been conducted on the basis of nanotechnology to achieve effective formulation of pharmaceutical medicines (Smoliga, 2014; Penalva et al, 2015; Reis et al, 2016; Jadhave et al 2016; Shindikar et al, 2016). One methods are Nano capsule formation by coating the unstable medicinal compounds by biodegradable (Venturini et al ,2011; Frozza et al, 2013; Friedrich et al ,2015; Conte et al, 2016). Regarding the advantages of Nano capsules, the main goal of this study is to use an effective formulation of resveratrol in a stable Nano capsule to improve the fluctuations problems in blood pressure. The effect of the capsulated resveratrol is studied in mice with metabolic syndrome by fat diet.

MATERIAL AND METHODS

Trans-resveratrol, PCL, Span 60 and Tween 80 obtained from Stigma Aldrich. Other chemicals and solvents were from analytical and pharmaceutical types. The Low Fat Diet (LFD) was prepared from Khorasan Seedling Company and to prepare High Fat Diet (HFD), fat-tail was used that had high levels of saturated fat. RV-NC was prepared by interfacial deposition method as described previously (Frozza et al, 2010). Briefly, to prepare the aqueous phase, polysorbate (0.0380 g) was dissolved in 53 ml of distilled water. The organic phase was prepared by vigorous stirring of RV, PCL, capric triglycerides, and sorbitanmonostearate in 27 ml of acetone at 40 ^{0}C. At the end, the organic phase was added to the aquatic phase and acetone was evaporated after 10 min and the suspension was concentrated under reduced pressure and filtered by 8 micrometer filter paper. Then, the non-loaded B-CN Nano capsules suspension was synthesized with the above method as the control formulation.

To determine the size, zeta potential and polydispersity of the Nano capsules, zeta sizer and particle sizes (20101 SA, made in Japan) with laser light scattering method at 25^{0}C were used. Before the experiment, the sample was diluted with MilliQ water or 0.01 µM NaCl and filtered by MILLIPORE 0.45 µM. The measurement was repeated for each formulation in triple mode. To determine PH, AL-1703 and MUNCHEN an immersed electrode in suspension were used at room temperature. The concentration of the loaded active substance (RV) in suspension Nano capsules was determined by HPLC using CLC-C8 column and equipped with a UV detector and using water and acetonitrile as mobile phase with the flow rate of 1.2 ml/min and inhibition time of 3.45 min. The capsulation efficiency was calculated as below:

$$\text{Encapsulation efficiency} == \left(\frac{\text{resveratrol load} - \textit{resveratrol in supernatant}}{\textit{resveratrol load}} * 100\right)$$

Here, resveratrol load and resveratrol in supernatant are active and free substance concentrations respectively. The free substance concentration was obtained by acetonitrile and the active substance extraction from suspension formulation was obtained by integrating ultrafiltration and centrifuge. 40 male mice (C57BL/6, 20-24 g, 4 weeks) were selected. The animals were kept in vitro under standard conduction such as free access to food and water in a room with controlled temperature (20-24 ^{0}C) and on a 12 h-light/dark cycle. The experimental protocol of this study was approved by Animal Ethical Committee of Zabol University of Medical Sciences. Before the experiment, all mice were acclimatized for an adaptation period a week and then, all groups except the control group (n=8, LFD), were kept under high fat diets (n=32, HFD) for 12 weeks. Measuring the parameters such as weight (each week), insulin levels and glucose were done by FG4000, Cayman kit by ELISA method, and ARKRAY, respectively.

The non-invasive blood pressure (BP) system (URIT, Poland) with a tail-cuff sphygmomanometer was used to measure systolic and diastolic (approximate measurement) blood pressure. A clear plastic tube used to placed mice and tail hole pieces secured at either end. A nervous, stressed animal may have diminished circulation in

the tail so the animals were placed in the holders at least 10 to 15 minutes prior to obtaining pressure measurements. At this step, mice which have consumed high fat diet randomly place in 4 groups which included; the groups treated with resveratrol Nano capsule (RSV-NC; 5 mg/kg/day), blank Nano capsule (B-NC; 5mg/kg/day), free resveratrol (RSV; 100 mg/kg/day), and metformin (MET; 250 mg / kg / day). The measured variables were done similarly at 3 steps: before and after induce syndrome and after treatment. The results are reported from AVONA as mean and standard deviation for at least 3 different experiments (Mean ± SD) and accordingly, significant difference between the groups can be observed ($P<0.05$). For between-group comparison, Tukey criteria are used as suitable criteria in making distinction between groups.

RESULTS AND DISCUSSION

RSV-NC and B-NC were synthesized using biodegradable materials such as poly- caprolactone (PCL) with no need to additional steps with interfacial deposition method. The physiochemical characteristics of Nano capsules are mentioned in Table 1. The zeta potentials of RSV-NC and B-NC were obtained as -7.15 and 6.21, respectively. The negative values indicate the existence of polysorbate 80 in formulation that leads to their increased spatial resistance in water/particle surface. Also, the Nano capsules suspensions were analyzed by DLS and monomodal stability in size distribution and polydispersity index were observed to be lower than 0.3, which shows the narrow size. Particle size for RSV-NC and B-NC were about 200 nm and according to resulting of Friedrich et al, 2015 is an acceptable size. In addition, the pH for the formulations of both Nano capsules was larger than 6. HPLC method showed the capsulation efficiency of RSV-NC to be %99.54±1.02. These results are consistent with the new findings in this formulation.

Table 1. The physiochemical characteristics of Nano capsules containing RV-NC and B-NC

Formulation		
	B-NC	R-NC
Size (nm)	205± 0.05	207±0.03
PDI	0.12±0.09	0.12±0.04
Zeta potential (mv)	-6.21± 0.45	-7.15± 0.19
PH	6.47± 0.02	6.22±0.04
Encapsulation efficiency (%)	-	99.54±1.02

Fat diet: Ruminant fat was used to induce metabolic syndrome in mice. The HFD and LFD components are presented in Table 2. The diet in this study has 30% carbohydrate, 45% fat and 25% protein that is almost similar to the diets in various societies.

The weight means of HFD and LFD groups before and after induced metabolic syndrome were compared. According to the statistical results obtained from ANOVA, the significance level between the two groups is lower than 0.05. This states that there is a significant difference between the weights of HFD (n=32) and LFD (n=8), due to the higher level of saturated fat, that the HFD group have received (fig.2). Analyzing the results by Tukey test show that, HFD subgroup (RV-NC, B-NC, RV and MET, n = 8), had higher weights which was because of receiving high levels of saturated fat for 12 than control group that used standard diet. According to the findings in fig 2, the weight means of RV-NC, RV and MET groups show a significant reduction during 4 weeks of treatment and among these, group RV-NC showed a weight loss in a shorter time.

The QUICKI index was the main index for the insulin resistance measurement, which is directly obtained from glucose and insulin values. In this study, the results of fig3, show that there is a significant difference between HFD group compared to the control group ($P<0.05$). HFD groups have the highest value in glucose and insulin and therefore have the lowest QUICKI index. Results of testing between groups, Tukey test, show that glucose and insulin levels in 5 groups of RV-NC, B-NC, RV, MET and control are different. In this study, B-NC group has the highest levels of insulin and glucose and the lowest values QUICKI index, in contrast to the control group.

Table 2. Composition of High Fat Diet (HFD) and Low Fat Diet (LFD)

	LFD		HFD	
	Present of total mass (g%)	Present of total kcal (kcal%)	Present of total mass (g%)	Present of total kcal (kcal%)
protein	28.1	30	25.84	25
carbohydrate	58.04	60	34.69	30
Fat	13.15	10	42.5	45
total (kcal/g)	3.02		4.26	

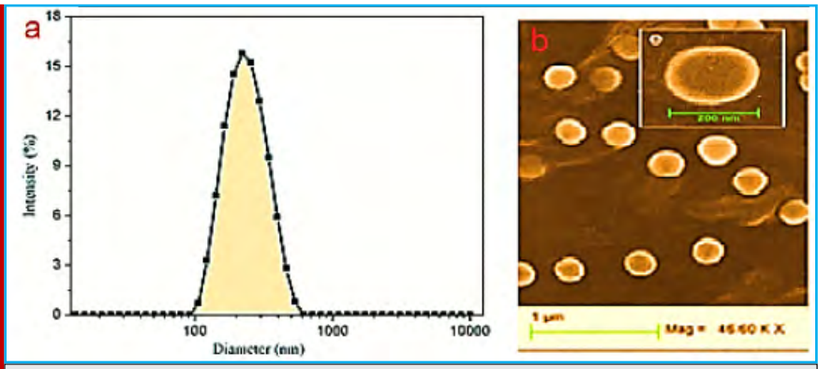

FIGURE 1. Size distribution graph for resveratrol – loaded Nano capsules (RV-NC) obtained by (a) DLS and (B) SEM photomicrographs.

In the RV-NC group, QUICKI index reach normal range, while the glucose and insulin values were normal. RV and MET groups have also the same result, relatively. Generally, it can be understood that RV-NC group operate better in increasing QUICKI index in shorter period of time.

Since the blood pressure is one of the main symptoms of the metabolic syndrome, this parameter was investigated in mice fed fat diet for 12 weeks and 4-week treatment compare to control group. The results of changes in values systolic and diastolic blood pressure which Statistical analysis by ANOVA, in Figure 4, section A and B respectively, show that there was significant difference between HFD group and LFD groups in values of systolic and diastolic blood pressure ($P<0.05$). After separation the animals HFD into 5 subgroups (RV-NC, B-NC, RV, MET) and conducted treatment phase, Tukey method for compare between groups were used. The results show a significant reduction of systolic and diastolic blood pressure in RV-NC, RV and MET groups. B-NC group which has used Nano capsules without pharmaceutical active ingredient, have the highest amount in blood pressure, while not observed in the control group significant changes over time. It appears that changes in Systolic blood pressures are more obvious than diastolic. It is clear that the group RV-NC in the regulation of blood pressure in Comparisons between groups of RV-NC, RV, MET, is better.

DISCUSSION

This study assessed the potential effects of resveratrol –loaded Nano capsules suspension on insulin to resistance (IR) and systolic and diastolic blood pressure in metabolic syndrome induced in mice. The results show that there is a major association between the resistance to the effects of insulin on both glucose uptake and insulin-induced vasodilatation in obese hypertensive patients, which are in accordance with the previous findings (Ferrannini et al, 1987; Laakso et al, 1989;

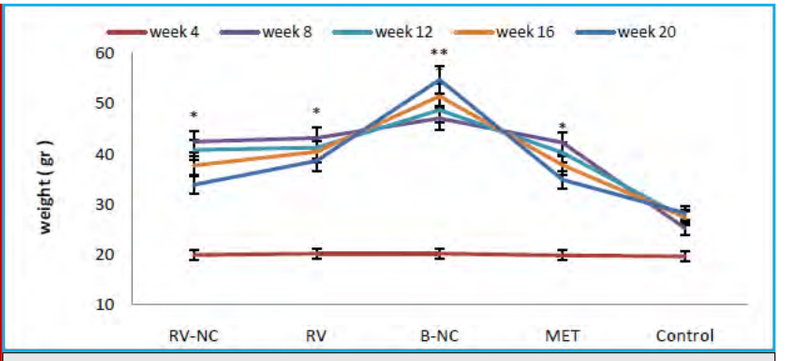

FIGURE 2. Weight changes in 5 different groups in 20 weeks. All data are expressed as mean ± standard deviation (n=8) and different letters show a significant difference at $p < 0.05$.

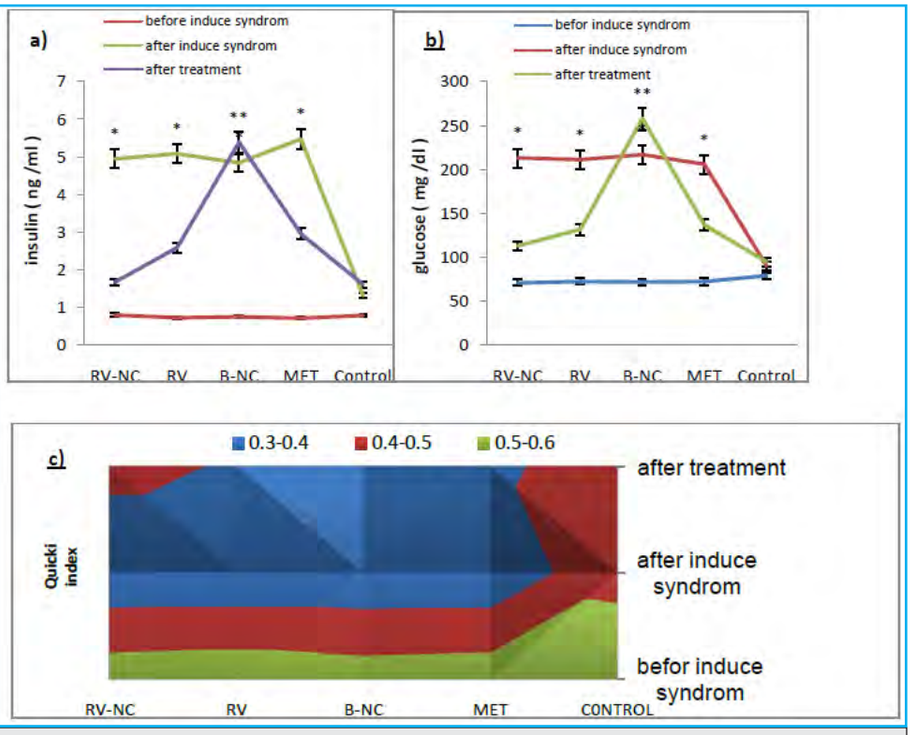

FIGURE 3. Changes in insulin (a), glucose (b) and QUICKI index (c) values in 5 different groups in three stages. All data are expressed as mean ± standard deviation (n=8) and Different letters show a significant difference at $p < 0.05$.

Natali et al, 1997; Lastra et al, 2010; Horita et al, 2011, Zhou et al, 2012).

The homeostasis model assessment-estimated insulin resistance (HOMA-IR) has been widely used for the estimation of IR in research (Matthews et al, 1985). It is calculated multiplying fasting plasma insulin (FPI) by fasting plasma glucose (FPG), then dividing by the constant 22.5, i.e. HOMA-IR=(FPI×FPG)/22.5 (Wallace et al, 2004). index that we used to determine IR is the quantitative IR check index (Quicki index) which that is a novel mathematical transformation of fasting blood glucose and insulin levels and useful index of IR in subjects with hypertension, obesity, type 2 diabetes, gestational diabetes, pregnancy, PCOS, premature adrenarche, hyperandrogenism, and nonalcoholic steatohepatitis (Katz et al, 2000; Hui et al, 2003).

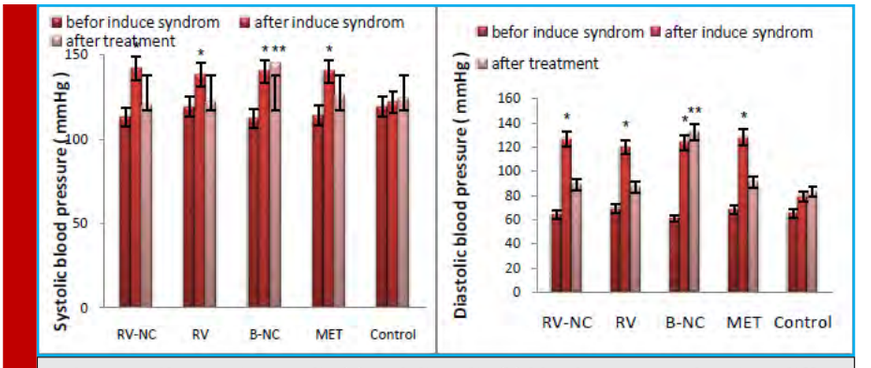

FIGURE 4. Changes in systolic (a) and diastolic (b) blood pressure values in 5 different groups in three stages. All data are expressed as mean ± standard deviation (n=8) and Different letters show a significant difference at $p < 0.05$.

Lifestyle factors such as excess body fat, excess dietary fat (total, Trans, and saturated fat), fake carbohydrates, smoking, stress and insufficient exercise are causes IR. This problem is a central part of a cluster of metabolic abnormalities called the metabolic syndrome. Candidate mechanisms whereby this metabolic syndrome might lead to hypertension include stimulation of sympatho-adrenergic activity, altered cellular electrolyte transport and composition, growth promoting effects, renal sodium retention and vascular hyper responsiveness (Lithell et al, 1998; Velliquette et al, 2003). In person with IR, the cells do not respond to insulin normally and glucose cannot easily enter the cells. As a result, the insulin level in blood will be high. Finally, the body will not be capable of building enough insulin to control blood glucose at normal level and diabetes, cardiovascular disorder and others occurs (Borkman et al, 1993; Vessby et al, 2001; Risérus et al, 2009; Sandeep et al, 2010). The diet used in this study consisted of proteins (25%), carbohydrates (30%) and saturated fat (45%) that is almost similar to the diets in most of the societies. The available diets for animal model almost contain 60% fat so that insulin resistance can be observed, but is not similar to the diets that are used by Individuals and cannot be generalized simply (Nishina et al, 1990; Surwit et al, 1995).

Several studies have been conducted on the edible containing the active ingredient in the prevention and treatment of insulin resistance and blood pressure regulation .The results show that edibles containing polyphenolic compounds (such as Red Grapes, Dark Chocolate and Blueberries) could be effective in this (Dauchet et al. 2005; Hu and Willett, 2002). Since Polyphenols such as resveratrol which that improve the risk factors of cardiovascular disorders (Poulsen et al, 2013), diabetes (Hausenblas et al, 2014*)* and pathologic conditions (Fernández et al,2011) is unstable in vivo and so use it expensive for patients (Joseph et al, 2006), Studies developed towards to produce new formulations to improve protecting and reaching acceptable level of bioavailability (Finley et al, 2010 and Francioso et al, 2014)

Nowadays, scientists could be using new technologies, especially nanoscience for drug delivery of active ingredients unstable to form of nanocapsules (Contri et al, 2016; Scognamiglio et al 2016 and Vivienne et al, 2016). Some of the advantages this method, Include the development of controlled-release system, maintaining the drug concentration in blood plasma for a long time, the possibility of developing drugs with very low dose and stability and efficacy impressive. In this study, we prepared protected form of resveratrol in the coating of biodegradable polymer PCL (poly-caprolacton) with a size of approximately 200 nm. However, the results of experiments in this plan highlight the successful performance of RV-NC compared to other groups to reduction of IR and regulation of blood pressure.

CONCLUSION

This is the first study on the effect of resveratrol loaded Nano capsule (RV-NC) on insulin resistance (IR) and blood pressure profiles in animal model metabolic syndrome. The results demonstrated that using high saturated fat in daily diet can cause IR and hypertension. On the other hand, RV-NC regulates blood pressure and reduces IR by reducing the amount of the fat in whole body. These findings suggest that further studies should be conducted on the effect of RV-NC on animal and human induced hypertension models, obesity, type 2 diabetes, gestational diabetes, pregnancy, PCOS, premature adrenarche, hyperandrogenism, and nonalcoholic steatohepatitis.

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Manipulating disease and pest resistance pathways in plants for enhanced crop improvement

Paramita Ghosh, Anjanabha Bhattacharya* and Bharat Char

Maharashtra Hybrid Seeds Company Private Limited, Jalna-Aurangabad Road, Dawalwadi, Jalna, Maharashtra, India

ABSTRACT

Plants are sessile organisms, therefore cannot escape challenges of their surrounding environment. The rich source of nutrients plant possesses attracts various organisms. Biotic stress results from array of organisms such as bacteria, fungi to various insects, pests and herbivores. Plants have evolved sophisticated mechanisms to protect themselves against invaders. In this review, we explore the plant surveillance system, different nodes in the defence pathways involved in plant protection and how it can be manipulated to get a resistant crop. Emerging technologies have provided us with a vast number of potential candidate genes from plants, pathogens and other organisms. We here, illustrate examples of technically useful solutions to make crops tolerant to pathogens and pests.

KEY WORDS: PLANT DEFENCE, BIOTIC STRESS, R GENE, DEFENCE SIGNALING TRANSDUCTION, NPR1, MAPK, GENETIC ENGINEERING

ABBREVIATIONS

MAMPs - Microbe Associated Molecular Patterns,SAR - Systemic Acquired Resistance, VOCS – Volatile Organic Compound, R gene – Resistance Gene, HR – Hypersensitive Reaction, ROS – Reactive Oxygen Species, MAPK – Mitogen Activated Protein Kinase, *avr* – Avirulence, ETI – Effector Triggered Immunity, NBS – Nuclear Binding Site, LRR – Leucine Rich Repeat , pv. – pathover, NO – Nitric Oxide, SA – Salicylic Acid, JA – Jasmonic Acid, ET- Ethylene, NPR1 – Non Expressor of PR Genes 1,PR – Pathogenesis Related, LTP –Lipid Transfer Protein, PPO – Polyphenol Oxidase, POD – Peroxidase, UV- Ultraviolet, HIPV – Herbivore Induced Plant Volatile, QTL – Qualitative Trait Loci, SIPK - Salicylic Acid Induced Protein Kinase, OS – Oral Secretion, FACs – Fatty Acid –Amino Acid Conjugates, WIPK – Wound Induced Protein Kinase, MEKK- Mitogen-Activated Protein Kinase Kinase, ISR – Induced Systemic Resistance, PRSV - Papaya Ring Spot Virus, ZFN – Zinc Finger Nucleases, TALENs – TAL Effector Nucleases, GM – Genetically Modified, RPP2 – Recognition of *peronospora parasitica* 2

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*Corresponding Author: anjanabha.bhattacharya@mahyco.com
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INTRODUCTION

Plants are nutrient rich organisms and therefore many invaders prey on their food reserve. Some of the invaders are significant threats to crop production, worldwide. In the process of co-evolution (Seidl and Thomma, 2017); plants, pathogens and insects have evolved various strategies to avoid each others' defence system. The goal of producing crops with durable and increased resistance to a broad spectrum of diseases and insects is therefore, a major focus in plant research.

In nature, plants are continuously challenged by different organisms, whereas, only few are successful in gaining entry into a prospective host. Plants have developed an elegant defence system with a wide variety of constitutive and inducible defences to protect themselves from damages of different biotic factors. Constitutive defences include many preformed barriers such as waxy epidermal cuticles, cell walls and bark (specialized morphological structures). Inducible defences include production of repellents, toxic chemicals, pathogen-degrading enzymes, anti-nutritional effects and deliberate cell suicide (Freeman and Beattie, 2008). Plants often do not produce toxic compounds or defence-related proteins until pathogens are detected due to the metabolic cost associated with the production and maintenance of such compounds. Plants have evolved to live in environments where they are very often exposed to different stress factors in combination. Plants have developed various mechanisms that allow them to detect precise environmental changes and respond to complex stress conditions, minimizing damage (Saskia and Jorunn, 2011).

NATURE OF ATTACKERS

Plant pathogens can be broadly divided into biotrops and necrotrophs. Bacteria and fungi can adapt to both lifestyles (Freeman and Beattie, 2008).Viruses are quintessential biotrophs, although they eventually kill the host cell. Insects, on other hand, cause damage by chewing and sucking. Plants respond to the insects by producing protease inhibitors and anti-feedants such as alkaloids [(Hanley et al., 2007; Jeffery and Jonathan, 2001). Nematodes can adapt to complex modes of parasitism by exhibiting variety of parasitic modes affecting the development responses of plants, causing galls, root knots or cysts (Jeffery and Jonathan, 2001; Davis et al., 2004; Roland and Maurice,2011) . Thus, plant immune system is highly polymorphic in their capacity to recognize and respond to different stress factors (Jeffery and Jonathan, 2001).

PLANT SURVEILLANCE SYSTEMS

Although plants lack immune system comparable to animals, they have developed sophisticated surveillance mechanisms, which can respond rapidly before harmed. These surveillance systems are linked to specific pre-programmed defence responses. Direct defences are mechanical protection on the surface of the plants which protects from all biotic factors (*e.g.*, hairs, trichomes, spines, thorns and thicker leaves) or toxic chemical production.

Basal resistance is the first line of pre-formed and inducible defences. It is also known as innate immunity (Freeman and Beattie, 2008; Owen and Zamir, 2010), and protect plants against entire groups of pathogens (Freeman and Beattie, 2008). Basal resistance is triggered when plants recognize microbe-associated molecular patterns (MAMPs). MAMPs include specific proteins, lipopolysaccharides, and components of cell wall commonly found in microbes. During evolution pathogens also have developed counter measures that are able to suppress basal resistance in certain plant species. If the basal defence is somehow suppressed, plants respond with hypersensitive response (HR) (Freeman and Beattie, 2008). In HR plants limit the pathogen's access to water and nutrients thereby sacrificing few cells in the infection site *i.e.* deliberate cell suicide (programmed cell death). HR is more pathogen specific than basal resistance. It is triggered in presence of disease-causing effector molecules. Once the hypersensitive response is triggered, plant tissues become highly resistant to a broad range of pathogens. This phenomenon is known as systemic acquired resistance (SAR) (Freeman and Beattie, 2008; Nelson et al., 2017), which represents readiness of plant metabolites to defend plants, in case of a heightened attack.

Mechanical damage caused by insects is not generally considered "true" plant disease although plants have developed surveillance systems designed to not only recognize insect pests, but also to respond with specific defence mechanisms. General wounding can be different from insect feeding in a way that elicitors are present in insect saliva. In response to insect chewing, plants release volatile organic compounds (VOCs), secondary metabolites and proteins that have toxic, repellent, and/or anti-nutritional effects on the herbivores (Freeman and Beattie, 2008; Saskia and Jorunn, 2011; Abdul Rashid War et al., 2012). Sometimes volatiles released by plants also attract beneficial predators (natural enemies) that prey on the destructive pests (Abdul Rashid War et al., 2012; Walling, 2000; Rashid and Chung, 2017). Plants become phenotypically plastic when induced defence is triggered as a result it decreases the chances of the attacking insects to adapt to the induced chemicals (Abdul Rashid War et al., 2012).

In addition, plants can defend themselves against viruses by a variety of mechanisms which include RNA silencing (Novina and Sharp, 2004, Csorba and Burgyan,

2016). Plants can recognize the foreign double stranded RNA or DNA, produced by viruses in the host cell during replication, and respond by digesting the genetic strands into non recognizable fragments and thereby stopping the infection. The interaction of plants with symbionts, pathogen, herbivores, and the natural enemies, both above and below the ground is the focus of a large amount of research effort and has great potential for utilization in crop protection.

With cultivation of huge areas of genetically identical crops, protection relies on a small number of inbred disease resistance genes per crop species and on the wide-spread application of pesticides. Unfortunately, an absolute control is very difficult to achieve through pesticides (Cesari, 2017), as pathogens can overcome disease resistance genes and/or become resistant to pesticides (Nelson et al., 2017; Zhonghua et al., 2005). Genetic manipulation can help solve the problem by inserting multiple genes as transgenes by careful selection from wild parent of the same plant species or from different plant species (Campbell et al., 2002). Therefore a search is on for genes that can confer a durable broad-spectrum resistance against biotic factors. To make it more environment friendly the gene product should be safe for all organisms and also reduce the need of harmful pesticides. However, the success so far achieved is very less. In majority of cases the tansgene results in unpredictable expression in different parts of the plants, this phenomenon is not due to the transgene itself, per se (Hammond-Kosack and Parker, 2003; Stuiverand Custers, 2001) . Therefore, optimization of transgene expression patterns needs close attention. Inducible expression of such gene is essential (Hammond-Kosack and Parker, 2003; Michelmore, 2003). A highly inducible promoter specific for defence gene expression can help the plant in directed recourse allocation by metabolic and transcriptional adaption during stress. Plant can optimize source sink relationship thus increasing yield or biological harvest index (Hammond-Kosack and Parker, 2003).

ENGINEERING PLANTS WITH INCREASED RESISTANCE AGAINST PATHOGEN AND INSECTS: TARGET GENES

(First generation strategies)

R gene

R genes (resistance genes) are important components of plant surveillance system. A diverse array of defence mechanism is triggered when R genes recognize pathogen or insects (Cesari, 2017). PR-gene induction, accumulation of inhibitory metabolites and oxidative burst response by production of reactive oxygen species, are some of the downstream responses triggered by R genes which lead to hypersensitive response (Owen and Zamir, 2010).

Pathogens possessing *avr* genes can overcome basal immunity of plants by blocking perception of PAMP or by inhibiting MAP kinase signaling cascade, which is known as effector- triggered susceptibility. In case of effector triggered immunity, the pathogen's effector molecules are recognized by R proteins either by direct or indirect interactions. Thus, enhancing the plant resistance and it is faster than PTI. To trigger ETI, R proteins must recognize specific avirulence proteins (Avr) in order to generate resistance. However, mutation in either *avr* gene or R gene can change the scene *i.e* it will result in compatibility and therefore loss of resistance. R genes encode proteins which have nuclear binding sites (NBS) and leucine rich repeat (LRR) domains (NBS-LRR

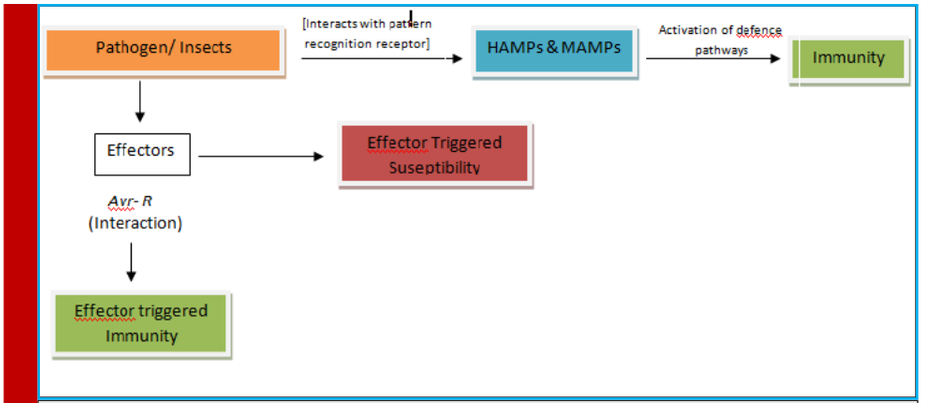

FIGURE 1. Shows pathogen triggered immunity when pathogen/herbivore associated molecular patterns are recognized by the cell receptors. However in presence of effectors, pathogens can surpass this immunity. In presence of R gene, effector triggered immunity induces defence response in plants.

proteins) (Cesari, 2017; Jeffery and Jonathan, 2001; Mari et al.,2013, Nelson et al., 2017).

Functional R genes conferring resistance against an array of different biotic factors such as bacteria, virus, fungus, nematodes and even insect pathogens have been isolated (Cesari, 2017, Zhao et al., 2005; Vossen et al., 2005; Reinink et al., 1989; Brotman et al., 2002). Even though the mode of action as well as the effector molecules of pathogens and insects are very different, R genes encode only a few classes of proteins. NBS –LRR class of proteins are the largest class of R gene which encodes 'nucleotide binding site with leucine rich repeat (Jeffery and Jonathan, 2001). It is reported that NB-LRR type R genes can confer resistance to multiple pathogens even though the pathogens belong to taxonomically distinct families (Mari et al., 2013). It is also termed as MDR or multiple disease resistance. In a maize recombinant inbred line (RIL) a QTL, *qMdr* have been identified for resistance to several diseases i.e, Norther blight, grey leaf spot and souther leaf blight. The molecular mechanism underlying the resistance is yet not known. In a research it is found that a gene, ZmCCoAOMT2, which encodes a caffeoyl-CoA O-methyltransferase is associated with conferring quantitative resistance to both southern leaf blight and gray leaf spot (Yang et al., 2017).

Bacterial effectors are delivered through type III secretion system, which can be up to 30 per strain, and by mimicking or inhibiting eukaryotic cellular functions colonization is achieved (Abramovitch et al. 2006). An example of a specific R-gene, *Rxo1* from maize conferred resistance to bacterial streak disease caused by *Xanthomonas oryzae* pv. Oryzicola(Zhao et al., 2005), when introduced in rice. In another instance, R gene *RCT1* from *Medicago truncatula* expressed in alfalfa conferred resistance to *Colletotrichum trifolii* (Yang et al., 2008), RPI-BLB2 from wild potato gave resistance to *Phytophtohora infestans* in day to day cultivated potato (Vossen et al., 2005). Some of the R gene work in pairs and are functional only when both genes are present (Mari et al.,2013). Some of the examples of such R gene pairs are *RPP2A/RPP2*(Sinapidou et al., 2004), *Pi5-1/Pi5-2* (Lee et al., 2009) *and Lr10/RGA2* (Loutre et al., 2009). Examples in wheat rust, *Sr31* from rye was effective against all *Pgt* races for many years until the appearance of Ug99 (Pretoriusetal.,2000).

Many single R genes responsible for resistance against insects are mapped in cereal crops, including wheat conferring resistance to Hessian fly (Hatchet et al., 1970). For decades, R genes have been used to control Hessian fly infestation in wheat. It is evident in support of gene-for- gene model in plant- insect interactions.

Some of the insect resistant R genes that are effective against aphids include: the lettuce *Nr* gene which gives resistance against aphid species *Nasanova ribis-nigri* (Reinink et al., 1989), the *Vat* gene from melon confers resistance against the melon/cotton *Aphis gossypii* aphid (Brotman et al., 2002), in another instance, the *Sd1* gene gives resistance against *Dysaphis devecta* aphid in apples (Walling, 2000; Roche et al. 1996), the *RAP1* gene gives resistance against the Pea Aphid in *Medicago truncatula* (Stewart et al., 2009), and the *Mi-1* gene in tomatoes (Rossi et al., 1998) found to be responsible for resistance against different organisms, the potato aphid *Macrosiphum euphorbiae*, the root-knot nematodes *Meloidogyne* spp., and the whitefly *Bemisia tabaci* (Nombela et al. 2003). The diverse resistance conferred by the *Mi-1* gene makes it is a very useful tool for integrated pest management. While, *Bph14 confers* resistance to the rice brown planthopper, *Nilaparvata lugens* (Zhang et al., 2009). However, a constitutive expression of a R gene can have a negative impact in absence of attackers. Constitutive expression of R gene can be detrimental to plants and therefore needs to be expressed with inducible promoters (Belbahri et al., 2001; Takakura et al., 2004).

For decades, R genes have been used in conventional breeding programme (Balconi et al., 2012) ; however, the resistance is only against a strain of pathogen or a particular species of insect. Traditional breeding strategies most often use only one R gene at a time. Pyramiding multiple R genes can promise a long lasting resistance as the pathogen has to accumulate mutation in multiple *Avr* genes to escape resistance. Effective combinations of R and APR gene by pyramiding or stacking can be considered for effective rust resistance (Jeffrey et al., 2014). However, it is a lengthy process to introduce a R gene into an elite cultivar by conventional breeding. R-genes from unrelated plant species can be introduced through genetic engineering, which often remain functional in the new host plant (Collinge et al., 2008) . The limitation of this technology being that resistance is conferred only against a single pathogen similar to breeding (Balconi et al., 2012). Additionally, R-gene only confers resistance against pathogens that essentially act as a sink for the host plant's metabolism *i.e.* biotrophs.

Shuffling of multiple R genes can also be considered rather than only pyramiding. Plant pathogen *Cladosporium fulvum* elicitors are recognition by *Cf* genes in tomato which belongs to the Hcr9 gene clusters (Brande et al., 2004). Studies have shown that Hcr9s are composed of sequences that have been generated by sequence exchange between individual homologues, Intra and intergenic recombination, gene conversion, point mutation, duplication and translocation. Therefore, shuffling multiple R genes might increase recognition specificities and engineering R gene for novel disease resistance specificities in plants can be achieved (Cesar, 2017). For example, gene shuffling done in tomato *Cf4* and *Cf9* R

Table I. List of R gene against different pathogens

R gene	Source (Donor)	Examples of transgenic crop	Against Pathogen	References
Rox1	Maize	Rice	Xanthomonas oryzae pv. oryzicola	Zhao *et al.*, 2005
RCT1	Medicago truncatula	Alfalfa	Colletotrichum trifolii	Yang *et al.*, 2008
RPI-BLB2	Potato (Solanum bulbocastanum)	Potato	Phytophtohora infestans	Vossen *et al.*, 2005
Bs2	Pepper	Tomato	Xanthomonas campestris	Tai *et al.*, 1999
Rpg1	Barley	Barley	Stem rust	Brueggeman *et al.*, 2002
Ve1 and Ve2	Tomato	Potato	Verticillium spp.	Kawchuk *et al.*, 2001
RRS1-R	Arabidopsis	Arabidopsis	Ralstonia solanacearum	Deslandes *et al.*, 2002
Pi-d2	Rice	Rice	Chinese rice blast	Chen *et al.*, 2006
RPW8	Arabidopsis	Arabidopsis, tobacco	Broad spectum resistance against powdery mildew	Xiao *et al.*, 2003
Pto	Tomato	Tomato	Pseudomonas syringae	Frederick *et al.*, 1998

genes lead to the identification of sequences required for the Avr-dependent HR in tomato (Brande et al., 2001).

SIGNAL TRANSDUCTION NETWORK

Plants can sense changes in their environment through signaling pathways (Pankaj and Atle, 2013). When pathogen elicitors interact with host receptors, signal transduction cascades are likely to be activated including oxidative burst (ROS), calcium fluxes, ion channel fluxes, NO production (Bollwell et al., 1999) and various protein kinases. Subsequently, transcriptional and/or post transcriptional activation of transcription factors takes place which lead to the induction of defence gene.

Plant hormones which play important role in defence are SA, JA and ET. SA is primarily involved in the protective response against biotrophic and hemi-biotrophic pathogens and systemic acquired resistance (SAR) (Grant and lamb, 2006). Some mutants insensitive to SA shows enhanced susceptibility to biotrophic pathogens. Methyl salicylate is a mobile inducer of SAR and is induced when the plant is infected with a pathogen in tobacco plants (Park et al., 2007). After pathogen challenge the elevated level of SA increases the expression of PR genes, therefore increasing resistance. Whereas the level of JA and ET are elevated against necrotrophic pathogen and herbivorous insects (Park et al., 2007).

Most often the SA and JA/ET defence pathways are antagonistic, however reports of synergistic interaction also exist (Kunkel and Brooks, 2002; Mur et al., 2006; Schenk et al., 2000) . Specific biotic factors regulate the positive or negative cross talk between SA and JA/ET pathways (Adie et al., 2007). In nature it is not one factor that affects the plant but several attackers, here plants have to employ complex regulatory mechanisms to cope with the complex situation. The mechanism by which plant is able to prioritize the responses is not known.

Non expressor of *PR* genes 1 (NPR1) is one of the important components of SA signaling. NPR1 plays an important role in SA-JA interaction (Dong, 2004). Downstream of NPR1 are several WRKY transcription factors which is also important is SA-dependent defence response. WRKY70 maintains the balance between the SA and JA pathways (Li et al., 2004; Li et al., 2006). Another key component which is involved in mediating the antagonism between SA and JA signaling in *Arabi-*

Table II. List of R gene against different insects

R gene	Source (Donor)	Against Insect	Reference
Nr gene	lettuce	Aphid species *Nasanova ribisnigri*	Reinink *et al.*, 1989
melon *Vat* gene	Melon	Melon/cotton *Aphis gossypii* aphid	Brotman *et al.*, 2002
Sd1 gene	Apple	*Dysaphis devecta* aphid	Roche *et al.*, 1996; Walling, 2000
RAP1 gene	Pea	Pea Aphid in *Medicago truncatula*	Stewart *et al.*, 2009
Mi-1.2 gene	Tomato	Potato aphid *Macrosiphum euphorbiae*, the root-knot nematodes *Meloidogyne* spp., and the whitefly *Bemisia tabaci*	Rossi *et al.*, 1998

dopsis is mitogen activated protein kinases (Petersen et al., 2000). In the second generation strategies, these signaling nodes will be discussed. The goal of effective and sustainable disease resistance can be achieved by the knowledge of signal transduction pathways (David et al., 2010), as the increased understanding has made it clear that successful pathogen process through pathogenicity factors (effectors). The disease resistance gene are mostly downstream genes and often do not act as specific receptors produced by pathogens and insects. A complex signaling network is also established when herbivorous insects attack a plant. To identify new molecules important for fine tuning of plant defence signaling, there is a need of dynamic modeling and simulation of signal transduction pathways (Beckers and Spoel, 2006; Erb et al., 2009).

Various plant protectant and defence gene are activated by the primary and secondary signals. The defence gene products include glutathione S-transferases, peroxidases, cell wall proteins, proteinase inhibitors, hydrolytic enzymes (e.g., β-1,3-glucanases and chitinases), pathogenesis-related PR proteins (Balconi et al., 2012).

PR proteins

Other potential candidates for manipulation are pathogenesis related (PR) genes, which shows promising activities against biotic factors *i.e.* pathogens as well as insect pests. Pathogenesis related (PR) genes could increase the level of pre-existing barriers (Owen and Zamir, 2010; Hammond-Kosack and Parker, 2003). Naturally occurring PR proteins are constitutively expressed at low levels and are induced to high levels challenged by pathogens or application of either salicylic acid or jasmonic acid (Ferreira et al., 2007) . PR proteins include several groups of unrelated proteins. Seventeen classes of PR protein have been examined, and numbered chronologically in order of discovery *i.e.* PR-1 to PR-17 (Balconi et al., 2012). PR-2 (b -1,3-glucanases), PR-3, -4, -8 and -11 (chitinases) target the pathogen cell wall (Owen and Zamir, 2010; Honee, 1999), PR-1 and PR-5 (thaumatin-like proteins and osmotins) are termed as permatins as they target the membrane, PR-10 has weak ribonuclease activity therefore may target pathogen RNA or play a role in defence against viruses, PR-6 proteins (proteinase inhibitors) may target nematodes, whereas the PR-7 protein (an endoproteinase) may be involved in microbial cell wall dissolution (Jorda et al., 2000) . The PR-9 family may enhance resistance to multiple pathogens by catalyzing lignifications which helps in cell wall reinforcement (Passardi et al., 2004). Since PR-10 family has weak ribonuclease activity it can be used against viruses (Park et al., 2004), PR-12 (defensins), PR-13 (thionins) and PR-14 (lipid transfer proteins) predicts antibacterial and antifungal activities (Epple et al., 1997) , some proteins generating hydrogen peroxide and are toxic to pathogen and pest, PR-15 (oxalate oxidases) and PR-16 (oxalate oxidase-like proteins) belongs to this family (Hu et al., 2003). PR-17 (uncharacterized) is detected in infected tobacco, wheat and barley (Christensen et al., 2002).

Most investigated PR proteins are chitinases and β1–3 glucanases (Owen and Zamir, 2010). Over-expression of chitinase have been moderately successful against fungal pathogens. Studies have found chitinase have role in insect resistance as well. The combined expression of chitinases and β1–3 glucanases have proven to enhance resistance by synergistic effect (Anand et al., 2003; Jach et al., 1995; Jongedijk et al., 1995; Zhu et al., 1994). Chitinases originating from *Trichoderma harzianum* (biocontrol agent), exhibit higher anti-fungal activity (Dana et al., 2006; Baranski et al., 2008; Kumar et al., 2009). Ectopic expression of thionins and defensins has conferred broad spectrum disease resistance, though the resistance is at low level (Punja, 2001). For example radish defensin RS-AFP2 (Kostov et al., 2009) when over-expressed in tomato resulted in up to 90% reduction in disease against agriculturally important pathogens. Lipid transfer proteins (LTP) are one of the important PR proteins which act as a potential mobile signal for systemic acquired resistance (SAR) in plants (Maldonado et al., 2002). LTP activates SAR over-expression of LTP might result in deleterious effect (Walters, 2007) , so far no such effect is observed. A highly inducible promoter can be used to over-express this gene in order to achieve the goal of disease resistance.

The plant defensive metabolites are termed as secondary metabolites play an important role in plant defense against herbivore and other interspecies defense, thus increasing the fitness of the plant. They can be either constitutively stored (phytoanticipins) as inactive forms or induced in response to the insect or microbe attack (phytoalexins)(King *et al*., 2014). Herbivore induced plant volatiles (HIPVs) play very important role in defense by either attracting the natural enemies of the herbivores or by acting as feeding and/or oviposition deterrent (Rashid and Chung, 2017). HIPV are released by healthy plants as well, however a different blend of volatiles is produced in response to herbivory and is very specific for a particular insect-plant system (Liu et al., 2012). For example, plants tend to release volatile compounds in response to aphid –attack to attract parasitoid wasps. In corn, plants release terpenoids in response to aphid attack. Many other volatile compounds like MeSa, C_6 volatiles etc influence plant-insect, pest and pathogen interaction.

Metabolite engineering can play an important role in developing plant with insect resistance. Increasing the flux of defence related secondary metabolites

by engineering the respective pathways can be of great importance in developing crops with insect resistance (Sanchez -Vallet et al., 2013). There are some reports of metabolic engineering of dhurrin, a cyanogenic glycoside in transgenic *A. thaliana* plants which, resulted in minor effects on the whole metabolome and transcriptome (Dudareva et al., 2013). Resistance to green peach aphid (*Myzus persicae*) feeding have been enhanced by metabolic engineering of raffinose in the phloem of *A. thaliana* (Jirschitzka *et al*., 2013). In another instance, manipulation of plant volatile emissions has enhanced the effectiveness of biological control agents. This can be used as a strategy to fight insect pests in an ecologically sound manner (Degenhardt *et al*., 2009).

SECOND GENERATION STRATEGIES

Master switch genes

Over-expression of a single defence-related gene is generally unable to provide high levels of resistance against a broad range of biotic factors like pathogen and herbivores. The knowledge of pathogen-induced signaling pathways in plants suggests that modifications of existing innate signaling pathways or expression of 'masterswitch' genes such as kinases and transcription factors (Owen and Zamir, 2010; Hammond-Kosack and Parker, 2003; Sarah and Paul 2005), which regulate a large number of defence genes could increase resistance against biotic factors (Owen and Zamir, 2010; Sarah and Paul 2005). The disadvantage encountered by this approach could be the harmful effect on plant development, due to potential yield loss which is common with over-expression of large number of genes at a time constitutively (Owen and Zamir, 2010). Therefore, the ideal candidates are the genes that activate partial pathways or augment pathways.

Transcription Factors

Transcriptome and QTL data analysis suggested transcription factors to be promising candidates for genetic engineering to increase disease resistance characteristics in plants (Sarah and Paul 2005) . They might behave as master switch gene by taking care of the expression of several genes in a single pathway. Therefore capable of making large changes in single trait causing very few disturbance on other traits (Doebley and Lukens, 1998). A good example is WRKY transcription factors (Owen and Zamir, 2010; Sarah and Paul 2005).

WRKY transcription factors are involved in SA- mediated defence pathways. Several WRKYs have the potential for increasing disease resistance, among them the most studied are *WRKY70* from *Arabidopsis* [50].Several other transcription factor families that have roles in plant defence could yield useful master switch genes like WRKY, ERF, TGA, MYB, Dof, GRAS, bHLH, GT1 and the Whirly factor Why1(Desvaux et al., 2004). The only limitation being, transcription factors mostly consist of large multigene families and identifying the best candidate can be difficult due to the functional redundancy (Eulgem et al., 2000). However, several of *Arabidopsis* WRKY has been identified have good functionality against pathogens (Sarah and Paul 2005).

MAP Kinase

Potential candidate master –switch genes which also play vital roles under different stress are protein kinases (Sarah and Paul 2005) . MAP kinase (MAPK) signaling is a necessary part of many defence-signalling pathways. When tobacco MAPK, SIPK is over-expressed it led to activation of defence responses and HR-like cell death showing the potential role of these genes (Zhang and Liu, 2001) . Enhanced resistance to virulent *P. syringae* and *Botrytis cinerea* was observed when MKK4a, MKK5a were over expressed transiently and MEKK1 was activated constitutively (Asai et al., 2002) . Other potential protein kinases are calcium dependent sensor proteins that changes Ca^{2+} defence response (Romeis et al., 2001) . In response to herbivore-induced cues such as insect oral secretions (OS) and oviposition fluid compounds, plants undergo a change in transcriptomes, proteomes, and metabolomes. The major components of the oral secretion of insects are fatty acid-amino acid conjugates (FACs) which activate the mitogen-activated protein kinase (MAPK) pathway. The MAPK pathway not only play an important role in signaling transduction in responses to a number of stresses including cold, heat, ROS, UV, drought, pathogen and insect attack but also regulate plant growth and development (Wu et al., 2007). On application of FACs in oral secretion of *M. sexta* leads to activation of several compounds/molecules of MAPKs, salicylic acid induced protein kinase (SIPK) and wound-induced protein kinase (WIPK), JA, SA and ethylene. In another case brown plant hopper *N. lugens induces* expression of putative *OmMKKI* (MAPK). Several FAC elicitors have been isolated from various lepidopteran species (Wu et al., 2007; von Dahl et al., 2007) .

NPR1

One of the most promising candidates of second generation strategy is *NPR1* (Cao et al., 1994). Pathogen or insect pest resistance can be achieved through signaling modification. The *Npr1* gene was discovered originally from various independent genetic screens. The *Arabidopsis* mutants *npr*-1 do not respond to inducers of systemic acquired resistance (SAR) such as salicylic acid (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997)

or lost the ability to accumulate PR transcripts and were also hypersensitive to biotrophic pathogens (Pieterse et al., 2004). NPR1 acts as a switch between the signaling pathways involving ethylene/jasmonic acid (ET/JA) (ISR) and salicylic pathway (SAR), therefore resistance to both necrotrophic and biotrophic pathogens depends on modulation of NPR1 gene (Li et al., 2004; Cao et al., 1994; Pieterse et al., 2004) . NPR1 is the key master switch as it constitutes a node which links SAR, ISR, SA, JA, ethylene, and also R gene-mediated resistance (Pieterse et al., 2004). The activation of NPR1 gene is through redox pathways by SA accumulation in the cytosol and then translocated to the nucleus, however without binding to DNA directly it acts through transcription factors, which in turn induces expression of several PR genes (Pieterse et al., 2004). *NPR1* is constitutively expressed at low levels, when challenged by pathogen or treated with SA, transcript accumulation increases up to two-fold. SA gives better defence against piercing and sucking insect pests than the chewing pests (Zhao et al., 2009).

SA-mediated expression of proteins by *NPR1* include the WRK70 transcription factors this lead to suppression of JA-dependent signaling events (Li et al., 2004; Ndamukong et al., 2007). However, nuclear localization of *NPR1is not required for direct regulation of* JA-pathways which indicates a dual function between the cytosolic and nuclear located *NPR1* (Glazebrook et al., 2003; Spoe et al., 2003; Yuan et al., 2007).

As both SA and JA dependant pathways are controlled by NPR1, it can be targeted to achieve broad spectrum disease resistance through genetic engineering. There are several instances where over-expression of NPR1 has resulted in resistance against both biotrophic (Cao et al., 1994; Lin et al., 2004). Necrotrophic (Lin et al., 2004; Makandar et al., 2006; Wally et al., 2009) pathogen in several plant species as well as against insect pest in tobacco plants. Over-expression of NPR1 resulted in quicker and higher intensity of PR proteins for longer duration. The function of NPR1 remained unchange when AtNPR1 was expressed in different crop like rice (Fitzgerald et al., 2004) , wheat (Makandar et al., 2006), carrot (Wally et al., 2009) tobacco (Meur et al., 2008) and tomato (Lin et al., 2004) indicating the conserved functionality of the signaling system as well as the NPR1 like proteins.

However, when AtNPR1 or the rice ortholog OsNH1 was expressed in transgenic rice, the constitutive expression of PR genes lead to stunted growth of plants and more light sensitivity apart from desired increase in disease resistance (Chern et al., 2005). Green tissue specific expression of AtNPR1 in rice reduced such developmen-

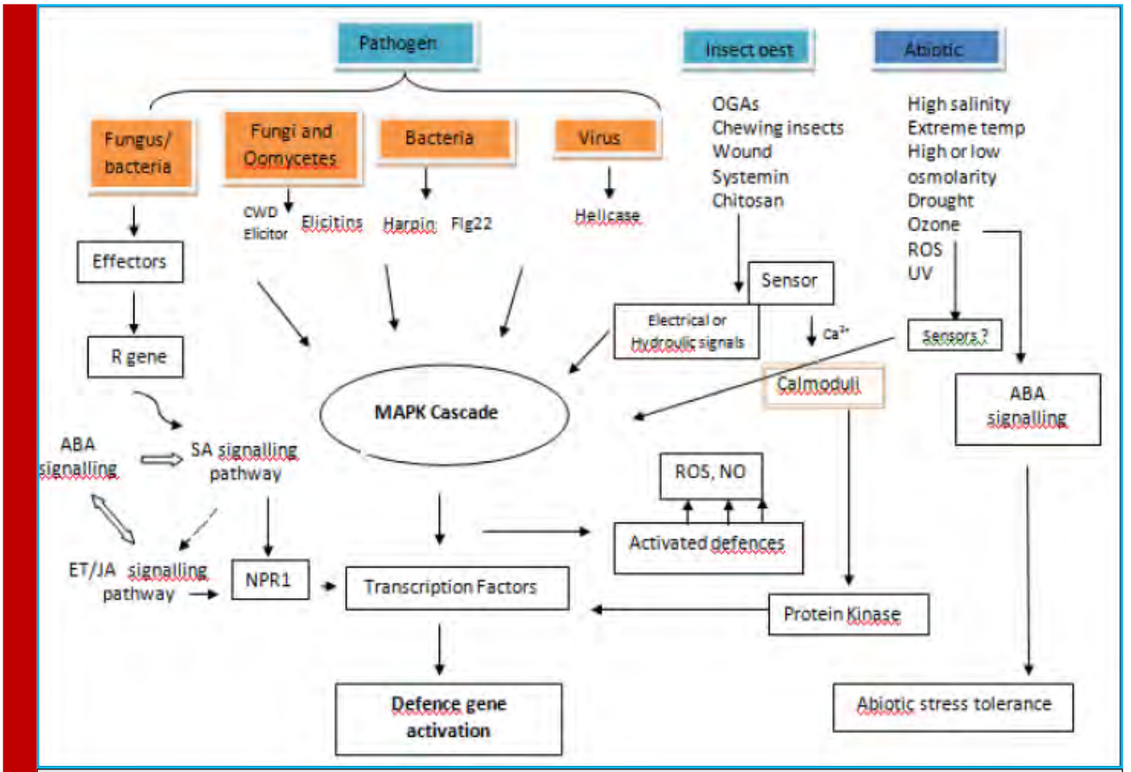

FIGURE 2. A summary of role of MAPK, NPR1 and transcription factors in plant defence.

tal abnormalities and conferred resistance to the sheath blight pathogen without compromising the growth and yield parameters (Molla et al., 2016)

MANIPULATION THE EXPRESSION OF TARGET GENES

Expressing *Avr* protein

De Wit (1992) proposed an interesting exploitation of R gene response, where a plant can be designed to express an active Avr protein under the control of a pathogen-responsive promoter for which the plant has a R gene. The induced Avr product would induce responses which would result in incompatible reaction to a wide range of pathogens (Dewit, 1992). The pathogen inducible promoter (*hsr203J*) in tobacco resulted in successful exploitation of HR elicitor cryptogein (Keller et al., 1999). The main benefit is resistance against a wide range of pathogens. However the real value of this strategy is yet to be exploited.

Synthetic modifications of PR proteins

To enhance the effectiveness of PR proteins, synthetic modifications such as linking a single chain antibody gene against a particular pathogen can be done (Peschen et al., 2004). The antibodies would then attach to the invading pathogen's cell wall and the antimicrobial proteins would effectively degrade the fungi. It has been demonstrated against *Fusarium graminearum*. It was highly effective against nine different species of the Fusarium genus, in *Arabidopsis*, however, not effective against unrelated pathogens (Peschen et al., 2004). This method has been also implemented in transgenic wheat, which reduced the disease symptoms against Fusarium head blight (Li et al., 2008).

Toxic gene products to engineer local cell death

One of the first strategies applied for increased disease resistance in plants was generation of an 'HR-like' local cell death artificially by expressing a toxic gene (Li et al., 2008). This strategy is only successful when 'HR' is restricted to infection sites otherwise uncontrolled cell death will occur even in uninfected tissues which is undesirable. Components of the pathogen can be expressed as toxic genes. But the promoters used so far have undesired background expression in uninfected tissues. Moreover, the toxicity level of the gene product needs to be studied well before the product is marketed.

RNAi

A useful tool inhibiting pathogen expression is through RNAi (Csorba and Burgyan, 2016; Novina and Sharp, 2004) technology. It inhibits the expression at both the transcriptional and post transcriptional levels in plants. RNAi has been exploited to develop many virus resistant plants (Fuentes et al.,2016). For example, papaya ringspot virus (PRSV) coat protein protected papaya in Hawaii has already been commercialized.

Stacking antimicrobial compounds

Expressing antimicrobial proteins, phytoalexins and enzymes in plant cell reinforcement or in the breakdown of pathogen infection structures has also been tried. The limitation of this strategy is resistance towards a specific pathogen. However to broaden the spectrum of resistance, stacking of antimicrobial peptides could be a reasonable approach (Van der Biezen, 2001).

Targeting inducible promoters

With the significant advances in sequencing technologies for transcriptome analysis, number of important crop genomes have been sequenced, which make it feasible for high throughput recognition of promoters and putative cis elements. Cis regulatory elements function as molecular switches in respose to various stress signals (Kazuko and Kazuo, 2005). Transcription factors interact with cis acting elements in the promoter region and forms a complex to initiate transcription thus can help in formation of initiation complex when activated and act as molecular switches to determine transcription initiation events. Therefore, it is important to determine the elements in the stress responsive promoters to understand the molecular switches of stress inducible genes. Apart from this, plant pathogen molecular interaction has shown that the promoter region also plays an important role in pathogen recognition (Patrick et al., 2009) . In gene for gene interaction pathogen effector interacts with the promoter region for activation of R gene. For example, some bacterial effectors like TAL effectors Avr BS3 and AVR Xa27 interact with the promoter region and activate the corresponding R genes (Patrick et al., 2009).

The current limitation of development of resistant transgenic crops using genetic transformation is unavailability of the right kind of promoter. Strong synthetic inducible promoters can be designed to address the issue of biotic stress. Promoters can be designed to not only recognize specific predators but also effector molecules from different pathogen and pests, thus giving a broad spectrum resistance against several biotic factors. It is also possible to use bidirectional promoters to activate two genes at the same time.

CONCLUSION

Durable pest and disease resistance so far has been achieved by traditional breeding and chemical applications. However, conventional breeding has prioritize

quality parameters and agronomic adaptation over resistant breeding. Therefore, new improved genomic tools are required to empower the process of genetic analysis and crop improvement. High through put sequencing and complete genome sequencing of many crops allows understanding of many metabolic pathways and disease resistance mechanisms. Understanding of omics are shedding light on the different compounds associated with plant defense. Using new technologies, it might be possible to achieve more durable and long term resistance through various genetic approaches. The wide spread application of pesticides can also be reduced through this technology. There are several success stories of plant genetic engineering which include herbicide resistant for weed control and insect resistance for lepidopteran insect control. However, transgenic disease resistance crop and resistance against sap sucking insects represent a very small portion of transgenic crops. Also the scope is wide with the advancement of genome editing tools like CRISPR-Cas9 and new digital phenotyping technologies, to develop a more sustainable agriculture that involves adaptation to changing climates. The global food demand needs to be fulfilled and therefore, it is the need of the hour to combat yield losses caused by diseases and sap sucking insect pests on a global scale. Also, an increased and stable yield is required to address decreasing land availability issues. Engineering disease resistance with new tools available needs to be made a priority.

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The effect of massage therapy on sleep quality in cardiac care unit patients

Arezoo Khosravi[1], Fariba Bolourchifard[2*], Mahnaz Ikhani[3] and Mohamad Amin Pourhoseingholi[4]

[1]Master of Medical Surgical Nursing, Student Research Committee, School of Nursing and Midwifery, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2]PhD in Nursing, Assistant Professor, Department of Medical Surgical Nursing, School of Nursing and Midwifery, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[3]Doctorate in Clinical Practice, Assistant Professor, Department of Medical Surgical Nursing, School of Nursing and Midwifery, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[4]PhD in Biostatistics, Associate professor, Gastroenterology and Liver Disease Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran

ABSTRACT

Low sleep quality is one of the most common complaints in patients admitted to CCU. This study aimed to determine the effect of Massage Therapy on Sleep Quality, admitted in the cardiac care unit. This research is a quasi-experimental study. 60 cardiac patients admitted to CCU at Shariati Hospital in Tehran were selected and randomly divided into two intervention and control groups. Members of the intervention group for a week, For one week, in 4 sessions, each session is 12 minutes twice a day, in the morning and in the evening or at least once before bed time were treated by massage, but the control group did not received the massage. Data collected by demographic characteristics questionnaire and Pittsburg sleep quality (PSQI) and were analyzed by using of statistical methods such as paired t-test and analysis of covariance. Results showed improved quality of sleep among the intervention group quality scores in the post-test significantly improved compared to pre-test (P= 0/001) but in the control group was not observed significant difference between the pre-test and post-test (P=0/520). Also, there was a significant difference between post-test score in two groups of intervention and control with control of pre-test effects (P=0/001). Therefore, massage therapy has improved the quality of sleep in the intervention group. According to effectiveness of massage therapy on sleeping quality in patients hospitalized in the coronary care unit, this method can be used to reduce undesirable effects of decreased sleep quality in patients.

KEY WORDS: MASSAGE THERAPY, SLEEPING QUALITY, CARDIOVASCULAR PATIENTS, CARDIAC CARE UNIT

ARTICLE INFORMATION:

**Corresponding Author: bolourchifard@gmail.com, bolourchi@sbmu.ac.ir*
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INTRODUCTION

Cardiovascular diseases have the highest mortality rates and will continue to be the leading cause of death in the world by 2020 (Shafie et al., 2013). At present, cardiovascular disease accounts for 38% of i the world (Moller, 2010), and the first cause of death in Iran. It has also been anticipated that, by 2030, the order of causes of death would be included ischemic heart disease, cerebro-vascular diseases, AIDS and chronic pulmonary diseases (Azizi, 2008).In spite of increasing awareness of cardiovascular diseases prevention and planning which governments have done in this regard, and given the aging population, the prevalence of cardiovascular diseases, and consequently the need for Coronary care unit (CCU) and cardiac intensive care beds are on the rise. As many as 2940 people across the country need to be admitted to cardiac care units (Talebi et al., 2009). This indicates an increase in the need for hospitalization and the number of beds in the cardiac care unit in Iran. Meanwhile, almost every disease with significant pain or discomfort, such as respiratory, cardiovascular, digestive and neurological diseases, can negatively affect sleep quality (Jahne et al., 2012). Sleep problems in patients with heart failure are more prevalent than those without this disease, and factors such as respiratory problems, increased age, medication, anxiety and depression play a significant role in this (Suna, 2015).

Sleep is a regular, repeatable, and reversible physiologic event in which a person experiences a decrease in consciousness, a relative loss of skeletal muscle (volitional) and a significant increase in the threshold of response to external stimuli. More than a third of human life span is spent in sleep. Therefore, any intermittent hypoxia and disorder in quantity, quality or pattern of sleep can have a significant negative effect on the person's physical and mental health and lead to the development of cardiovascular complications (Wang, 2010). For example, Gvstafsn writes on the findings of his studies: trouble in falling asleep is an independent risk factor for cardiac events in men. He also believes that there is a link between inadequate sleep and many clinical manifestations of coronary artery disease such as angina, cardiac arrhythmia, increased blood pressure, respiratory problems, the risk of developing myocardial infarction and sudden death (Bayley, 2010). Sleep also affects the cardiovascular system regulation, so that at the time of awakening the heart of a healthy person, on average, 70 to 80 beats per minute, while at bedtime it is reduced to 60 times per minute. Find (Fontana and Pittiglio, 2010). Conversely, sleep deprivation increases heart rate and increases the myocardial need for oxygen (Matthews et al., 2010).

Nerbass et al. (2010) found that although coronary artery bypass graft surgery is a common operation (practice) with a low mortality rate and relieves angina symptoms in a desirable manner, but recovery from post-cardiac surgery is followed by symptoms and signs of pain and psychological distress, and sleep problems. Considering all the emphases and warnings about the effects of sleep deprivation in hospitalized patients, especially in cardiac care units, many of the patients admitted to these units experience problems caused by sleep and rest disturbances (Zolfaghari et al., 2013), and sleep disorders in patients admitted to intensive care units are highly prevalent (Habibzade et al., 2011). Although sleep problems can be somewhat controlled by medication, but due to the problems and complications of drug therapy (Cinder, 2007), the use of non-medical methods that can reduce sleep problems in cardiovascular patients is logical. In order to solve these problems, various nursing practices have been used as complementary therapies to help patients to meet their psychological and physical needs that among which massage therapy is an effective nursing intervention in relaxing, reducing stress, relaxing the mind and body in patients (Oshundi et al, 2013).

After a preliminary study on cardiac patients, Cutshall and colleagues suggested that massage be used as a complementary therapy to help reduce pain and anxiety in these patients. Nelson et al. (2008) found that massage with release of endorphins prevents from the transmission of pain messages and Wilkinson (2009) suggests that relaxation and eliminating anxiety can be due to reduced muscle spasm and thus reduce pain (Watson, 2011). Also, Castro and colleagues reported during their research that massage therapy in patients with fibromyalgia via decreased muscle tendon restriction, reduces anxiety and improves sleep quality and physical performance in these patients. Some research has shown that massage therapy is effective in improving sleep quality and reducing fatigue in patients during the recovery period after coronary artery bypass graft surgery (Narbass et al., 2010).

Kavehia et al. (2013) investigating the effects of massage therapy on psychological outcomes in post-cardiac surgery patients stated that massage therapy is effective in reducing pain and improving psychological outcomes in patients undergoing cardiac surgery. Massage therapy with parasympathetic stimulation can lead to effects such as lowering heart rate, reducing respiration, facilitating and returning to normal conditions in cardiovascular patients (Morskaa et al., 2010). Therefore, considering the low sleep quality of patients admitted to CCU, the complications of sleep medications, the need to use complementary and simple therapies in nursing, safe and inexpensive methods, it was decided to conducting a study aimed to determine the effect of massage on the quality of sleep in the patients admitted to intensive cardiac units, a step toward helping these patients

through nursing interventions is taken. For this purpose, the aim of this study was to evaluate the effect of massage therapy on the quality of sleep in patients admitted to cardiac care units (CCU).

MATERIALS AND METHODS

This research is a semi-experimental study with university ethics committee approval. 60 patients hospitalized in the cardiac care units of selected hospitals of Tehran University of Medical Sciences in 2016, according to inclusion of research (complete consciousness, aged 18 to 85 years, the lack of use of a variety of complementary therapies during the previous three months, non-use of sedative and narcotic drugs, healthy areas of massage, and permission from the doctor, lack of severe neuropathy, mental retardation, blindness and deafness, lack of history of arthritis, joint rheumatoid arthritis and joint disorders and absence of coagulation disorders and diabetes) were selected using convenience and purposeful sampling method and randomly even numbers were assigned in the experimental group and odd numbers assigned to control group.

The method of doing research was that the researcher received confirmation from the committee of research and graduate education and the Ethics Committee of Shahid Beheshti University of Tehran and obtaining a referral from the university and presenting it to the management and office of nursing at Shariati Hospital in Tehran, and obtaining permission after their introduction to the head nurse and the staff of the cardiac care unit and patients, the purpose of the research was explained to them. Before initiating massage therapy, the researcher first provided the patient and his or her environment for intervention, in such a way as to preserve the patient's privacy and not feel insecure. Then, the subjects completed the questionnaires. For non-literate students, a researcher or another family member read the content to the patient and the questionnaire was completed. For the intervention (experimental) group, in addition to the routine pharmaceutical and non-pharmaceutical methods of unit, Shiatsu massage was performed in the following way:

A total of six points and each point for two minutes were massaged. The points under massage proposed by an expert and acupressure specialist, included the point of ht7, the point at the radial and proximal angles relative to the wrist line at the plantar and tendon level of fifth finger flexor, point kidney 3 point and surface between the ankles and the achilles tendon in the horizontal direction, the anmien point at the angle between the mastoid and the mandible is located one centimeter directly above the hair extension and below the midline and posterior of the head to cover the important points of the control of insomnia (Fig. 1).

The massage of every point lasts for two minutes, which included one minute of vertical and direct pressure, which began with a very gentle pressure, and the pressure increased to some extent that the patient would report a pleasant sensation of diffusion of a stream or lightness, or that the therapist's nail is discolored due to pressure, or the patient feel uncomfortable with the increase in pressure, the process is continued. Then massage the point is done for one minute. At the time of acupressure, you need to focus on quiet breathing and fingertip pressure. This intervention lasted for one week in 4 sessions and 24 minutes per session twice daily in the morning and afternoon, or at least once before bedtime. After the intervention, the sleep quality questionnaire was completed again. Patients in the control group had all the conditions of the patients in the experimental group, but no intervention was provided for them, and patients received only routine pharmacological and non-pharmacological care and the questionnaire was completed on the first and last days by them.

The tools used in this study included demographic questions (age, gender, marital status, duration of illness, etc.), and the Pittsburgh Sleep Quality Index (PSQI) which has been designed by Bays et al. in 1989 to assess

FIGURE 1. Points in Shiatsu massage

the quality of sleep in the psychiatric institution of Pittsburgh. This index consists of 19 questions in 7 dimensions (subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, Sleep disturbances, use of sleeping medication and daytime dysfunction). Each part is scored from Zero (No problem) to 3 (there is a serious problem). The total score is between zero and 21, with higher scores indicating low sleep quality and vice versa. Bays et al. (1989) obtained internal consistency of the questionnaire using Cronbach's alpha of 0.83. In the Iranian version of this questionnaire, reliability was obtained by Cronbach's alpha of 0.46 and by the split-half method of 0.52 (Heidari, Ehteshamzade and Marashi, 2010).

The software SPSS version 19 was used to analyze the data. Also, paired t-test was used to compare the mean of the groups. To compare the mean score of sleep quality before and after the intervention in two groups, covariance analysis was used. The significance level was considered as $p < 0.05$.

RESULTS

The mean age of the experimental group was 58.38 with a standard deviation of 19. 21 and the mean age of the control group 52.36 with a standard deviation of 17.55. The sample consists of 38 (63.33%) males and 22 females (36.66%) that each experimental and control group (n = 30) equally includes 19 males and 11 women. In the experimental group, 3 (10%) were single and 27 (90%) were married. In the control group, 4 (13.3%) were single and 27 (86.7%) were married. Finally, the mean duration of the disease was 3.30 months in the experimental group with a standard deviation of 6.25and in the control group, 5.48 months, with a standard deviation of 5.48.

The mean and standard deviation of the pre-test and post-test of the studied variables in the intervention (experimental) and control groups are presented in Table 1.

As the results of the table above reveal, the post-test score of the intervention group was reduced by 2.93 in comparison with the pre-test score. In the intervention group, the post-test score increased by about 40% compared to the pre-test score.

The calculated Z to evaluate the normal distribution of data for pre-test and post-test of the sleep quality score was 1.29 and 1.07, respectively, which was not statistically significant (Table 2). Therefore, parametric tests can be used to examine the research hypotheses.

Paired t-test was used to evaluate the effect of massage therapy on the quality of sleep in patients admitted in cardiac care units (Table 3).

As shown in table (3), the sleep quality score of the intervention group in the post-test has been decreased and according to the scoring of the questionnaire, the decrease in score means improving sleep quality. The t-value calculated for changes in the mean in post-test of the intervention group compared to the pre-test score was 4.82, which was statistically significant (p = 0.001). In contrast, the calculated t value for the comparison of

Table 1. Mean and standard deviation of pre-test and post-test of sleep quality score in two groups of intervention (experimental) and control

variable	Group	Number	test periods	mean and standard deviation
Sleep quality	Intervention	30	Pre-test	13.96(2.930
			Post-test	11.03(1.27)
	control	30	Pre-test	13.06(2.75)
			Post-test	13.46(1.77)

Table 2. Kolmogorov-Smirnov test for data normalization

Variable	Mean (SD)	Z Kolmogorov-Smirnov	p-value
Pre-test score of sleep quality	13.51 (2.58)	1.29	0.069
Post-test score of sleep quality	12.25 (1.96)	1.07	0.20

Table 3. Paired t-test results to examine the intra-group mean changes of intervention and control groups in the quality of life score

Variable	Group	Pre-test		Post-test			
		Mean	SD	Mean	SD	t	p-value
	Intervention	13.96	2.93	11.033	1.27	4.82	0.001
	Control	13.06	2.75	13.46	1.77	0.64	0.52

Table 4. One-way covariance analysis to compare the mean score of sleep quality in two intervention groups with control

	Source	sum of squares	degree of freedom	mean square	F	P	eta coefficient	observed power
Sleep quality	Group	83.07	1	83.07	34.56	0.001	0.377	0.99
	Error	137.003	57	2.40				
	Total	9231.00	60					

the pre-test and post-test mean of the control group was 0.64, which was not statistically significant (p = 0.52).

To evaluate the consistency of variances between two groups, Levin test was used (F = 0.73, df1 = 1, 58 = df2, p = 0.08). The insignificancy of Levine's test is that the variance in sleep quality score is identical in both groups and covariance can be used.

One-way covariance analysis was used to compare the mean of intervention and control groups. The results of one-way covariance analysis are presented in Table (4).

The results of one-way covariance analysis showed that the calculated f value for the comparison of the means in the intervention and control groups is 34.56, which is statistically significant (p = 0.001). Therefore, the results of this analysis showed that the intervention, namely, massage therapy, is effective on the quality of sleep in patients admitted to cardiac care units.

DISCUSSION

The aim of this study was to determine the effect of massage therapy on sleep quality of patients hospitalized in intensive care units. The findings showed that sleep quality in the intervention group improved after massage therapy. This finding is consistent with the results obtained in some previous studies. For example, Shafiee et al. (2013) examined the effects of massage therapy on the quality of sleep after surgery in patients undergoing coronary artery bypass graft surgery. The mean of quality of life scores in the experimental group and control group was 22.5 ± 3.6 and 22.3 ± 3.8, respectively. The difference was not statistically significant. After intervention, mean of quality of life scores of the patients in the experimental (intervention) group and control group was 5.5 ± 4.7 and 11 ± 2.15, respectively, which showed a significant difference (p < 0.001). Also, the results showed that the use of massage therapy can improve the quality of sleep after surgery in patients undergoing coronary artery bypass graft surgery and considering the simplicity and low cost of this method, this method may be considered as a suitable supplement for medication and postoperative interventions in these patients. The differences between this study and the present study can be explained by the difference in the type of massage, the place where the work was carried out, the society and the research environment and the disease. The aim of this study was to investigate the effect of massage on the quality of sleep in patients with heart disease. In addition, the lack of improvement in sleep quality in the control group in the post-test compared with the pre-test is consistent with Arab and colleagues (2012), in a study entitled "the effect of acupressure on quality of life in patients undergoing hemodialysis".

Regarding the comparison of sleep quality of patients admitted to CCU in intervention and control groups, the findings showed that the intervention, namely, massage therapy, was effective on the quality of sleep in patients admitted to intensive care units. This finding is consistent with the study conducted by Narbas entitled "the effects of massage therapy on the quality of sleep after coronary artery bypass graft surgery. This research was performed on 57 patients undergoing coronary artery bypass surgery that were divided into two groups of control and massage group after discharge from the intensive care unit. Participants in the control group and the massage group were three nights without massage and three nights under the massage therapy. Patients were evaluated the next morning. The results of the study in the experimental group showed that sleep quality in the intervention group has been increased.

Some of the theories that look at massage offer assumptions (hypotheses) about the effectiveness of this method. For example, according to the gate control theory of pain, this method has been shown to increase the secretion of endorphins and enkephalins, thereby controlling pain, as well as improving the function of the immune system and eliminating toxins from the body. Also, based on the theory of nerve impulses, massage can inhibiting the afferent nervous messages and closure of nerve valve on the posterior horn of the spinal cord, inhibits the transmission of pain. It seems that by massage, the pituitary and hypothalamus glands are stimulated and endorphin is secreted as an intravenous narcotic similar to morphine by them, thus these neural mediators reduce the pain (Rigi et al., 2015).

Other researchers also believe that increased blood supply (increased intake of food, oxygen, and removal of cellular waste) and sensitization of muscles in relation to neural waves, of direct effects of massage therapy on the body. On the other hand, this technology can indirectly affect fatigue by reducing pain, improving depression and relaxation (Domingos & Barg, 2015). Massage reduces anxiety and tension, relieves pain and causes

physical relaxation, leading to two-way energy transfer between the patient and the therapist, and as a general manipulation of the soft tissues of the body for restoring the metabolic balance of these tissues is used. The short-term use of massage for hands, feet, neck and shoulders can have therapeutic effects. But many massage therapists focus on foot massage because of the lack of enough time to massage the whole body, which has the benefits of physical and mental relaxation, reducing anxiety and improving sleep (Shabani et al., 2005).In general, based on the literature on the effects of massage therapy, it seems that the effect of this treatment on the quality of sleep can be the direct and indirect effects of massage therapy. Its direct effects are due to changes in the hormonal and neuro-transmitter, and its indirect effects are due to the reduction of some of the variables affecting the quality of sleep such as reduced fatigue, stress and ... as a result of massage therapy.

Each research has limitations that can influence its outcomes. Some of the limitations of this research are: 1) lack of complete control of disturbing variables such as personality, physical and psychological variables as well as social, economic and cultural variables; 2) lack of suitable research facilities such as research room, proper chair and other necessary facilities in the hospital and therapeutic center; and 3) the limited power of generalizing the results due to the limited research sample group.

It is suggested that in future researches the effectiveness of massage therapy on other groups of patients with problems in sleep quality and other variables such as stress and anxiety of heart patients be investigated. It is also suggested that the effectiveness of massage therapy on the quality of sleep is compared with other therapies that are available to improve the quality of sleep, so that therapies with greater efficacy can be used to improve sleep quality. In addition, teaching this method of treatment to nurses, using this treatment to improve the quality of sleep in patients admitted to intensive cardiac units and preventing the negative consequences of poor sleep, such as heart problems, are some of the practical suggestions of the present study.

CONCLUSION

According to the results of this study and the effect of massage therapy on the sleep quality of patients admitted to the cardiac care unit, this method can be used to reduce the adverse effects of decreased sleep quality in these patients.

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Effect of antibiotic sensitivity on different cultured tissues and its significance in genetic transformation of cabbage *Brassica oleracea*

Geetika Gambhir*, Pankaj Kumar and D K Srivastava

Department of Biotechnology, Dr Y.S.Parmar University of Horticulture and Forestry Nauni, Solan-173230, Himachal Pradesh, India

ABSTRACT

Successful plant transformation requires highly efficient regeneration protocol and suitable selection system. In this regard, the effect of kanamycin and cefotaxime was studied on cultured hypocotyl, cotyledon, leaf and petiole tissues of cabbage to look at the suitability of kanamycin resistance as a selectable marker and cefotaxime in controlling excessive bacterial growth during genetic transformation studies. Kanamycin sensitivity (0-60 mgL^{-1}) was checked by fresh weight of the explants (leaf and petiole) which was measured at the interval of 7 days till 35 days. Explants showed decrease in fresh weight as concentration of the kanamycin increased resulting in full or partial inhibition of shoot regeneration. A negative correlation was observed between the concentration of kanamycin and fresh weight of the explants at different intervals of time. Effect of different concentrations of cefotaxime (0-500 mgL^{-1}) was studied on the regeneration potential in cotyledon and hypocotyl explants of cabbage and found no much effect of cefotaxime on regeneration potential. Effect of different concentrations of cefotaxime and kanamycin (50 mgL^{-1}) was studied on the growth of agrobacterial cells and regeneration potential of cotyledon and hypocotyl tissues after cocultivation. In both the explants, growth of agrobacterial cells were controlled at concentration of 400 mgL^{-1} cefotaxime and maximum per cent shoot regeneration in cotyledon (35.55 %) and hypocotyl (48.15 %) was obtained on the best MS shoot regeneration medium supplemented with 400 mgL^{-1} cefotaxime.The results indicate that kanamycin and cefotaxime act as an effective selective agents during genetic transformation studies.

KEY WORDS: CABBAGE, CEFOTAXIME, COTYLEDON, HYPOCOTYL, *IN VITRO* REGENERATION, LEAF, PETIOLE, KANAMYCIN

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**Corresponding Author: geetikabt@gmail.com*
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INTRODUCTION

Brassica oleracea is an extremely diverse species of nutritionally rich and economically important vegetable crops including cabbage, cauliflower, broccoli, Kohlrabi and kale. *Brassica oleracea* L. var. *capitata* (Cabbage) is one of the most important vegetable in India, as India is next only to China in area and production of vegetables and occupies prime position in production of cabbage (FAO). It is cultivated in 0.245M ha with the total production of 5.167 M mt and average productivity of 22.9mt/ha (NCPAH India, 2012). But quality and quantity of cabbage produce is challenged by many biotic and abiotic stresses including infestation of insects and pest. The major pest affecting cabbage production is the diamondback moth (*Putella xylostella*) (Jin *et al.,* 2000). Total yield loss of cabbage in India due to infestation of insect and pest is about 16.87-58.83% (Dhandapani *et al.,* 2003). Massive quantities of synthetic insecticides are used, giving rise to major concerns about food and nutritional security and environmental pollution in addition to the high chemical and labor costs. Modern biotechnological tools could be of much significance to alleviate the negative effects of chemicals and synthetic pesticides. Application of plant genetic engineering using transgenic technology expressing foreign genes (insect resistant gene) could be an important aspect of integrated pest management. Two requirements for successful transformation are the ability to introduce desirable genes into the genome and the capacity to regenerate plants from the transformed cells (Kanwar and Kumar, 2011). As a preliminary step in efficient genetic transformation experiments involving the antibiotic sensitivity experiment (kanamycin resistance as a selectable marker and cefotaxime in controlling excessive bacterial growth).Genes encoding antibiotic resistance and herbicide tolerance are widely employed asselective markers to identify the rare transformed explants (de Vetten *et al*., 2003; Miki & McHugh, 2004; Kumar and Srivastava, 2016a). Selective agent concentration to be used in gene transfer should beoptimized prior to transformation to determine agent effective on shoot and rootregeneration and to determine lethal dose for each agent. The continued presence of *Agrobacterium* interferes with the growth, development,and rooting rates; and even it causes the necrosis of transformed explants (Tang *et al*., 2004). Moreover, elimination of *Agrobacterium* from transformants is a prerequisite inpreventing the possibility of gene release when these plants are transferred to soil (Barrett *etal*., 1997). Bacterial presence on putative transgenics may also result in false positivesduring molecular analyses. Most commonly used antibiotics for elimination of variousstrains of *Agrobacterium* are carbenicillin, cefotaxime and timentin (Nauerby *et al*., 1997; Kumar and Srivastava, 2016b). The purpose of this study was to check the toxic level of kanamycin and cefotaxime on various explants of cabbage.

MATERIAL AND METHODS

MEDIUM PREPARATION

MS salts (macro and micro salts) and vitamins supplemented with 100mgL^{-1} meso-inositol, 3.0 % sucrose and 0.8 % agar-agar for solidification of media were used as basal medium. The selective media for kanamycin and cefotaxime sensitivity experiment was prepared by adding kanamycin (kanamac, Macleods Laboratory Pvt. Ltd., Mumbai, India) and cefotaxime into pre-sterilized molten MS regeneration medium (best shoot regeneration medium) of cabbage cv. Pride of India, under aseptic conditions by filter sterilization through 0.22µm pore size, Whatman® membrane filter. Different concentrations (0, 10, 20, 30, 40, 50 and 60mgL^{-1}) of kanamycin was added into the medium to study the effect of antibiotic on the relative growth of cultured explants/tissues (leaf and petiole) of cabbage. Different concentrations (100, 200, 300, 400 and 500mgL^{-1}) of cefotaxime were added into the medium to study the effect of antibiotic on the regeneration potential of cotyledon and hypocotyl explants.Different concentrations (0, 100, 200, 300, 400, 500mgL^{-1}) of cefotaxime with 50mgL^{-1} kanamycin were added into the medium to study the effect of antibiotics on the growth of agrobacterial cells and regeneration potential of cotyledon and hypocotyl explants.

INOCULATION OF EXPLANTS ON SELECTIVE MEDIA

The leaf and petiole explants were excised from glasshouse grown 20-25 days old seedlingsof cabbage, surface sterilized by 0.1% of mercuric chloride for 3 mins followed by 3-4 washing with double distilled water and cut into small pieces and weighed on an electronic balance under aseptic conditions in the laminar flow cabinet. The initial fresh weight of the explants was recorded. The explants were cultured on the normal regeneration medium as control and on the selective regeneration medium containing different concentrations of kanamycin

The cotyledon and hypocotyl explants were excised from asepticallygrown seedlings (seven to nine days old), cut into small pieces and cultured on selective shoot regeneration medium (MS medium + 2.0mgL^{-1}Kn + 0.50mgL^{-1}NAA) for cotyledon explants and (MS medium + 1.5mgL^{-1} Kn + 0.25mgL^{-1} IAA) for hypocotyl explants containing different concentrations of cefotaxime, cefotaxime with 50mgL^{-1} kanamycin. The growth and differentiation of the explants were recorded.

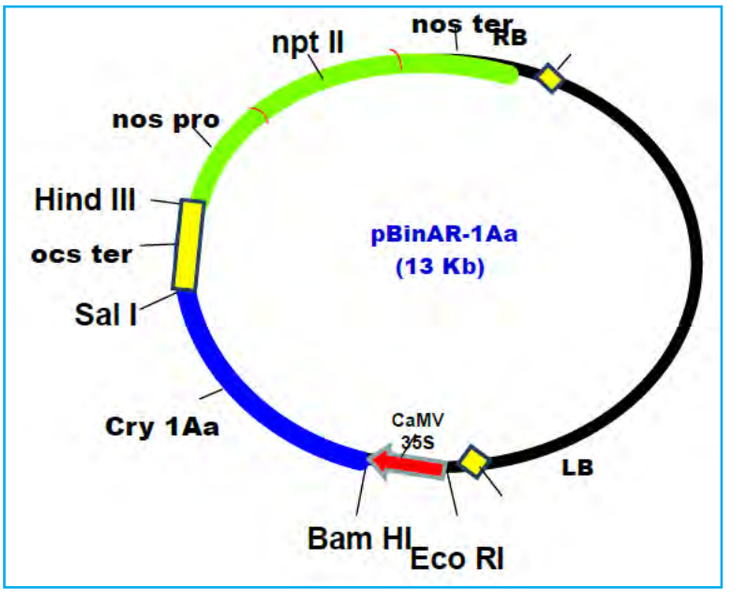

FIGURE 1. (a) The detailed diagram of binary vector pBin AR-1Aa containing *cryIAa* (insect resistance) gene along with *npt-II* (kanamycin resistance) gene for selection in both bacteria and plant. (b) Schematic diagram of gene construct: T-DNA of pBinAR-1Aa containing transcriptional fusion of CaMV 35S promoter with the coding region of *cryIAa* gene and NOS promoter with the coding region of *npt-II* gene.

MORPHOLOGICAL OBSERVATIONS AND MEASUREMENT OF RELATIVE GROWTH OF CULTURED TISSUES

Morphological changes were observed in leaf and petiole explants from 0 to 35 days in culture. Fresh weight of both the explants (leaf and petiole) was measured at the interval of 7 days, from 0 day to 35 days in culture. Relative growth at 7 days interval was calculated on the normal as well as on the selective media.Morphological changes were observed in these tissues/explants at interval of seven days till shoot regeneration in culture. The effect of cefotaxime and kanamycin on the growth of agrobacterial cells and plant cells were recorded till shoot regeneration.

RESULTS

KANAMYCIN SENSITIVITY IN LEAF AND PETIOLE TISSUES OF CABBAGE

The leaf and petiole explants were excised from *in* vivo grown seedlings, surface sterilized and cut into small uniform pieces. Fresh weight of these explants were measured under Laminar air hood with proper aseptical conditions and then inoculated on MS selective shoot regeneration medium having different concentrations of kanamycin (0, 10, 20, 30, 40, 50 and 60 mgL^{-1})

The explants in control medium i.e. MS shoot regeneration medium without kanamycin were very healthy and showed appropriate growth on this medium. But, on the selective media at concentration as low as 10mgL^{-1} kanamycin, the colour of explant/tissue had changed to pale greenish yellow. In control experiment, adventitious shoot bud regeneration was observed after 30-35 days in culture, whereas no shoot regeneration or shoot bud formation was observed even after 5 weeks in leaf explants but in petiole explants little callusing at the initial stage but later on it turned into brownish black was recorded on culture in MS selective medium containing 10mgL^{-1} kanamycin. A gradual decline in fresh weight of leaf explants were recorded with increased concentration of kanamycin (10 to 60mgL^{-1}) from 7–35 days. The maximum decline in fresh weight was observed at 50mgL^{-1} kanamycin in leaf explants, whereas in case of control (MS regeneration medium without kanamycin) there was a

Table 1. Effect of different concentrations of kanamycin on the relative growth (fresh weight) of leaf explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) on MS Basal full strength + 1.5 mg/l BAP+ 0.50mg/l NAA.

S. No.	Days	Average fresh weight of leaf explants/callus of cabbage (mg)						
		Kanamycin concentrations (mg/l)						
		0	10	20	30	40	50	60
1.	0	45.00±0.58	42.00±0.00	50.00±1.15	43.00±0.29	27.30±0.61	31.00±1.15	30.00±1.15
2.	7	95.00±0.58	87.20±0.12	92.00±1.15	81.00±0.29	62.40±0.61	51.00±1.15	45.00±1.15
3.	14	130.00±1.73	118.20±0.12	120.50±1.15	105.00±0.00	81.40±0.61	63.00±1.44	54.00±0.58
4.	21	201.00±1.73	177.2±0.69	171.00±2.52	150.00±0.00	105.7±0.61	76.00±1.44	64.00±0.58
5.	28	299.00±1.15	263.20±0.69	252.50±1.53	220.00±0.00	125.70±0.61	85.00±1.44	70.00±0.58
6.	35	448.00±1.15	388.20±0.69	350.50±1.53	289.00±0.00	131.20±0.61	81.50±1.44	65.00±0.58
$CD_{0.05}$		1.434						
SE±		1.014						

maximum increase in fresh weight of explants (Table 1 and 2) (Fig. 2A-F).

Statistical analysis of the recorded data showed that there was a significant difference between fresh weights of all six treatments of kanamycin concentrations (0 to 60 mgL^{-1}). Negative coefficient of correlation was observed between different concentrations of kanamycin and fresh weight of explants/tissue. It indicates that kanamycin has an inhibitory effect on the growth of cultured tissues as it is a potent inhibitor of protein synthesis.(Fig.1 (A-F))

EFFECT OF CEFOTAXIME ON SHOOT REGENERATION FROM COTYLEDON AND HYPOCOTYL EXPLANTS

In cotyledon explants, the maximum per cent (88%) shoot regeneration with average number of shoots (2.66) were obtained on MS shoot regeneration medium with 300 mgL^{-1} cefotaxime. With the increase in the concentrations of cefotaxime, percent shoot regeneration was same till 300 mgL^{-1} cefotaxime but increased at 300 mgL^{-1} cefotaxime then the percent shoot regeneration

Table 2. Effect of different concentrations of kanamycin on the relative growth (fresh weight) of petiole explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) On MS Basal Full Strength + 2.0mg/l Kn + 0.25mg/l NAA.

S. No.	Days	Average fresh weight of leaf explants/callus of cabbage (mg)						
		Kanamycin concentrations (mg/l)						
		0	10	20	30	40	50	60
1.	0	5.66±0.41	5.00±0.29	5.60±0.00	5.40±0.12	5.00±0.58	5.80±0.23	5.20±0.12
2.	7	17.17±0.41	11.00±0.29	14.20±0.00	12.46±0.12	11.32±0.58	7.92±0.23	6.68±0.12
3.	14	52.67±0.41	39.10±0.29	32.87±0.20	23.36±0.12	19.32±0.58	14.62±0.23	11.28±0.12
4.	21	125.50±0.87	103.70±0.29	92.30±0.21	65.26±0.12	41.12±0.58	22.62±0.23	16.28±0.12
5.	28	215.50±0.87	158.90±0.22	126.00±0.21	80.36±0.12	50.69±0.79	24.82±0.23	16.08±0.12
6.	35	366.40±0.87	257.50±0.22	188.00±0.21	110.10±0.12	58.32±0.58	24.52±0.23	14.98±0.12
$CD_{0.05}$		0.5396						
SE±		0.3816						

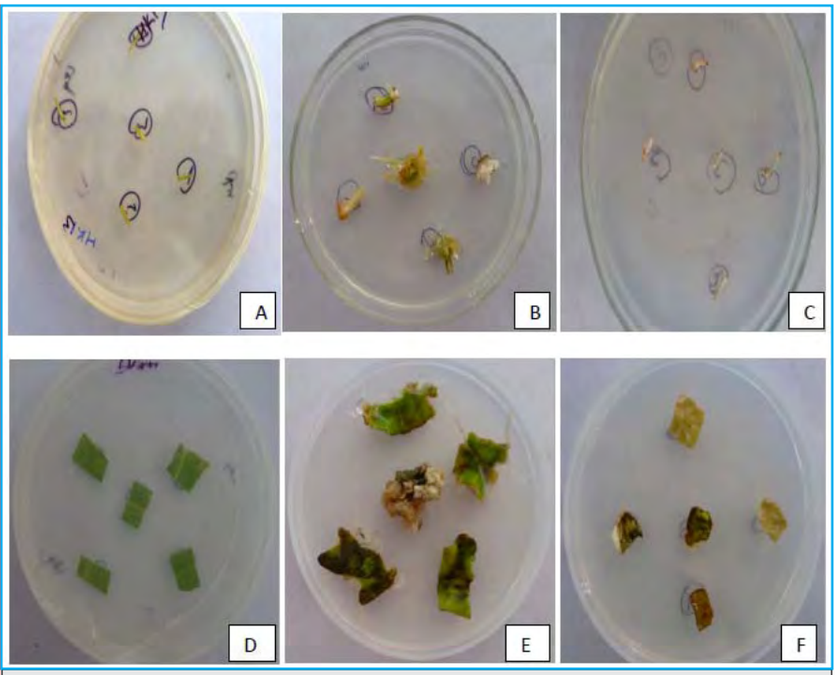

FIGURE 2. (a) Petiole explants cultured on selective medium (MS basal + 2.0mg/l Kn + 0.25mg/l NAA + 50mg/l Kanamycin) at day 0 in culture. (b) Petiole explants showing shoot initiation on selective medium (MS basal + 2.0mg/l Kn + 0.25mg/l NAA + 10mg/l Kanamycin) at 35 days of culturing. (c) Petiole explants turned brown (dead) on selective medium (MS basal + 2.0mg/l Kn + 0.25mg/l NAA + 50mg/l Kanamycin) at 35 days of culturing. (d) Leaf explants cultured on selective medium (MS basal + 1.50mg/l BAP + 0.50mg/l NAA + 50mg/l Kanamycin)at day 0 in culture. (e) Leaf explants showing callus initiation on selective medium (MS basal + 1.50mg/l BAP + 0.50mg/l NAA + 10mg/l Kanamycin) at 21 days of culturing. (f) Leaf explants turned brown (dead) on selective medium (MS basal + 1.50mg/l BAP + 0.50mg/l NAA + 50mg/l Kanamycin) at 35 days of culturing.

Table 3. Effect of different concentrations of cefotaxime on the relative growth of cotyledon explants of cabbage (Brassica oleracea L. var. capitata cv.Pride of India) (without co-cultivation)

Sr. No.	Cefotaxime concentration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	MS basal medium + 2.0mg/l Kn + 0.5mg/l NAA +0 mg/l	2.597	84.44(66.87)
2	MS basal medium + 2.0mg/l Kn + 0.5mg/l NAA +100 mg/l	2.460	84.44(66.87)
3	MS basal medium + 2.0mg/l Kn + 0.5mg/l NAA +200 mg/l	2.579	84.44(66.87)
4	MS basal medium + 2.0mg/l Kn + 0.5mg/l NAA +300 mg/l	2.666	88.88(70.73)
5	MS basal medium + 2.0mg/l Kn + 0.5mg/l NAA +400 mg/l	2.423	80.00(63.44)
6	MS basal medium + 2.0mg/l Kn + 0.5mg/l NAA +500 mg/l	2.597	84.44(66.87)
$CD_{0.05}$	0.156	5.66(3.435)	
SE±	0.078	2.59(1.632)	

Table 4. Effect of different concentrations of cefotaxime on the relative growth of hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) (without co-cultivation)

Sr. No.	Cefotaxime concentration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	MS basal medium+ 1.5mg/l Kn + 0.25mg/l IAA +0 mg/l	2.460	90.73(73.47)
2	MS basal medium+ 1.5mg/l Kn + 0.25mg/l IAA +100 mg/l	2.393	87.03(69.59)
3	MS basal medium+ 1.5mg/l Kn + 0.25mg/l IAA +200 mg/l	2.460	87.03(69.59)
4	MS basal medium+ 1.5mg/l Kn + 0.25mg/l IAA +300 mg/l	2.367	81.49(64.56)
5	MS basal medium+ 1.5mg/l Kn + 0.25mg/l IAA +400 mg/l	2.466	87.03(69.59)
6	MS basal medium+ 1.5mg/l Kn + 0.25mg/l IAA +500 mg/l	2.533	87.03(69.59)
$CD_{0.05}$	0.086	6.589(4.091)	
SE±	0.039	3.024(1.915)	

decreased. All the observations were recorded after 45 days of inoculation of explants. Whereas, in case of hypocotyl explants, very interesting and different shoot regeneration was observed on the same medium on different segments of hypocotyl explants. The maximum per cent (90.73%) shoot regeneration with average number of shoots (2.460) were obtained on MS shoot regeneration medium with 0 mgL^{-1} cefotaxime. Percent shoot

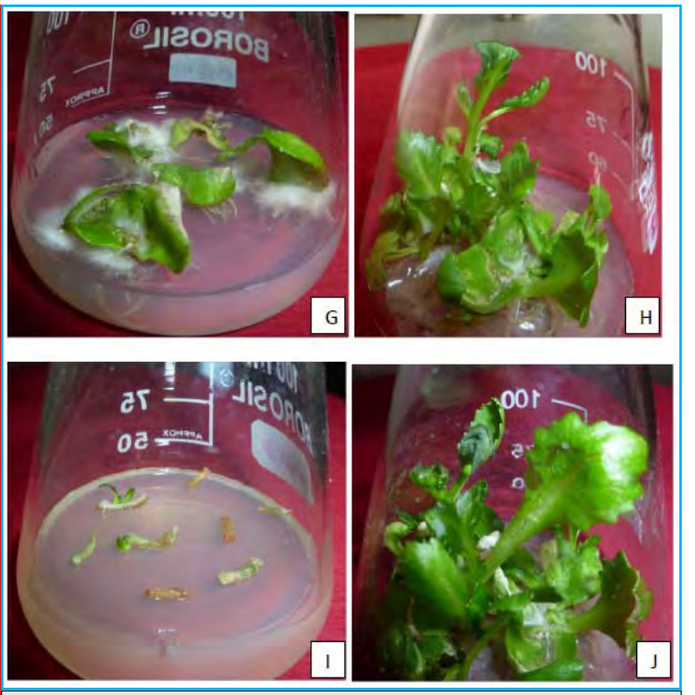

FIGURE 2(*Continued*). (g) Cotyledon explants showing callus initiation on selective medium (MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 500mg/l Cefotaxime) after 13 days of culturing. (h) Shoot elongation from cotyledon explants on selective medium (MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 500mg/l Cefotaxime) after 45 days of culturing. (i) Hypocotyl explants showing shoot initiation on selective medium (MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 500mg/l Cefotaxime) after 22 days of culturing. (j) Shoot elongation from hypocotyl explants on selective medium (MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 500mg/l Cefotaxime) after 50 days of culturing.

Table 5. Effect of various concentrations of cefotaxime and kanamycin (50 mg/l) on the regeneration potential of cotyledon explants in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)(after co-cultivation)

S. no.	Cefotaxime concentration	Average number of shoots per explants	Per cent shoot regeneration
1.	MS basal medium + 2mg/l Kn+ 0.50mg/l NAA + 50mg/l Kanamycin +0 mg/l	0.000	0.00 (0.00)
2.	MS basal medium + 2mg/l Kn+ 0.50mg/l NAA + 50mg/l Kanamycin +100 mg/l	0.000	0.00 (0.00)
3.	MS basal medium + 2mg/l Kn+ 0.50mg/l NAA + 50mg/l Kanamycin +200 mg/l	0.000	0.00 (0.00)
4.	MS basal medium + 2mg/l Kn+ 0.50mg/l NAA + 50mg/l Kanamycin +300 mg/l	0.400	16.66(19.16)
5.	MS basal medium + 2mg/l Kn+ 0.50mg/l NAA + 50mg/l Kanamycin +400 mg/l	2.930	35.55(36.59)
6.	MS basal medium + 2mg/l Kn+ 0.50mg/l NAA + 50mg/l Kanamycin +500 mg/l	1.35	22.22(28.07)
$CD_{0.05}$	0.330 3.140(1.998)		
SE±	0.1502.024(1.347)		

regeneration decreased in medium supplemented with 300 mgL^{-1} cefotaxime whereas remain constant at 100, 200, 400 and 500 mgL^{-1} cefotaxime but maximum average number of shoots (2.466) were observed in medium supplemented with 400mgL^{-1} cefotaxime(Table 3 and 4& Fig. 1(G-J))

EFFECT OF CEFOTAXIME AND KANAMYCIN (50MGL^{-1}) ON THE REGENERATION POTENTIAL OF CABBAGE AFTER CO-CULTIVATION

The effect of varying concentrations of cefotaxime with same concentrations of kanamycin was studied on the regeneration efficiency and their capability to control the overgrowth of agrobacterial cells after co-cultivation. At lower concentrations (0, 100, 200, 300 mgL^{-1}) of cefotaxime showed overgrowth of agrobacterial cells and at higher concentrations of cefotaxime, the agrobacterial cells growth was completely inhibited. Per cent shoot regeneration (35.55%) and (48.15%) and average number of shoots (2.93) and (1.770) per plant was found maximum in 400mgL^{-1} cefotaxime with 50 mgL^{-1} kanamycin for cotyledon and hypocotyls explants respectively. At lower concentrations of cefotaxime, all the explants died due to uncontrolled growth of agrobacterial cells. At the concentration of 300 mgL^{-1} cefotaxime the density of agrobacterial cells started decreasing(Table 5 and 6).

DISCUSSION

Kanamycin resistance gene is most widely used selectable marker for plant cell transformation and sensitivity of a particular species to kanamycin is a key element in the development of any new transformation system in

Table 6. Effect of various concentrations of cefotaxime and kanamycin (50 mg/l) on the regeneration potential of hypocotyl explants in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) (after co-cultivation)

S. No.	Cefotaxime concentration	Average number of shoots per explants	Per cent shoot regeneration
1.	MS basal medium + 1.5mg/l Kn + 0.25mg/l IAA+ 50mg/l Kanamycin+0 mg/l	0.000	0.00 (0.00)
2.	MS basal medium + 1.5mg/l Kn + 0.25mg/l IAA+ 50mg/l Kanamycin+100 mg/l	0.000	0.00 (0.00)
3.	MS basal medium + 1.5mg/l Kn + 0.25mg/l IAA+ 50mg/l Kanamycin+200 mg/l	0.000	0.00 (0.00)
4.	MS basal medium + 1.5mg/l Kn + 0.25mg/l IAA+ 50mg/l Kanamycin+300 mg/l	0.700	20.33 (26.38)
5.	MS basal medium + 1.5mg/l Kn + 0.25mg/l IAA+ 50mg/l Kanamycin+400 mg/l	1.770	48.15(43.94)
6.	MS basal medium + 1.5mg/l Kn + 0.25mg/l IAA+ 50mg/l Kanamycin+500 mg/l	1.250	35.18(36.37)
$CD_{0.05}$	0.410 4.510(3.098)		
SE±	0.190 2.740(1.701)		

which a kanamycin resistance gene will be employed. The genetically engineered *Agrobacterium* strain which we have used in the present study has two genes i.e. *cry IAa* and *npt-II*.Kanamycin sensitivity of cultured tissue of leaf and petiole explants of cabbage had shown similar results, i.e. both explants are highly sensitive to kanamycin even as low as 10 mgL^{-1} concentration of kanamycin. The non-transformed tissues did not survive on the selective medium containing Kanamycin during transformation experiment. Srivastava (1997) reported that cells which are not transformed get killed on selective media in such a manner that they become toxic to adjacent transformed cells, resulting in inhibition of the whole callus. Similar results were also reported by Eimert and Siegemund (1992). The alternating culture and repeated selection seem to be necessary for differentiation of transformed shoots against the inhibitory effect of kanamycin and elimination of escapes. The explants in control medium were very healthy and showed appropriate growth on the medium, but on selective medium at concentration as low as 20 mgL^{-1} kanamycin, the color of the explants/tissues had changed to greenish yellow and finally turned brown after 35 days of culture of broccoli (*Pankaj et al*., 2017).

Only 35 mgL^{-1} of kanamycin totally inhibit shoot differentiation from co-cultivated thin cell layer explants of *Brassica napus* (Charest *et al*., 1988), 20mgL^{-1} inhibits shoot induction from stem segments (Pua *et al.,* 1987) and 50 mgL^{-1} inhibits shoot regeneration of *Brassica oleracea* (Bhalla and Smith, 1998; Dixit *et al*., 1998; Bhattacharya *et al*., 2002; Sharma and Srivastava, 2003; Singh and Srivastava, 2003; Cao *et al.,* 2008; Deng-Xia *et al.,* 2011; Kumar and Srivastava, 2016b, Sharma and Sirvastava 2017).Kang *et al.* (2002) reported that for cotyledon explants of Chinese cabbage, shoot induction was not significantly affected by kanamycin at 1.0mgL^{-1} but the number of shoots formed was significantly reduced at 2.0mgL^{-1} and no shoot were regenerated from any explants at 6.0mgL^{-1} or higher and similar results were obtained in case of hypocotyl explants. Paul *et al.* (2005) reported that hypocotyl explants of cabbage showed inhibition in growth on medium containing 20mgL^{-1} kanamycin. Bhalla and Smith (1998) also reported that exposure of regenerated green shoots to a higher kanamycin concentration on medium containing low sucrose was used to eliminate non-transformed shoots. Bhau and Wakhlu (2001) reported callus of *Coryphantha elephantidens* showed less or no inhibition in callus growth at lower concentration of kanamycin whereas at higher concentration (10, 15, 20 mgL^{-1}) observed inhibited callus growth. In contrast some of the monocotyledons indicate a high level of natural resistance to kanamycin. More than 800mgL^{-1} is required to inhibit growth of cell suspension cultures of several species of Graminae (Hauptman *et al*., 1988). Oz *et al*. (2009) used higher concentration of kanamycin (200 mgL^{-1}) for inhibition of non-transformed tissues of chickpea. Kanwar and Kumar (2011) used higher concentrations of kanamycin (100 mgL^{-1}) for selection of transformed callus/ tissues of *Dianthus caryophyllus* L.

The effect of different concentrations of cefotaxime has been studied separately on the regeneration potential of cabbage. In the present investigations, maximum per cent shoot regeneration was obtained on the best shoot regeneration medium with 300 mgL^{-1} cefotaxime in cotyledon explants. It has been observed that the increase in the concentration of cefotaxime showed no much effect on regeneration potential.Cefotaxime has potential to increase the growth, regeneration and embryogenesis *in vitro*. Cefotaxime promoted growth and morphogenesis in callus cultures of wheat and barley (Mathias and Boyd, 1986; Mathias and Mukasa, 1987). Yepes and Aldwinekle (1994) evaluated the effect of antibiotics on morphogenesis of apple. Similar studies were also carried out by Humara *et al.* (1999) and they observed that 250 µg ml^{-1} cefotaxime enhanced the shoot regeneration capacity. Danilova and Dolgikh (2004) reported stimulatory effect of the antibiotic cefotaxime on plant regeneration in maize tissue culture which enhanced its morphogenesis. The highest increase in the number of regenerated shoots was observed at the antibiotic concentration of 150 mgL^{-1}. Kaur *et al.* (2008) obtained enhanced *in vitro* shoot multiplication and elongation in sugarcane used at the rate of 250 and 500 mgL^{-1} cefotaxime in the medium. In hypocotyl explants, no increase in shoot regeneration potential was observed on medium supplemented with different concentration of cefotaxime. Borrelli *et al.* (1999) reported similar results that cefotaxime did not affect callus growth in durum wheat. Whereas, Ahmad *et al.* (2012) observed that with the increase of cefotaxime concentration the transformation frequency was lowered and most of the explants of *Solanum tuberosum* L. were dead.

CONCLUSION

Selection and identification of transformed cells and tissues are vital steps of genetic transformation which prove to be helpful in improving the selection and transformation efficiency. This study thus reports an efficient antibiotic selection protocol for *Agrobacterium*-mediated cabbage transformation.

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Trend step changes of seasonal and annual precipitation over Kermanshah during a 60-year period using non-parametric methods

Amir Hossein Hashemian,[1,3] Mansour Rezaei,[2,3]* Hajar Kashefi,[3] Meghdad Pirsaheb[1,4] and Hassan Kharajpour[5]

[1]*Research Center for Environmental Determinants of Health (RCEDH), Kermanshah University of Medical Sciences, Kermanshah, Iran*
[2]*Social Development and Health Promotion Research Centre, Kermanshah University of Medical Sciences, Kermanshah, Iran*
[3]*Department of Biostatistics, Faculty of Health, Kermanshah University of Medical Sciences, Kermanshah, Iran*
[4]*Department of Environmental Health, Faculty of Health, Kermanshah University of Medical Sciences, Kermanshah, Iran*
[5]*PhD student of Meteorology, Department of Geography, University of Kharazmi, Tehran, Iran*

ABSTRACT

One of the approaches usedtoinvestigate trend step changes in precipitation isstatistical techniques.The main objective ofthis paper is toidentify and analyze thetrend ofchanges in annual and seasonalprecipitation overthe studied area.The current study employs monthly precipitation data from 1951 to 2010 derived from a weather station in Kermanshah. Using non-parametric Mann-Kendall test and graphic based methods depicting total annual and seasonal (cold and warm seasons) precipitation, the research analyzes trend changes of Kermanshah over different time series. Primarily, data changes in studied seasons and years are analyzed. Then, type and time of these changes are identified. Averages of monthly precipitation overKermanshah during warm and cold seasons are determined as 47.9±33.64 Mm and 169.68±54.56 Mm, respectively. There is no significant trend in annual precipitation over Kermanshah. Analysis of averages precipitation of warm and cold seasons indicates no significant trend; however, warm season seems to follow a decreasing trend in general. Yet, there are leaps in the average levels of both annual and seasonal precipitation. The obtained results show that general trend of change in average precipitation during warm seasons is downward with leaps of average during the studied period. But, in general, there is no significant trend of change in the averages of annual and seasonal precipitation during the studied time.

KEY WORDS: CHANGES OF PRECIPITATION, TREND ANALYSIS, ANNUAL, SEASONAL, KERMANSHAH, NON-PARAMETRIC MANN-KENDALL TEST

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INTRODUCTION

Precipitation is the most important input data in hydrological cycle which needs to be considered mostly in runoff, drought, groundwater, flood and sediment studies. Now a day, global warming, caused by increasing greenhouse gases, and its effect on climate change is a scientific fact accepted by many researchers. Almost all processes in the biosphere are affected by climate change and the effect of this phenomenon on the environment and water resources is a matter of great concern. In order to be prepared against adverse effects of climate change and to reduce its resulting damages, it is necessary to study common trends of change in weather variables in each area so as to adopt proper policies and plans for development and management ofwater resources Katirai et al., (2007) Aziz and Burn (2006) and Chen et al., (2007).

To detect trends of weather variables in different time intervals various test may be used which can be divided into two groups: parametric and non-parametric tests. Parametric tests have more trend analysis potentials than non-parametric tests and require random (independent) data with normal distribution. On the other side, non-parametric tests are consistent with random data and are not sensitive to normal distribution. Mann-Kendall and Spearman are examples of these tests used in trend analysis of weather variables.

In general, trend analysis of climate change, changes in precipitation trend in particular, is among issues that have been considered by researchers of climate and hydrology science, in recent years. Regardless of climate status of a region (wet or dry), precipitation trend analysis of a region may aid executives and managers associated with water issue to make better decisions about implementation of future development projects. Considering that large parts of Iran is located in belt of arid and semi-arid regions of earth , on one hand, and important role of precipitation in supplying water resources of the country, on the other hand, has put more emphasis on gaining greater awareness of trends of precipitation over Iran. Broad investigations have been carried out to identify the process of precipitation over the whole world and Iran. With respect to the significant impact of precipitation on climate system numerous studies have been conducted, including the studies of Matyasovszky et al. 1993, Angel and Huff (1997) Keily et al (1998) Gellens (2000) Piccarreta et al. (2004) Xu et al. (2003) Turgay and Ercan (2005).

All of these studies, trend analysis of precipitation intervals is carried out using non-parametric tests. Trend analysis of precipitation in different time intervals using parametric and non-parametric methods has attracted the attention of many domestic researches, as well. Kamali (11) investigated precipitation trend of different stations during statistical period from 1986-1996 and found that precipitation trend was both increscent and decrescent depending on the region. He indicated that increscent trend has been more frequent Iran than decrescent trend.

Javeri investigated temporal changes in temperature and precipitation over Iran using statistical tests with fixed and variable model and proved that the variation is significant and these changes appear in the form of random displacements, changes in trend, seasonal fluctuations, and periodical changes. Accordingly, in term of temporal changes in temperature and precipitation, Iran is divided to five different zones. In this study, to measure seasonal and annual trend of precipitation data, two non-parametric tests, Mann-Kendall test and Sen's Estimator, are used and the results are compared. Proving the significance of precipitation trend in a given time interval cannot be decisive evidence on climate changes in a region on its own, however, it strengthens such a hypothesis. It is caused bymultiplicity offactors controlling the climate system (KamaliGh 1996., Javeri 2003 Serrano et al., (1999)

Kermanshah Province, situated in western Iran, spreads over an area of 25,000 km2 (9,560 square miles, roughly the size of Vermont), or 1.5 percent of the total area of the country (Fig.1). It lies between latitudinal 45.5° and 48° E, longitudinal 33.7° and 35.3° N .The province is bound on the south by Ilām Province, on the southeast by Lorestān Province, on the east by Hamad ān Province, on the north by Kordestān Province, and on the west by Iraq, with about 250 km of international borderline. The capital city of this province is Kermanshah (Ahmadi et al 2010).

The province is bound on the south by Ilām Province, on the southeast by Lorestān Province, on the east by Hamadān Province, on the north by Kordestān Province, and on the west by Iraq, with about 250 km of international borderline. Considering the geographical location of Kermanshah, studies of climate change during the past decades and identifying that it follows a trend or not, with respect to recent droughts and growing population, may affect making proper policies to deal with drought and proper consumption. Such a study has not been done in this way in the metropolis of Kermanshah, so far.

MATERIAL AND METHODS

To examine the trend of change in precipitation of Kermanshah and find a proper model for it, monthly precipitation data of synoptic meteorological station (mm) in a 60-year period (1951-2010) are derived from Mete-

FIGURE 1. Map of geographical location of Kermanshah

orological Organization of Iran. Seasonal precipitation is a collection of monthly rainfall and annual precipitation is a collection of seasonal rainfall. The obtained data are restored using correlation method and regression model. Data homogeneity is evaluated using Run test so as to be sure homogeneity of data in a 60-year period. In the first place, this test is employed to indicate that time series are non-parametric. In doing so, statistical series are arranged in ascending order. In this test, having no defined trend indicates that data are random. If we find a trend, data are not random. To show that data are random, the following test was carried out as per Mitchell et al (1996):

$$T = \left[\frac{4p}{n(n-1)}\right] - 1$$

Where T is Kendall's statistic, n is total statistical years, and P is total number of ratings bigger than $n_i$placed under it and can be determined through the following relation:

$$P = \sum_{i=1}^{n} n_i$$

The following equation tests the significance of T:

$$(T)_t = \pm t_g \sqrt{\frac{4n+10}{9n(n-1)}}$$

Where t_g is critical value of normal standard (z) with the test probability level which is 1.96 at the confidence level of 95%. If $-T, < T < +T,$ series are random with no trend. $T < -T_t$ indicates a downtrend and if $T < +T_t$ a rising trend is governs the time series (16). If n represents a 60-year period, the obtained values would be ± 0.089.

To determine the trend direction, type, and time, graphic Kendall test has to be carried out. In calculating thestatistics using graphical sequential mann-kendalltest for detection of change time, in two phases of beginning to end, and vice versa, plotted in one graph, change point appears well. Detailed (short term) procedures, change in position, or starting point of the series are examined using time series graphs of u(t) and u′(t). If you graph u and u′ sequences indexed by i, when the trend is significant, the two lines intersect, outside the range of±1.96, at the starting point and move in opposite direction. This intersection point is referred to as a leap. While, if there was no trend, the two sequences (u and u′) would move on roughly in a parallel direction or intersect each other in a several points in a way that result in no change in direction. U graph is plotted based on year and u′ is defined to show its significance of leap point. Where -1.96<u<1.96, series are random and no certain trend can be defined. But, u>1.96 and u<-1.96 indicate existence of a positive and a negative trend, respectively. This study considers a two-dimensional data matrix (12×60: 60 = studies years, 12=number of months).

If data series indicate a certain trend, the actual slope (change rate per unit of time), can be obtained using a simple non-parametric methodof Sen's slope estimator. First, obtain slope of each pair of consecutive data series using the following equation:

$$Q_i = \frac{(x_j - x_k)}{j-k} \quad \text{for} \quad i=1,2,\ldots,n$$

Where, X_j and X_k are data values in time j and k, respectively, which differ one unit of time, Q_i is the median value, and n is slope of line estimated by Sen's slope estimator. Sen's slope estimatoris obtained through the following equation:

$$Q_{med} = Q_{(n+2)/2}$$

Where n is an even number, Sen's slope estimatoris achieved as:

$$Q_{med} = (Q_{n/2} + Q_{(n+2)/2}) / 2$$

If you measure Q_{med} with mutual test at the confidence level of 100(1-α)%, it is possible to obtain the actual value of the line slope. Considering zero between two derived slopes, no trend can be attributed to the time series with this confidence level. Otherwise, significant trend of time series, at the considered level of confidence, is proved. Total monthly precipitation data in 1996, from January to May, is not available so average precipitation value of other months are used. Months of the year are divided into two groups, warm and cold months, and trends in each group are examined and compared separately.

RESULTS AND DISCUSSION

Average precipitation over Kermanshah per month (±Standard Deviation) is 36.18 mm (±42.52) and the highest amount of precipitation per month during the statistical period (60 years) is 494.8 mm recorded in 1974. The least amount of precipitation is zero and recorded during summer. Mann- Kendall test results for total annual precipitation are calculated and drawn. During the studied period, no trend is detected in average precipitation over Kermanshah weather station within the significant levels of the test since u and u′, at no time interval, intersect outside the meaningful range±1.96 (Chart 1). From 1968 to 1975 the graph falls above 1.96; changes which indicate leapsin total annual precipitation during these years.

Distribution of annual precipitation during the studied years is drawn. The least amount of annual precipitation is 215.8 recorded in 1995 and the highest amounts of precipitation is 785.5 recorded in 1996 and 783.9 recorded in 1957 (Chart 2).

According to the results of Mann-Kendall diagram, no significant trend is detected in annual precipitation data recorded during the studied period (60 years).Sen's slope estimator confirms the obtained results. The value of statistic in Z is 0.03. With respect to obtained values for the highest (1.67) and lowest (-2.26) amount of *Q*, with confidence level of 95%, it may be concluded that null hypothesis of this test is confirmed and no trend in detected in the precipitation data recorded during the 60-year period. In Sen's slope estimator test, null hypothesis is: there is no trend is the studied period. Considering the obtained P-value (>0.05) and available

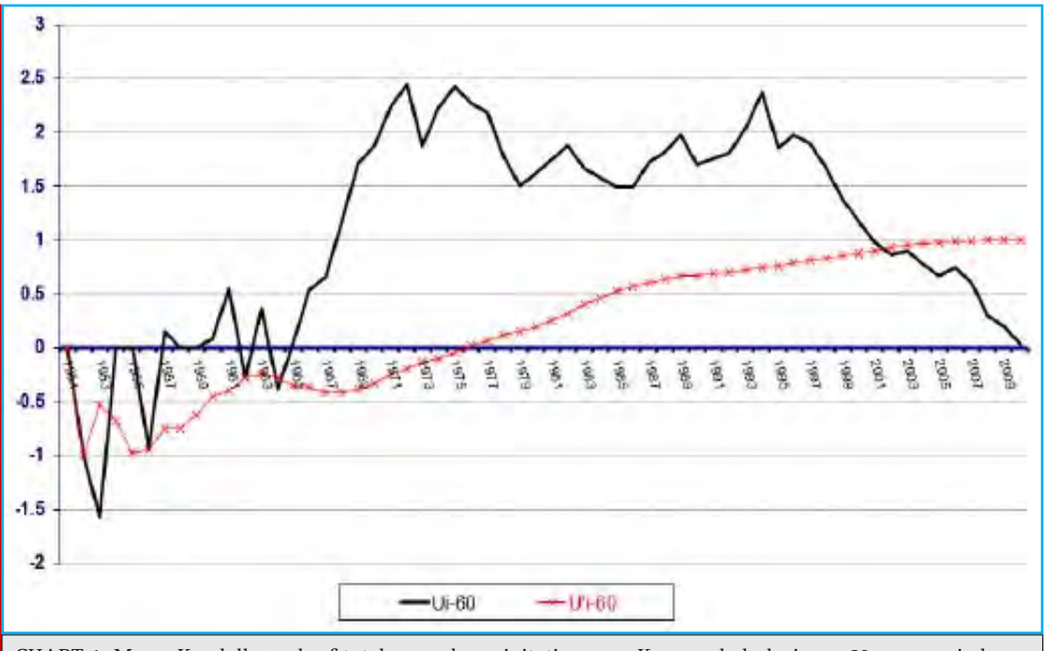

CHART 1. Mann-Kendall graph of total annual precipitation over Kermanshah during a 60-year period

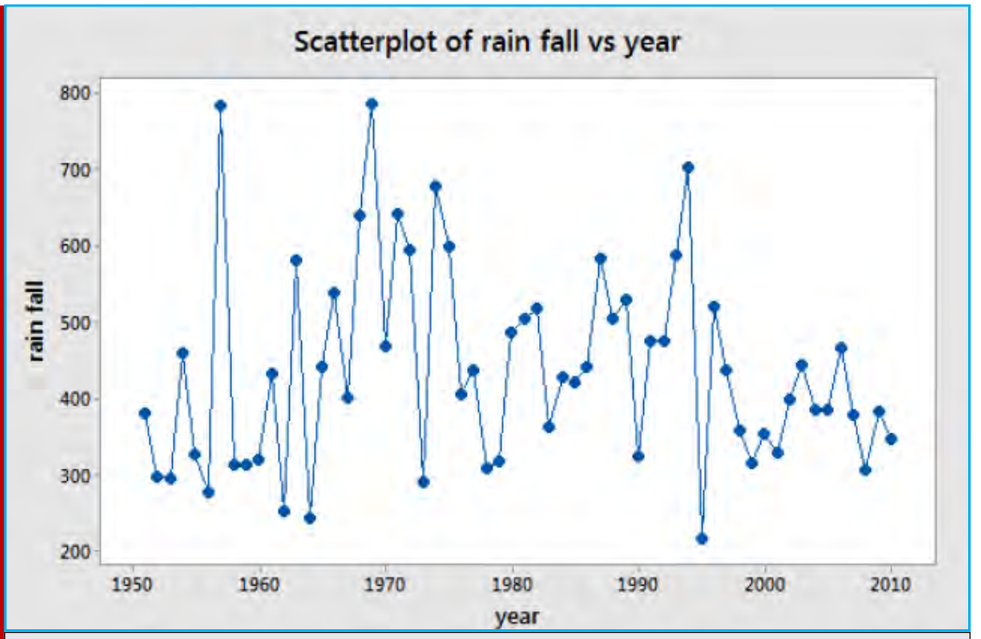

CHART 2. Distribution of total annual precipitation over Kermanshah

data, the assumption of existence of a trend in the studied period is rejected. In other words, no trend can be attributed to existing data. With regard to the highest and lowest slope values, zero depends on the interval between these two values. Therefore, null hypothesis of the test is confirmed based on this confidence interval (Table 1).

Average precipitation over Kermanshah per month (± standard deviation) is 47.9 Mm (±33.64) and the highest amount of precipitation per month during the statistical period (60 years) is 297.6 mm recorded in spring of 1963. Average monthly precipitation over Kermanshah during cold seasons is 169.68 mm (±54.56) and the highest amount of monthly precipitation during cold seasons is 494.8 mm recorded in winter of 1947. The least amount of precipitation is zero and recorded mostly in summers.

Mann – Kendall test results of average monthly precipitation are analyzed and drawn for both warm and cold seasons. During warm seasons of the study period, the average amount of precipitation over Kermanshah weather station indicates no statistically significant trend in the significant range; however, from 1951 to 1968, a rising trend is detected with sharp leaps and falls and positive phase of change is witnessed in precipitation. In 1969, a leap from average is occurredand data are considerably increased. Again, in 1977 the precipitation goes back to normal phase. Since 1978 precipitation level follows a decreasing trend which again has its own ups and downs and does not go beyond the significant level (Chart 3).

Diagram of distribution of average monthly precipitation over Kermanshah during warm seasons of a 60-year period is drawn. The lowest and highest averages of monthly precipitation during warm seasons are 0 and 148.8, respectively, recorded in 1963 (Chart 4).

According to the results of Mann-Kendall diagram for warm seasons, no significant trend is detected in monthly precipitation data recorded during the studied period (60 years).Sen's slope estimator confirms the obtained results. The value of statistic in Z is 0.69. With respect to obtained values for the highest (1.11) and lowest (-63.1) amount of Q, with confidence level of 95%, it may be concluded that null hypothesis of this test is confirmed and no trend in detected in the precipitation data recorded during the 60-year period (Table 2).

Considering the obtained P-value (>0.05) and available data, the assumption of existence of a trend in the studied period is accepted. In other words, no trend can

Table 1. Results of Sen's slope estimator test for total annual precipitation over Kermanshah

variable	Time interval	Number of years	Z statistic	p-value	Qmax	Qmin
Amount of precipitation	1951-2010	60	0.03	0.488	1.67	-2.26

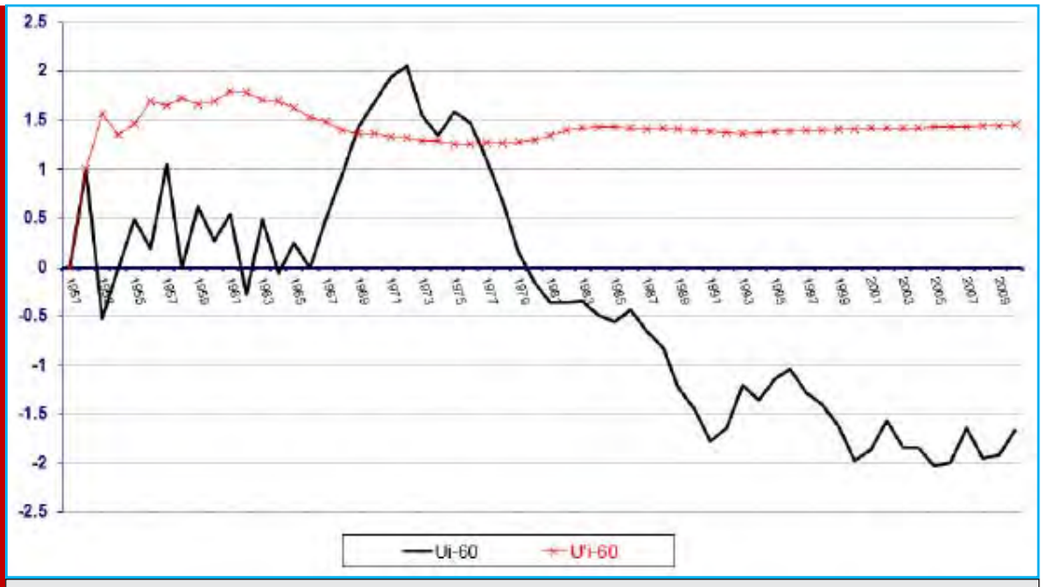

CHART 3. Mann – Kendall diagram of average monthly precipitation over Kermanshah during warm seasons of a 60-year period

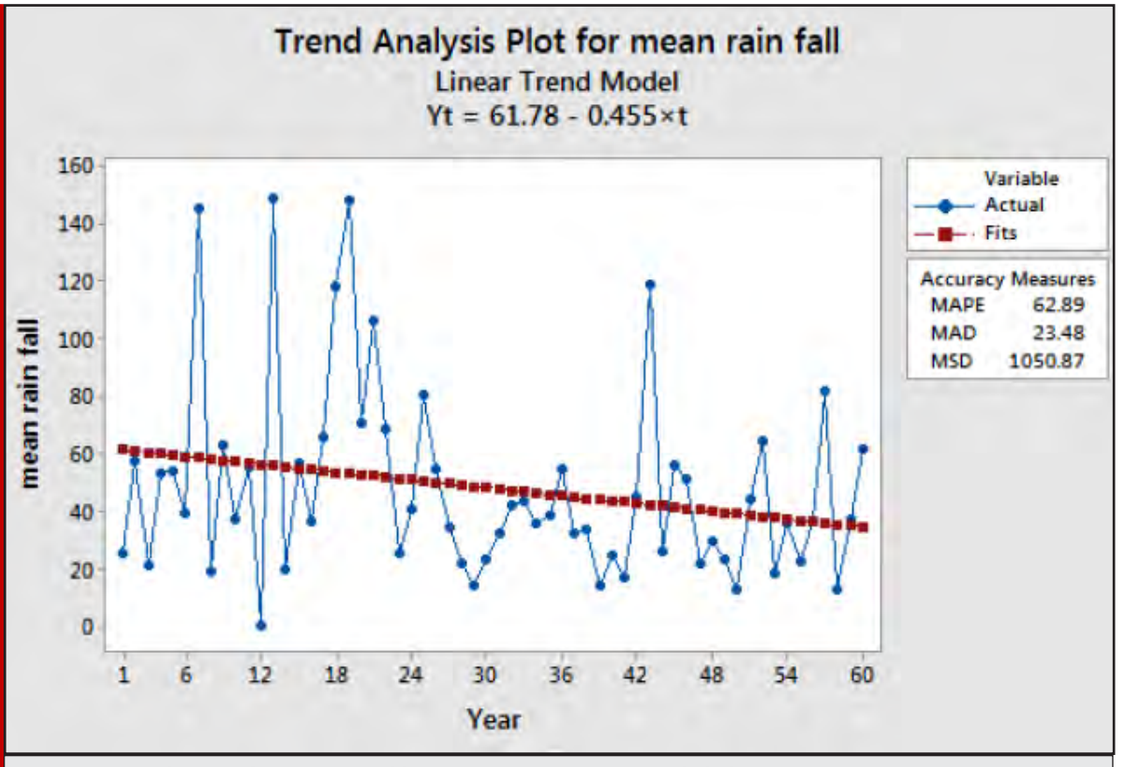

CHART 4. Distribution of average monthly precipitation over Kermanshah during warm seasons

Table 2. Results of Sen's slope estimator test for total monthly precipitation over Kermanshah

variable	Time interval	Number of years	Z statistic	p-value	Qmax	Qmin
Amount of precipitation	1951-2010	60	1.56	0.059	0.06	-0.712

be attributed to existing data. With regard to the highest and lowest slope values, zero depends on the interval between these two values. Therefore, null hypothesis of the test is confirmed based on this confidence interval (table 2). Although, with regard to Sen and Mann-Kendall statistical indicators, no significant trend is detected in average monthly precipitation during warm seasons, the general trend of time series is decrescent (Chart 4). The linear equation of times series obtained by trend analysis test is $y_t = 61.78 - 0.455 \times t$ (t is the time difference since the beginning of time series). The linear equation obtained by Sen's slope estimator is below:

$$f(year) = -0.297 \times (year - first\ Data\ Year) + 50.2 .$$

Forecasts of the two employed tests for the average precipitation in warm seasons are very close and similar.

To examine existence of trend in precipitation during cold seasons, Mann-Kendall test and Sen's slope estimator are used, the results of which are as follows. During cold seasons of the studied period, no trend is detected in average precipitation over Kermanshah weather station within the significant levels of the test. In 1967, a leap from average is occurred and this sudden change continues in an upward positive direction until 1995. Then, until the end of the study period, precipitation follows a downward trend (Chart 5).

The lowest and highest averages of monthly precipitation during cold seasons are 51.6 and 325.35, respectively (Chart 6).

According to the results of Mann-Kendall diagram, no significant trend is detected in annual precipitation data recorded during the studied period (60 years).Sen's slope estimator confirms the obtained results. The value of statistic in Z is 0.69. With respect to obtained values for the highest (1.11) and lowest (-0.63) amount of *Q*, with confidence level of 95%, it may be concluded that null hypothesis of this test is confirmed and no trend in detected in the precipitation data recorded during the 60-year period (table 3).

Considering the obtained P-value (>0.05) and available data, the assumption of existence of a trend in the studied period is accepted. In other words, no trend can be attributed to existing data. With regard to the highest and lowest slope values, zero depends on the interval between these two values. Therefore, null hypothesis of the test is confirmed based on this confidence interval (Table 3).

Generally, a comparison between average precipitations during warm and cold seasons indicate that

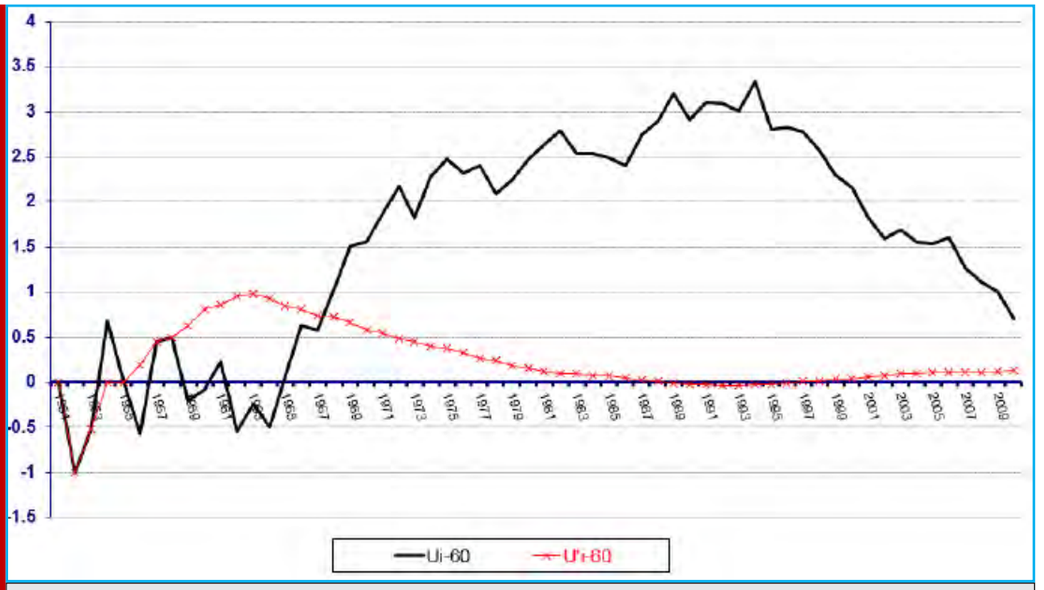

CHART 5. Mann – Kendall diagram for average precipitation over Kermanshahduring cold seasons of a 60-year period

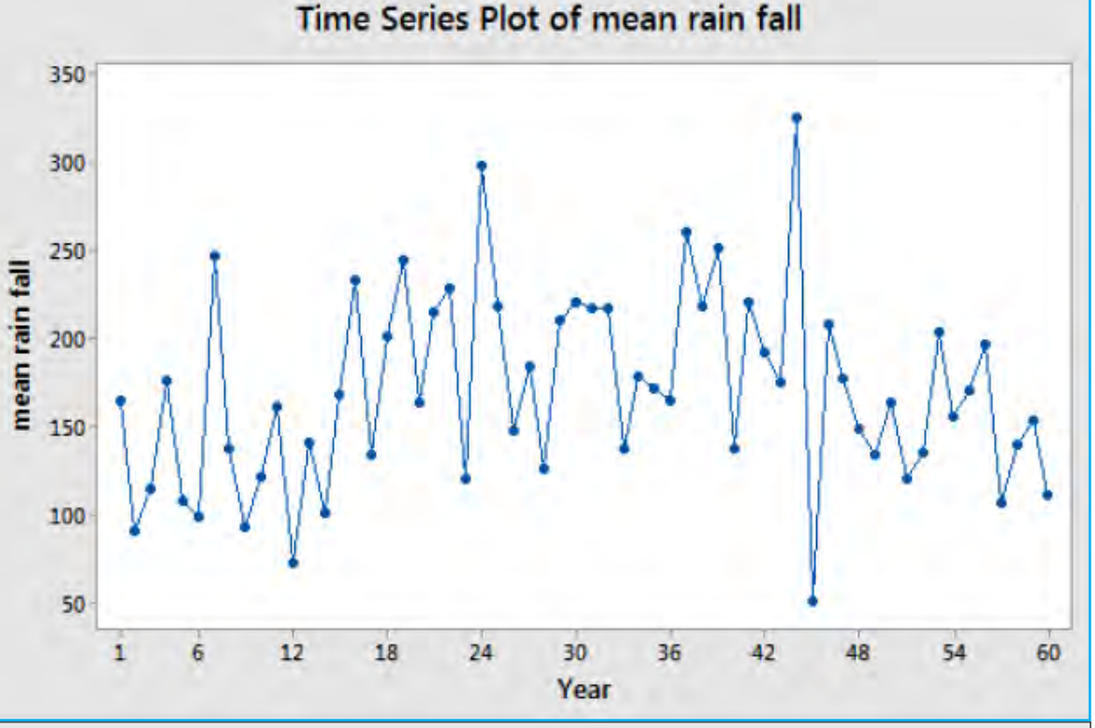

CHART 6. Distribution of average monthly precipitation over Kermanshah during cold seasons

changes in warm season starts a downward trend since 1970. But an upward trend of precipitation in cold seasons changes to a downward direction since 1993. In the beginning years of the study period, changes in average monthly precipitation during warm seasons is more than cold seasons; however, the more we approach the end of the study period, the less evident are these ups and downs.

1. Statistical studies of precipitation have been largely considered since 1980. Statistical studies of Kane and Trivedi (1988), Karl (1988)and Katsoulis and Kambetzldis (1989) are among these. When compared with Iran with annual precipitation of about 260 mm, Kermanshah, with average annual precipitation of 434 mm, is regarded as an area with high rate of precipitation. Trend step changes in precipitation are evident in different stations.

But in most stations, the trend of change seems not be statistically significant. Average monthly precipitation over Kermanshah during warm and cold seasons indicates no significant trend of change. The results of this study are different from the findings of the study carried out by Jahanbakhsh et al. in which they measured changes in precipitation and temperature of Karkha region. This difference may be due to differences in geographical location of the two regions (19). But, Hejam et al. research is consistent with the current study. They examined trend of changes in seasonal and annual precipitations over several meteorological stations and because of lack of trended and un-trended series they could not attribute a certain trend to seasonal and annual precipitations of the studied region, (Jahanbakhsh et al., (2010) Negaresh et al (2012) .

Table 3. Results of Sen's slope estimator test for average precipitation over Kermanshah during cold seasons

variable	Time interval	Number of years	Z statistic	p-value	Q min	Q max
Average precipitation	1951-2010	60	0.69	0.246	-0.63	1.11

Investigation seasonal (warm and cold) trend of precipitation indicated that average precipitation during warm seasons of the 60-year period is generally downward; however, this trend is not significant at 0.05. The results of this study are consistent with the results obtained from the study of Alijani et al. (2012). During summer, cloud formation and precipitation is not possible over a wide range of Iran. Therefore, this season is the driest season. In the 60-year period, because of lack of precipitation during summer, changes in average precipitation during warm seasons was similar to springs which was different to that of Katirai et al report. They found a decreasing trend for spring precipitation in Iran. Although, they examined precipitation trend of the whole country of Iran from 1960 to 2001 but we only studied Kermanshah with more detail and during a longer period. Negaresh et al. (2012), in a statistical investigation of changes in precipitation over Saqqez, found similar results and decreasing trend for precipitation during summer. Although we found no significant trend for precipitation during warm seasons, direction of changes in precipitation trend was downward.

The current study found no trend for precipitation during cold seasons and in this regard our findings are consistent with Alijani et al 2012 . studies of precipitation during cold seasons indicated no trend, either, and these findings are consistent with the study of Negaresh et al. (2012). In case of finding a trend, precipitation forecast for coming years would be possible.

CONCLUSION

In general, annual precipitation over Kermanshah, during a 60-year period, from 1951 to 2010, follows no specific (downward or upward) trend. General changes in trend of precipitation during warm seasons, although non-significant, follow downward directions. Also, some leaps of change are witnessed in annual and seasonal trend of precipitation.

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Impact of integrated nutrient management on growth and fruit physical attributes in Cape gooseberry, *Physalis peruviana*

Niharika Verma, Deepa H Dwivedi, Sachin Kishor and Namrta Singh

Department of Applied Plant Science (Horticulture), Babasaheb Bhimrao Ambedkar University, Vidya Vihar Rae Bareli Road Lucknow- 226025, U.P., India

ABSTRACT

Cape gooseberry (*Physalis peruviana* L.) commonly known as Rasbhari is an important minor tropical Solanaceae fruit crop of India. An experiment was conducted at Horticulture Research Farm, Babasaheb Bhimrao Ambedkar University, Lucknow during winter season 2016-2017, to study the impact of integrated nutrient management on growth and physical attributes of fruits in Cape gooseberry. The experiment comprised six treatments T1 (control)), T2 ((NPK 100 % RDF), T3 (FYM 100%) T4 (vermicompost 100%), T5 (50% NPK + 50% FYM) and T6 (50% NPK + 50% vermicompost) and was laid out in randomized block design with three replications. The observation revealed that the application of 50% vermincompost+50% NPK (T6) was better for improvement of plant height (34.55 cm), stem diameter (8.4 cm), number of leaves per plant (49.5), number of branches per plant (13.5), leaf length (8.7 cm) and leaf width (6.5 cm) along with the fruit physical attributes i.e. fruit weight with husk (7.0 g), fruit weight without husk (6.7 g), fruit size (3.2 cm equatorial diameter), fruit size (3.1 cm polar diameter), fruit volume (6.9 ml) and fruit specific gravity (1.2) which were found to be increased with the treatment.

KEY WORDS: CAPE GOOSEBERRY, RASBHARI, FYM, NPK AND VERMICOMPOST

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**Corresponding Author: deepahansraj1999@gmail.com*
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INTRODUCTION

Cape gooseberry (*Physalis peruviana* L.) of family Solanaceae is an important minor tropical fruit crop of India. It is commonly known as Rasbhari, resembles tomato in shape (Girapu and Kumar, 2006) and is native to the Andes region of South America. It is used in making jam, sauce, pies, puddings, chutneys and ice cream and is eaten fresh in fruit salads and cocktails. It is an excellent source of Vitamin A and C among other nutrients (Chaves *et al.*, 2005). However, its cultivation is restricted to a limited area in India due to low production potential, poorly developed package of practices, etc. Thus, fertilizer application plays an important role in harnessing optimum and good quality fruits in Cape gooseberry. Although chemical fertilizers, particularly nitrogenous and phosphatic fertilizers, contribute a lot in fulfilling the nutrient requirement of plants but continuous use of these fertilizers affects the soil health adversely and deteriorates physicochemical properties of soil. Hence, organic manures may increase soil fertility and thus, the crop production potential possibly by changes in soil physical and chemical properties including nutrient bioavailability, soil structure, water holding capacity, cation exchange capacity, soil pH, microbial community and activity etc. Some organic sources can increase crop yields due to nutrient release during decomposition and mineralization. They may also improve soil physical properties such as moisture retention, bulk density and aeration (Singh *et al.,* 2013, Sharma *et al.,* 2013 and Gond *et al.,* 2017).

Organic materials, besides enhancing P availability, even supply some P. This is because of their high tissue concentration of N compared to other nutrients. The objective of the present study was to assess the impact of integrated nutrient management on growth and fruit physical attributes in Cape gooseberry.

MATERIALS AND METHODS

The experiment was conducted at Horticultural Research Farm, Department of Applied Plant Science, Babasaheb Bhimrao Ambedkar University, Lucknow during November 2016 to May 2017. The soil type of the experimental plot is estimated as being saline having pH 8.2 and low organic carbon (Dwivedi *et al.*, 2012). It is located at 26°50'N latitude and 80°52'E longitudes. The experiment comprised six treatments T_1 (control)), T_2 ((NPK 100 % RDF), T_3 (FYM) 100%) T_4 (vermicompost 100%), T_5 (50% NPK + 50% FYM) and T6 (50% NPK + 50% vermicompost) and was laid out in randomized block design with three replications. Organic manures were incorporated in the experimental plots before transplanting. Standard package of practices for Cape gooseberry (Chattopadhyay, 1996) were followed for the entire crop season. The observation on vegetative and fruit parameters growth was recorded as per standard methods on three plants selected randomly in each treatment. Fruit size (equatorial diameter in cm), fruit size (polar diameter cm), fruit volume (ml) and fruit specific gravity were recorded following the methods described by (Rangna 1986) taking five fruits selected randomly from fruits harvested from three plants from each replication. The observed data were analyzed statistically using analysis of variance as formulated at 5% level of significance (Sahu and Das, 2014).

RESULTS AND DISCUSSION

The plant height and stem diameter increased significantly by application of organic and inorganic fertilizers as compared to control (Table.1). The maximum plant height (34.5 cm), stem diameter (8.4 cm), number of leaves (49.5) per plant, number of branches (13.5) per plant, leaf length (8.7 cm) and leaf width (6.5 cm) were recorded in plants treated with 50% vermicompost and 50% NPK (T_6) followed by 50% NPK+ 50% FYM (T_5). Vermicompost has high microbial activity due to presence of fungi, bacteria and actinomycetes (Tomati, *et al.,* 1988) and these microbes are reported to produce plant growth regulators (PGRs) such as auxins, gibberellins, cytokinins, ethylene and abcisic acid (Frankenberger and Arshad, 1995).

Syntheses of these plant growth hormones are known to regulate growth process which affect plant (Singh and Singh, 2009) *viz,* number of leaves, leaf length, leaf width and leaf area which may be due to the cell division caused by cytokinins. N being a constituent of protein and chlorophyll plays a vital role in photosynthesis. It enhances accumulation of carbohydrates which, in turn, increase growth of plants, while phosphorus is known to promote cell division as well as photosynthetic activity and flowering (Mahmoud and Amara, 2000). Vermicompost and FYM both provide high amount of N (148 kg/ha) as compared to other organic sources (Dwivedi *et al.*, 2015). Higher nutrient availability and increased nitrogen from organic manures influence in mobilization of the nutrients upon addition of the compost which is further enhanced physical, chemical and biological properties of the soil.

The fruit weight with husk (7.0 g), fruit weight without husk (6.7 g), fruit size (equatorial diameter 3.2 cm and polar diameter 3.1 cm), fruit volume (6.9 ml) and fruit specific gravity (1.2) were recorded highest by the application of 50% vermicompost and 50% NPK (T_6) followed by treatment comprising of 50% NPK+ 50% FYM (T_5) as shown in Table 2. Similar results were obtained by (Yadav et *al.,* 2010 and Shukla *et al.,* 2009) in tomato. This increase in fruit size and weight during the present investigation might be due to the increase in photosyn-

Table 1. Improving vegetative growth trough integrated nutrient management in Cape gooseberry

Treatment	Plant height (cm)	Stem diameter (cm)	Number of leaves/plant	Number of branches/plant	Leaf length (cm)	Leaf width (cm)
T_1 (Control)	26.8	5.1	37.5	10.5	6.5	6.5
T_2 (RDF 100%)	28.9	5.4	38.9	12.1	6.9	5.2
T_3 (FYM 100%)	34.5	6.0	40.1	10.9	7.7	5.5
T_4 (Vermicompost 100 %)	32.6	5.6	42.4	10.9	7.4	5.2
T_5 (50% NPK+FYM 50%)	32.2	8.1	48.0	12.8	8.1	6.0
T_6 (50% vermincompost+50% NPK)	34.5	8.4	49.5	13.5	8.7	6.5
SE (m) ±	0.45	0.39	0.65	0.36	0.23	0.22
CD (P=0.05)	1.38	1.19	1.99	1.11	0.72	0.68

Table 2. Improving fruit growth trough integrated nutrient management in Cape gooseberry

Treatment	Fruit weight with husk (g)	Fruit weight without husk (g)	Fruit Size (equatorial diameter in cm)	Fruit Size (polar diameter cm)	Fruit volume (ml)	Fruit specific gravity
T_1 (Control)	4.5	3.9	1.7	1.9	4.4	0.9
T_2 (RDF 100%)	6.3	4.7	2.4	2.0	5.3	1.0
T_3 (FYM 100%)	5.4	4.6	2.9	2.1	5.9	1.1
T_4 (Vermicompost 100 %)	6.6	5.4	2.7	2.4	5.6	1.1
T_5 (50% NPK+FYM 50%)	6.1	5.7	3.0	2.8	6.1	1.1
T_6 (50% vermincompost+50% NPK)	7.0	6.7	3.2	3.1	6.9	1.2
SE (m) ±	0.44	0.42	0.14	0.11	1.40	0.17
CD (P=0.05)	1.34	1.28	0.42	0.35	0.46	0.08

thetic activity of plants fertilized with vermicompost, azotobacter and inorganic fertilizer, which in turn might have favored an increased accumulation of dry matter. Fruit size, weight and berry volume are highly correlated with dry matter content and application of organic and inorganic fertilizers and might have balance the level of hormone and nitrogen fixers known for accumulation of dry matter and their translocation (Kachot *et al.,* 2001). Similar results were obtained by (Yadav et *al.,* 2010 and Shukla *et al.,* 2009) in tomato.

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PCR-based detection of microsporidia in silkworms using non-conventional RNA polymerase primers

Gourab Roy, Kalidas Mandal and G. Ravikumar*

Seri-Biotech Research Laboratory, Central Silk Board, Carmelaram Post, Kodathi, Bangalore, 560 035, India.

ABSTRACT

Microsporidia are obligate intracellular, spore-forming parasites that infect both invertebrates and vertebrates. They infect silkworms causing the deadly pebrine disease leading to heavy crop loss in sericulture. Because of the horizontal and vertical transmittance, outbreaks should be detected at an early stage and persistent infections should also be identified to prevent further transmittance. So far, microscopic examination method remains the conventional detection method for screening of microsporidia in sericulture. Molecular diagnosis tools have an advantage over microscopic detection as they are more specific, sensitive and aid in early detection. Microsporidia detection by PCR method using primers designed from SSU-rRNA is widely used. In this study, we developed a PCR assay for the detection of microsporidia using primers designed from the conserved regions of RNA polymerase gene. Under optimized PCR conditions, the assay yielded a ~650 bp DNA fragment from microsporidia infected silkworms, *Bombyx mori* and *Antheraea mylitta*. Sequence analysis of the amplified products has shown homology to various microsporidia including *Nosema bombycis* and *N. antheraea*. No non-specific products were observed. This method could help in early detection of microsporidia infection at any developmental stage of the silkworm and thereby reducing the crop loss.

KEY WORDS: DETECTION, MICROSPORIDIA, PCR

INTRODUCTION

Silkworm, *Bombyx mori* is one of the most important domesticated insects, which produces luxuriant silk thread in the form of cocoon by consuming mulberry leaves during larval period. In India the bulk of the commercial silk produced is mulberry silk whereas, Eri, Tasar & Muga silk contribute to a lesser extent. These silkworms are susceptible to various diseases resulting in substantial crop loss which is estimated to be 40% in India (Singh et al., 2012). The common pathogens infecting them are microsporidians including *Nosema*

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**Corresponding Author: ravikumarpillai@gmail.com*
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bombycis, nucleopolyhedrovirus (NPV) and densovirus (mainly DNV1&2), infectious flacherie virus (IFV), cytoplasmic polyhedrovirus (CPV) and bacteria. The microsporidians cause pebrine disease; NPV causes grasserie; and DNVs, IFV and bacterial pathogens cause flacherie diseases. Among all, the microsporidians disease is responsible for the significant economic loss in the sericulture industry. Microsporidiasis remained a threat to silk industry since time immemorial, because of its unique and recurrent occurrence and is the only disease transmitted both horizontally and vertically (Bhat et al., 2009). Several species and strains of microsporidia have been isolated from infected silkworms among which pebrine caused by *Nosema bombycis* is the most prevalent. Other microsporidian species (*Vairimorpha, Pleistophora, Thelohania* etc.) which differ in their spore morphology, sites of infection and virulence, have also been isolated from silkworms (Kawarabata, 2003, Gupta et al., 2017).

Since the control of disease is often met with limited success, early detection of pathogens is essential to control of emerging, reemerging, and in preventing the spread of infectious diseases. Microsporidian are easily detected by light microscopy when infections are heavy and spores are present. However, early infections without spores, or light infections with low numbers of spores are easily missed. This limitation has made it difficult to conduct investigations into microsporidian prevalence and transmission. To overcome these difficulties, PCR- based techniques have been developed to detect the major pathogens of silkworms with great specificity and sensitivity (Hatakeyama and Hayasaka 2003, Hamiduzzaman et al., 2010, Ravikumar et al., 2011, Fu et al., 2016).

Due to the availability of sequence information and the presence of conserved and variable sequence regions within the SSU rRNA genes, PCR-based methods have typically used primers of this gene for the detection of microsporidians (Franzen and Muller, 1999). Herein, we report that primers designed from the RNA polymerase of microsporidians can also be used to detect microsporidians from silkworms. To our knowledge, this is the first report on the detection of microspordians using its RNA polymerase primers from silkworms.

MATERIALS AND METHODS

SILKWORM AND MICROSPORIDIAN INFECTION

The silkworm rearing and microsporidian infection were essentially performed as reported by us (Ravikumar et al, 2011). Silkworms, *B. mori* (Pure Mysore) were fed on mulberry leaves. For Microsporidian infection, 3rd instar day 1 larvae were orally fed with 2000 spores/larva and periodical observations were taken. Control larvae did not receive microsporidian infection. On 4th and 8th day post infection (p.i.), larval mid gut tissues were dissected out and used for DNA extraction, followed by PCR. DNA was also extracted from pupa, adult and eggs of infected and normal silkworms.

DNA EXTRACTION

DNA extracted from the mid gut of infected and control using Hi-Pure DNA extraction Kit (Himedia) according to manufacturer's protocol. DNA from mulberry leaves and pebrine infected *A. mylitta* DNA were used as reported earlier (Ravikumar et al., 2011). The DNA was analyzed in 1% agarose gel electrophoresis and quantified using a Nanodrop (Thermo Corporation) spectrophotometer.

PCR AND CLONING

A set of primers were designed from the conserved region of available microsporidian RNA polymerase sequences from NCBI database. The primers used were: Sense: 5'-CCICAYTTYCCIAARGARGAYTA-3' and antisense: 5'-AARGAYITIGARGGIACIAAYGA-3'. (I: deoxyinosine; R: A, G; Y: T, C). PCR reactions were carried out using 1X Taq buffer, 2.5 mM dNTPs, 25 mM MgCl2, 0.5U Taq DNA polymerase (Fermentas) and 100 ng of DNA. The DNA from control silkworms and mulberry DNA were employed as negative controls. PCR reactions were carried out (Eppendorf) using the following cycles: 94°C for 2 min, 30 cycles of 94°C for 40 s, 48°C for 30 s, and 72°C for 30s and 1 cycle of 72° C for 5 min. PCR products were analyzed in 1% agarose gel electrophoresis, stained in Sybergreen (HiMedia) and visualized under UV transillumination. The PCR products were cloned in pJET blunt end cloning vector (Fermentas) and positive clones were confirmed by colony PCR. Purified plasmids were sequenced at Eurofins, Bangalore, followed by BLAST analysis (NCBI).

RESULTS AND DISCUSSION

Results are presented in Figure 1. PCR amplifications have resulted in discrete and desired product. DNA extracted from the microsporidian infected silkworm yielded specific amplification products of ~ 650 bp (Lane 1-4) using RNA polymerase primers. No non- specific products were observed. The negative controls; DNA from normal silkworm and the plant DNA from mulberry showed no PCR products, indicating the specificity of the PCR. The banding intensity on day 8th was higher to that of on 4th day showing the proliferation of pathogen at an advanced stage of infection. Further confirmation of the PCR products was done by sequencing and BLAST analysis. BLAST showed 92- 99 % homology to RNA polymerases of various isolates of

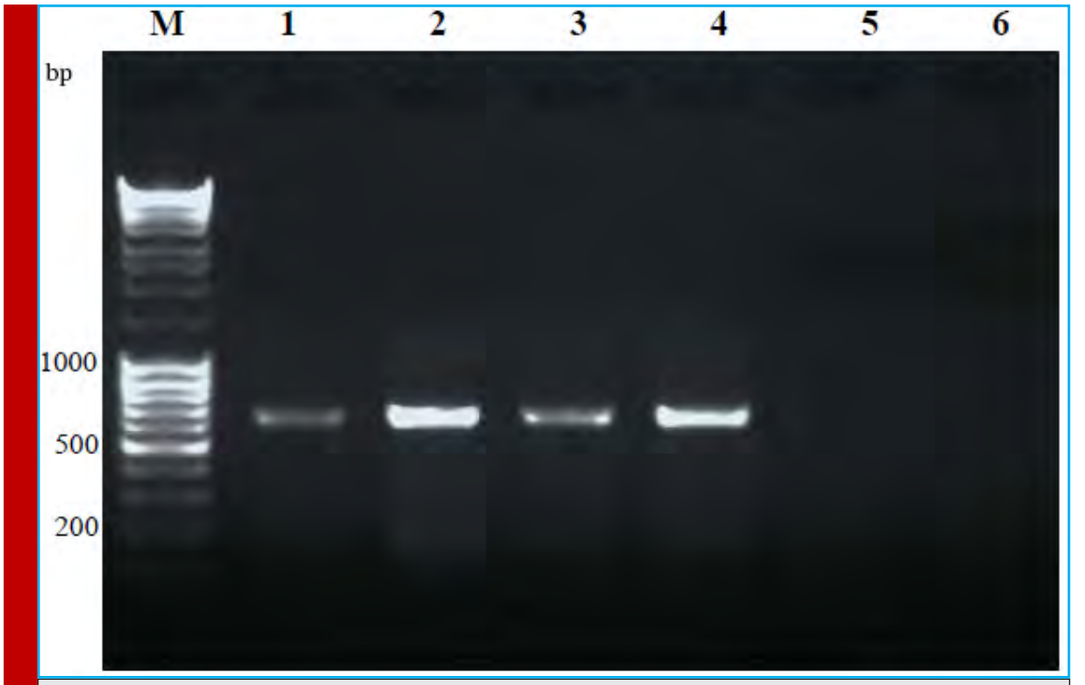

FIGURE 1. PCR amplification of microsporidia DNA (~650bp). M: Molecular weight marker, Lane 1&t2: DNA from microsporidia infected *B. mori* larvae, day 4 and 8 p.i., respectively; Lane 3&t4: DNA from microsporidia infected *A. mylitta* larvae day 4 and 8 p.i. Lane 5&t6: DNA from uninfected *B. mori* and mulberry leaves, respectively, were used as controls.

N. bombycis, *Nosema* Sp, *N. antheraea*, *N. ceranae*, *N. pernyi* and other microsporidia. The same results were obtained from other developmental stages, pupa, adult and eggs (data not shown) of the silkworm. In addition, the same primer sets could detect microsporidian of tasar silkworm *A. mylitta* showing the efficacy of the RNA polymerase-based primers in detecting microsporidian of other silkworm species than *B. mori*. The conserved regions of RNA polymerase gene was effectively utilized for the detection of microsporidia in the present work and it can be used for detection of microsporidia of other insects/organisms also. Highly conserved SSU-r RNA gene primers were successfully used for the detection and classification of microsporidia across organisms with high specificity and sensitivity (Weiss and Vossbrinck, 1999; Jehle et al., 2006, Ravikumar et al., 2011). PCR diagnosis of *N. pernyi* using SSU-rRNA primers provided increased specificity and sensitivity when compared with light microscopy in *Antheraea pernyi* (Jiang et al., 2011). For the effective control of pebrine disease, outbreaks should be detected at an early stage and persistent infections should also be identified to prevent further transmittance of the disease.

In our study, microsporidia were detected by PCR at 4th day of p.i, whereas the spores were visible under microscope only on day 8 and afterwards. Hence, this method can be useful in the early detection of microsporidia which is critical in reducing crop loss in sericulture. Further, real-time quantitative PCR assay can be used with RNA polymerase primers for increased sensitivity. The results of this study suggest that RNA polymerase primers from microsporidia can be used for pebrine detection in sericulture. To the best of our knowledge, this is the first study in which PCR was used for the successful detection of microsporidia using RNA polymerase primers in silkworms.

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Genome-wide comparative analysis of the codon usage pattern in *Flaviviridae* family

Anjusha Mune[1], Ajay Pandey[2] and Khushhali Menaria Pandey[1]*

[1]*Department of Biological Science and Engineering, MANIT Bhopal (M.P.) India*
[2]*Department of Applied Mechanics, MANIT Bhopal (M.P.) India*

ABSTRACT

Flaviviridae family is a group of viruses that cause several deadly diseases like Dengue Fever Virus, Zika Virus, Japanese Encephalitis Virus, and Hepatitis C Virus etc. The codon usage analysis can serve as a tool to understand about the molecular evolution and regulation of viral gene expression. The objective of this study is to find the key determinants of codon usage in the family. In this study, the codon usage pattern for 114 genomes of *Flaviviridae* family (with four genus *Flavivirus, Hepacivirus*, Pegivirus, *Pestivirus*) was analysed through codon usage indices (like NC, RSCU, ENC, PCA) and multivariate statistical methods. Our results show that among the four genus *Flavivirus* and *Pestivirus* show similarity in preferred base on the count of being AG rich. On the other hand, *Pegivirus* and *Hepacivirus* show similarity in preferred base on the count of being GC rich. The overall codon usage bias in the entire family is slightly biased. RSCU analysis showed that *Flavivirus* and *Pestivirus* prefer AG ending codons, whereas *Pegivirus* and *Hepacivirus* show preference to GC ending codons. Many unclassified members show similarity with members of genus *Flavivirus* in choices of codon. The ENC -GC3 plot show that mutation pressure is dominating evolutionary driving force in making codon usage preferences. The study represents comprehensive analysis of codon usage pattern and help to better understand the mechanism of codon usage bias.

KEY WORDS: EFFECTIVE NUMBER OF CODON, MUTATION PRESSURE, NUCLEOTIDE CONTENTS, PRINCIPAL COMPONENT ANALYSIS, RELATIVE SYNONYMOUS CODON USAGE

INTRODUCTION

Flaviviridae family is composed of fast evolving RNA viruses. The members of the family are positive single stranded RNA viruses that are causative agents for number of neglected tropical diseases in humans and animals. *Flaviviridae* family is mainly classified into four genera: *Flavivirus, Hepacivirus, Pegivirus* and *Pestivirus* (Lobo *et al.*, 2009; Lu *et al.*, 2017). The viral genomes vary from 9 to 13 Kb and contain a single known ORF

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**Corresponding Author: kmpbiomanit@gmail.com*
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that codes for a polyprotein, which is processed co- and post-translationally by host and viral proteases into at least 10 functional, individual polypeptides (Blitvich and Firth, 2015; Brand *et al*., 2017).

Flavivirus genus comprises arthropod born viruses (arboviruses) which are transmitted to host by vectors (mosquitoes or ticks) via blood feeding. Birds and mammals are usual primary hosts. Members of this genus are further classified into groups on the basis of vector. They are mosquito born, tick born, and non-known arthropod vector (NKV) (Velazquez-Salinas *et al*., 2016). *Flavivirus* includes viruses which cause several diseases including: Dengue fever, Japanese encephalitis, Murray valley encephalitis, West Nile fever, Zika fever. These viruses are distributed worldwide but individual species are restricted to particular epidemic areas (Moosavi *et al*., 2011; Huang *et al*., 2014; Zhang *et al*., 2017).

Hepacivirus are the group of viruses mainly transmitted by blood contact in mammals (horses, rodents, bats, cows and primates); its best species being Hepatitis C virus. Genus *Pestivirus* infect mammal's members of family *Bovidae* (cattle, sheep, and goats) and *suidae* family (various species of swine). Compared to the other viruses in the family *Pestivirus* encode two unique gene products, namely $N^{pro}$and E^{rns}. These unique proteins are involved in repression of the host type I IFN response. The genus *Pegivirus* commonly causes persistent infection in a broad range of mammals (humans, non-human primates, pigs, horses and a range of rodent and bat species).Less information is available on transmission of these viruses in different host species (Theze *et al*., 2015; Zhou *et al.,* 2012; Tautz *et al.,* 2015).

Many new viruses have been documented but their relationship with other virus, mode of transmission and vector association is not clear, hence they are not assigned under any genus and we consider them unclassified. The genetic code comprises 64 codons that can be divided into 20 groups. Each group corresponds to each of the standard amino acids and consists of one to six codons (Butt *et al*., 2013; Chen, 2013; Gu *et al*., 2003). Alternative codons within the same group coding for the same amino acid are often termed 'synonymous' codons. Most amino acids can be translated by more than one codon. This redundancy is an important factor that provides accuracy in production of protein. These synonymous codons are not used randomly. There are some codons that are used more often than other codons. This phenomenon is referred to as codon usage bias (Tao *et al*., 2009; Moratorio *et al*., 2013; Wang *et al*., 2016; Van *et al*., 2016).

Studies on codon usage have determined several factors that could influence codon usage pattern, including mutational pressure, natural or translational selection, secondary protein structure, replication and selective transcription, hydrophobicity of the protein, and the external environment. Among these, the major factors responsible for codon usage variation among different organism are considered to be compositional constraints under mutation pressure and natural selection. Numbers of previous studies on codon usage of different viruses have highlighted mutation pressure as the major factor in shaping codon usage patterns compared with natural selection. But with increasing understanding of codon usage it appears that although mutational pressure is still a dominating force, it is certainly not the only one when different viruses are considered (Sharp *et al.,* 1988; Cristina *et al.,* 2015; Xiang *et al.,*2015, Butt *et al.,* 2016).

Analysis of codon usage patterns of *Flaviviridae* would not only provide a base for better understanding of biased usage of synonymous codons, the evolution and pathogenesis of *Flaviviridae*, but also improve our understanding of the regulation of viral genes expression and aid vaccine design, where the efficient expression of viral protein may be required to generate immunity. In order to gain insight into these matters, we have analysed codon usage and base composition of the 114 species of *Flaviviridae* family. The patterns of preferred codons for each individual amino acid in each species were identified.

MATERIALS AND METHODS

Total 114 complete genome sequences of viruses of *Flaviviridae* family were downloaded from the National Centre for Biotechnology (NCBI) database (http://www.ncbi.nlm.nih.gov) in FASTA format. The accession numbers and other detailed information of the selected genomes were listed in [supplementary material Table 1]. Open reading frames (ORF) of all the genomic sequences were identified by using NCBI ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/).

In order to understand the frequencies of occurrence of each nucleotide in ORFs, composition analysis was conducted. The overall frequency of occurrence of the nucleotides (A %, C %, U %, and G %) was calculated along with the frequency of each nucleotide at the third site of the synonymous codons (A_3, C_3, U_3 and G_3). Also the overall GC, AU and GC_3 content were calculated to investigate the compositional properties. The codons AUG and UGG are the only codons for Met and Trp, respectively, and the termination codon UAA, UAG, and UGA do not encode any amino acids. Therefore, these five codons are excluded from the analysis.

The ENC is a very effective estimator to measure the magnitude of codon usage bias in the coding sequences of members of *Flaviviridae* family. The ENC value ranges from 20 (when only one synonymous codon is chosen

by the corresponding amino acid) to 61 (when all synonymous codons are used equally) (Lu *et al*., 2005). In an extremely biased gene where only one codon is used for each amino acid, this value would be 20; if all codons are used equally, it would be 61; and if the value of ENC is greater than 40, the codon usage bias is regarded as low (Wright F, 1990). We calculated ENC to measure the degree of departure from equal use of synonymous codons of ORF of members of *Flaviviridae* family. The values of ENC were obtained by EMBOSS CHIPS program. These ENC values were further analysed as suggested by Wright's ENC – plot (Zhang *et al*., 2011). The ENC values are plotted against GC_3 as a method to understand the pattern of codon usage. The viruses, whose codon choice is constrained only by a mutation bias, will lie on or just below the curve of the predicted values. The predicted values of ENC were calculated as

$$ENC = 2 + s + \frac{29}{s^2 + (1 - s^2)}$$

Where *s* represents the given $(G+C)_3$ % value.

Relative synonymous codon usage (RSCU) Analysis: The RSCU values of codons in each ORF for all the member of *Flaviviridae* family were calculated by the given formula to determine the characteristics of synonymous codon usage. The synonymous codons with RSCU values > 1.0 have positive codon usage bias and were defined as preferred codons, while those with RSCU values < 1.0 have negative codon usage bias and were defined as less- preferred codons. When the RSCU value is 1.0, it means there is no codon usage bias for that amino acid and the codons are chosen equally or randomly. Moreover, the synonymous codons with RSCU values >1.6 and < 0.6 were treated as over-represented and under-represented codons, respectively (Wong *et al*., 2010; Ma *et al*., 2013).

$$RSCU = \frac{g_{ij}}{\sum_j^{ni} g_{ij}} n_i$$

Where g_{ij} is the observed number of the *i*th codon for the *j*th amino acid, which has n_i types of synonymous codons.

The correlation analysis was performed between each general nucleotide composition (U%, A%, C%, and G %) and each nucleotide composition in the third site of codon (U_3%, A_3%, C_3%, and G_3%) and the value A%, T%, C%, G% and A_3%, T_3%, C_3%, G_3% were compared with GC%,, GC_3% and ENC Using statistical software SPSS 19 for windows. It is used to identify the relationship between nucleotide composition and synonymous codon usage pattern of viruses *Flaviviridae* family.

Principal component analysis (PCA): In this study PCA was performed to analyse the major trend in codon usage pattern among members of *flaviviridae* family. Principal component analysis is one of the most frequently used multivariate statistical techniques (Su MW *et al*., 2009; Yadav and Swati D, 2012; Kanaya *et al*., 2001; Wang *et al*., 2011). PCA is an orthogonal linear transformation that is used to transform the original data set into a new coordinate system. It involves a mathematical transformation procedure that transforms some correlated variable (RSCU) into a smaller number of uncorrelated variables called principal components. The greatest variance represented by the data lies on the first coordinate, thus known as the first principal component (PC), the second greatest variance is on the second PC, and so on. One can use top 2 or 3 PCs to represent the data instead of the large number of original variables (in this case, 59 Variables). In this study PCA was done by constructing a 114 × 59 RSCU data matrix. In the matrix each row denotes the codon usage pattern of a specific virus, demonstrated by its RSCU value. Each member of *Flaviviridae* family was represented as a 59 dimensional vector and each dimension corresponds to the RSCU value of one sense codon, which only included several synonymous codons for a particular amino acid, excluding Met (AUG), Trp (UGG) and three stop codon.

RESULTS AND DISCUSSION

COMPOSITIONAL PROPERTIES OF ORFs OF 114 FLAVIVIRIDAE GENOMES

The nucleotide contents (A, U, C, G and AU, GC %) and each nucleotide contents in the third site of codon (A_3, U_3, C_3, G_3 and GC_3%) in the orf of members of *Flaviviridae* family do not show similarity and are found to be quite different from each other. [Table 1 supplementary material].The genome of genus *Flavivirus* is enriched for purines (A and G) compared to Pyrimidines (U and C) with high frequency of base G ranging from 21.52-34.01. The purine richness is maintained throughout the genus without getting affected by the vector choice of that virus. But the content of G is higher in tick born viruses as compared to others. The effect of purine richness can be observed on the selection of codons as the most preferentially used codons are A - ended or G – ended codons with higher preference to A – ended codons except AUC for Ile in *Flavivirus*. Members of genus *Pestivirus* are also enriched for purine bases, with high A content ranging from 31.54-36.94 unlike *Flavivirus* .Most preferentially used codons are A - ended or G – ended codons with higher preference to A – ended codons except AGU for Ser in *Pestivirus*. The genus *Pegivirus* and *Hepacivirus* are rich in GC content with High content of G base in *Pegivirus* ranging from 27.67-32.41 and content of base C is almost similar in both the genus. The GC and GC_3

Table 1. List of Over-represented and Under-represented codons of four genus and unclassified members of Flaviviridae family.

Genus	Over-represented codons	Under- represented codons
Flavivirus (mosquito born)	UUG and CUG (Leu), GUG (Val), GGA (Gly), UCA (Ser), CCA(Pro), ACA(Thr), AGA and AGG (Arg),	UUA (Leu), GUA (Val), CCG (Pro), GCG (Ala), GGU (Gly)
Flavivirus (tick born)	CUG (Leu), AUC (Ile), GUG (Val), CCA (Pro), AGA and AGG (Arg), GGA (Gly), AGU and AGC (Ser)	UUA and CUA (Leu), GUA (Val), UCG (Ser), CCG (Pro), GCG (Ala), CGU and CGA (Arg)
Flavivirus (NKV)	UUG (Leu), UCA (Ser), CCA (Pro), AGA & AGG (Arg), GGA (Gly), ACA (Thr), GUG (Val)	GUA (Val), CGC & CGU (Arg)
Pestivirus	CUA and CUG (Leu), AUA (Ile), GUG and GUA (Val), UCA, AGC and AGU (Ser), CCA for Pro, GCA for Ala, AGA and AGG for Arg , GGG for Gly, ACA (Thr)	UCG (Ser), GCG (Ala), CGU, CGC, CGA and CGG (Arg), UCG (Ser) and CCG (Pro).
Pegivirus	UUG and CUG (Leu), AUC (Ile), GUG (Val), UCU and UCC (Ser), ACU and ACC (Thr), CGC and CGG (Arg)	GUA (Val), UUA and CUA (Leu), AUA (Ile), and AGA (Arg) and GGA (Gly).
Hepacivirus	AUC (Ile), CUC (Leu), GGC (Gly), AGG (Arg), UCC (Ser)	UUA and CUA (Leu), GUA (Val), CGA (Arg)
Unclassified	UUG (Leu), GUG (Val), CCA (Pro) GGA (Gly)	GUA (Val), UUA (Leu)

compositions also highlight the richness of these nucleotides in *Pegivirus* and *Hepacivirus*.

As a result of this they prefer using G - ended or C - ended codons with higher preference to C – ended codons. The unclassified group of virus show similarity with genus *Flavivirus*, enriched for purines, hence most preferentially used codons are A- ended or G with higher preference to A – ended codons. Two out of the four genus, *Flavivirus* and *Pestivirus,* are AG rich and show similarity in preferred base. On the other hand, *Pegivirus* and *Hepacivirus* are GC rich and show similarity in preferred base. We observed that the four possible nucleotides are not used at equal frequencies. *Flavivirus* and *Pestivirus* genus show low C content whereas *Pegivirus* and *Hepacivirus* genus show low A content. The base U is observed to be stable in the entire *Flaviviridae* family.

EFFECTIVE NUMBER OF CODON USAGE (ENC)

Different species have different tendencies to prefer specific codons, symbolized by Effective number of codons values. To investigate the overall codon usage pattern of *Flaviviridae* family, the ENC values for each orf is calculated and compared among the four genus [Supplementary material Table 1]. The values were analysed and compared within a genus and between different genus. Overall, the observed ENC values range between 44.99 (*Norway rat Pestivirus*) to 58.97 (*Aedes Flavivirus*) with the average being 53.74 across the *Flaviviridae* family. We also observed the values across 4 Genus. The codon bias of *Flavivirus* genus was on average 53.54 and ranged from 58.97 (*Aedes Flavivirus*) to 47.30 (*Tamana bat virus*) with standard deviation of 2.14. The overall codon bias of *Hepacivirus* genus was on average 55.07 and ranged from 57.02 (*Hepatitis GB virus B*) to 51.27 (*Norway rat Hepacivirus*) with standard deviation of 1.85. The overall codon bias of *Pegivirus* genus had an average value of 54.17 and ranged from 57.37 (*Human Pegivirus 2*) to 50.59 (*Rodent Pegivirus*) with standard deviation of 2.48.The overall codon bias of *Pestivirus* had an average value of 50.71 and ranged from 54.08 (*Atypical porcine Pestivirus 1*) to 44.99 (*Norway rat Pestivirus*) with standard deviation of 2.19. Finally, the codon bias of unclassified members was represented by an average value of 55.89 and ranged from 58.15 (*Anopheles flavivirus variant 1*) to 52.30 (*Bamaga virus*) with standard deviation of 1.75.

Among the entire genus *Flavivirus* showed highest variation in ENC value and members of unclassified group have shown the least variation. The codon variation of *Flavivirus* genus is higher than the variation of other three genus, implying that the evolution speed of these viruses is higher than the speed of the remaining viruses of the family. Conceptual value is comprised between 21 (if only single codon is used for each amino acid) and 61 (if all codons are used with equal frequency). In general, the overall codon bias of the four genus and unclassified members of *Flaviviridae* viruses is considerably weak. This is in agreement with previous reports about some other RNA viruses, for example BVDV (ENC=51.42), H5N1 (ENC=50.91) and SARS-covs (ENC=48.99), NDV (ENC=56.15) (Wang *et al.*, 2011; Zhou

et al., 2005; Gu *et al.*, 2003; Wang *et al.,* 2011). The possible explanation of weak codon bias in RNA virus is that a weak bias is helpful for efficient replication of virus in host cells. (Zhong *et al.*, 2007)

MUTATION PRESSURE AFFECTS THE CODON USAGE PATTERN

Mutational pressure and natural selection are considered the two major factors that shape codon usage patterns (Jenkins and Holmes, 2003). A general mutational pressure, which affects the whole genome, would certainly account for the majority of the codon usage among certain RNA viruses (Tatarinova *et al.*, 2010). To identify whether the evolution and variation pattern of codon usage had been driven alone by mutation pressure or also contributed by natural selection, we compared the correlation between overall nucleotide composition (A, U, C, G) and nucleotide composition at the third position of codon (A_3, U_3, C_3, G_3) and correlation between overall nucleotide composition (A, U, C, G, A_3, U_3, C_3, G_3) and GC, GC_3 and ENC for individual genus using Pearson s correlation [supplementary material (Tables 2-3).

In genus *Flavivirus* GC and GC_3 show significant positive correlation with G(*r*=0.87, *P*<0.01) (*r*=0.80, *P*<0.01), C (*r*=0.75, *P*<0.01) (*r*=0.80, *P*<0.01) and G_3 (*r*=0.86, *P*<0.01) (*r*=0.85, *P*<0.01), C_3 (*r*=0.75, *P*<0.01) (*r*=0.82, *P*<0.01).and negative correlation with A (*r*=-0.84, *P*<0.01) (*r*= -0.85, *P*<0.01), U (*r*= -0.70, *P*<0.01) (*r*= -0.65, *P*<0.01), and A_3 (*r*= -0.76, *P*<0.01), (*r*= -0.81, *P*<0.01), U_3(*r*= -0.69, *P*<0.01) (*r*= -0.68, *P*<0.01). ENC shows positive significant correlation with C (*r*= 0.73, *P*<0.01) and C_3 (*r*= 0.70, *P*<0.01), and negative correlation with A (*r*= -0.69, *P*<0.01) and A_3 (*r*= -0.65, *P*<0.01) and non-significant correlation with U, G, and U_3. A shows positive correlation with A_3, negative correlation with C_3 and G_3, and non-significant correlation with U_3. U shows significantly negative correlation with C_3 and G_3, positive correlation with U_3 , and non-significant correlation with A_3. G and C show significantly negative correlation with A_3 and U_3 and significantly positive correlation with C_3 and G_3. When we study correlation vector wise, tick born and NKV viruses show significant correlation in comparison with mosquito borne viruses of the genus. In genus *Pestivirus* an interesting and complex correlation was observed.

To sum up, the GC, GC_3 and ENC have highly positive significant correlation with C (*r*=0.84, *P*<0.01) (*r*=0.86, *P*<0.01) (ENC=0.87), C_3 (*r*=0.90, *P*<0.01) (*r*=0.94, *P*<0.01) (ENC=0.89) and G (*r*=0.94, *P*<0.01) (*r*=0.91, *P*<0.01) (ENC=0.74),G_3 (*r*=0.98, *P*<0.01) (*r*=0.97, *P*<0.01) (ENC=0.82) And significantly negative correlation with A (*r*= -0.99, *P*<0.01) (*r*= -0.99, *P*<0.01) (ENC= -0.92), A_3 (*r*= -0.97, *P*<0.01) (*r*= -0.99, *P*<0.01) (ENC= -0.90) and U (*r*= -0.89, *P*<0.01) (*r*= -0.84, *P*<0.01), U_3(*r*= -0.82, *P*<0.01) (*r*= -0.80, *P*<0.01) (ENC= -0.66, *P*<0.05). A_3 and U_3 show significantly positive correlation with A and U, and significantly negative correlation with C and G, whereas C_3 and G_3 show significantly negative correlation with A and U and significantly positive correlation with C and G. In genus *Hepacivirus* A_3 shows positive correlation with A and has non-correlation with U, C, G and GC and GC_3. Similarly, A shows non-correlation with U_3, C_3, G_3 and GC, GC_3 and ENC. GC and GC_3 show significantly negative correlation with U (*r*= -0.94, *P*<0.01) (*r*= -0.97, *P*<0.01) U_3 (*r*= -0.92, *P*<0.01) (*r*= -0.96, *P*<0.01) and highly positive correlation with G (*r*=0.95, *P*<0.01) (*r*=0.94, *P*<0.01), G_3 (*r*=0.96, *P*<0.01) (*r*=0.97, *P*<0.01) and C (*r*=0.98, *P*<0.01) (*r*=0.95, *P*<0.01), C_3 (*r*=0.96, *P*<0.01) (*r*=0.99, *P*<0.01). ENC of Hepacivirus show non-correlation with A, U, C, G, U_3, G_3.

In genus *Pegivirus* the GC and GC_3 show significantly positive correlation with G (*r*=0.83, *P*<0.01) (*r*=0.83, *P*<0.01), C (*r*=0.84, *P*<0.01) (*r*=0.71, *P*<0.05), C_3 (*r*=0.82, *P*<0.01) (*r*=0.84, *P*<0.01) and significantly negative correlation with U (*r*= -0.77, *P*<0.05) (*r*= -0.91, *P*<0.01) and A_3 (*r*= -0.89, *P*<0.01) (*r*= -0.73, *P*<0.05). ENC have highly significant correlation with A, U, C, G, A_3, C_3 and non-correlation with U_3 and G_3.A shows significant correlation with A_3 but does not show significant correlation with U_3, C_3, G_3. U shows significant correlation with U_3, C_3 and G_3, and non-significant correlation with A_3.C show significant correlation with A_3 and C_3 and non-significant correlation with U_3 and G_3. G shows significant correlation with G_3 and non-significant correlation with A_3, U_3, and C_3.The members of *unclassified group* do not show significant correlation with other nucleotides, they show significant positive correlation with the same type of nucleotide like A show positive correlation with A_3. The GC and GC_3 show positive correlation with C (*r*=0.73, *P*<0.01) (*r*=0.62, *P*<0.05) and G (*r*=0.81, *P*<0.01) (*r*=0.67, *P*<0.01) and negative correlation with A (*r*=-0.75, *P*<0.01) (*r*=-0.69, *P*<0.01) and U (*r*=-0.78, *P*<0.01) (*r*=-0.59, *P*<0.05). ENC does not show significant correlation with any nucleotide. This analysis collectively indicates that mutational pressure is most likely responsible for the patterns of nucleotide composition and, therefore, codon usage patterns in all four genus of *Flaviviridae* family.

VARIATION OF RELATIVE SYNONYMOUS CODON USAGES IN FLAVIVIRIDAE FAMILY

In order to investigate the extent of codon usage bias in *flaviviridae* family, all RSCU values of different codons in genus *Flavivirus* (69), *Hepacivirus* (14), *Pegivirus* (8), *Pestivirus* (11) and unclassified members (12) were calculated. The heat map [supplementary material Fig. 1]

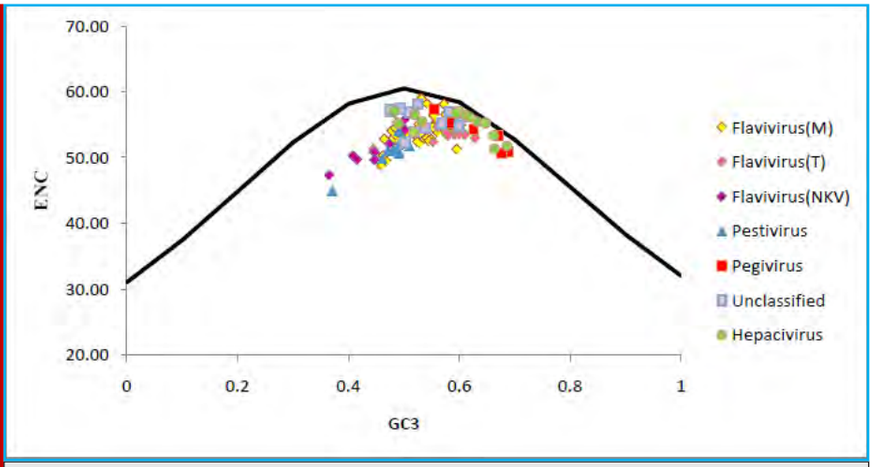

FIGURE 1. The ENC vs GC3 plots of four genus and unclassified members (*Pestivirus, Pegivirus, Hepacivirus, and Flavivirus*) of Flaviviridae family.

show the RSCU results of all codons in the 114 viruses of *Flaviviridae* family. Green represent lower RSCU value, black represent moderate RSCU, and red represents greater RSCU values. The common over - represented and common under - represented codons are listed for each genus of *Flaviviridae* family [Table 1]. As we know genus *Flavivirus* is classified into three groups on the basis of vector. The over - represented and under - represented codons are identified vector wise for this genus.

Viruses in this genus show similarity in choice of codon with their subtype or genotype like the four serotype of Dengue show similar choice of codons. Preferred codons in four serotype are UUG and CUG for Leu, AUA for Ile, GUG for Val, UCA for Ser, CCA for Pro, ACA for Thr, GCC and GCA for Ala, AGA and AGG for Arg, GGA for Gly. Less preferred codons are GUA for val, UCG for Ser, CCG for Pro, ACG for Thr, GCG for Ala, CGU and CGC, CGA and CGG for Arg, GGU and GGC for Gly. Similarly, westnile 1 and westnile 2 virus show similar choice of codon usage UUG, CUC and CUG for Leu, AUC for Ile, GUG for Val, UCA for Ser, CCA for Pro, ACC and ACA for Thr, GCU and GCC for Ala, AGU and AGC for Ser, AGA and AGG for Arg, GGA for Gly. The less preferred codons are UUA for Leu, GUA for Val,UCG for Ser, CCG for Pro,GCG for Ala, CGU and CGA for Arg, GGU for Gly. In genus *Hepacivirus* Equine and Bovine show similar choices for preference of codon in comparison to the other members of the group. The unclassified members of *Flaviviridae* family show similarity with genus *Flavivirus* like, Lammi virus shows similarity with mosquito born *Flaviviruses* especially with *West Nile* virus in choice of preferred and less preferred codon. *Nhumirim* virus shows similarity with nkv group of *Flaviviruses* especially with *Paraiso* virus show similarity in preferred codons CCC, CCA & CCG for Pro, GCG for Ala, CGA & CGG for Arg, AGU for Ser and GGA & GGG for Gly. *IIomantsi* virus and *Donggang* virus show similarity in preferred codon CCC & CCG for Pro, GCG for Ala, CGA & CGG for Arg, AGU for Ser and GGA & GGG for Gly. *IIomantsi* ,*Lammi*and *Nienokoue* viruses show higher degree of similarity in choice of preferred codon with mosquito born *Flavivirus*. GUG for Gly is the only common codon in the entire *Flaviviridae*.

In general, the amount of the over - represented codon is more than the amount of under - represented codon in the four genus of family and this feature is consistent with all the 114 ORF`s, suggesting that the evolution process of viral genome of all four genus is similar to some degree and the codon usage bias is weak which is supporting the results we observed from ENC values. The nucleotide composition also plays an important role in choosing preferred codons, therefore *Flavivirus* and *Pestivirus* shows preference to A and G ending codons, as they are rich in purines. And *Hepacivirus* and *Pegivirus* show preference to G and C ending codons as they are rich in GC content.

In addition, the RSCU values of the eight codon containing CpG (CCG, GCG, UCG, ACG, CGC, CGG, CGU, and CGA) in four genus were analysed. All of these eight codons were not preferential codons and were found suppressed in genus *Flavivirus* and *Pestivirus*. In genus *Pegivirus* and *Hepacivirus* six codons are under - represented except CGC and CGG. The explanation for CpG scarcity in these viruses is attributed to their property to escape the host immune response. A high CpG content leads to increased unmethylated CpGs which has immunostimultory property and therefore are easily recognized by the host's innate immune system as a pathogen signature. This is injurious to the small DNA (or RNA) viruses. Thus high mutational rates are observed in CpGs

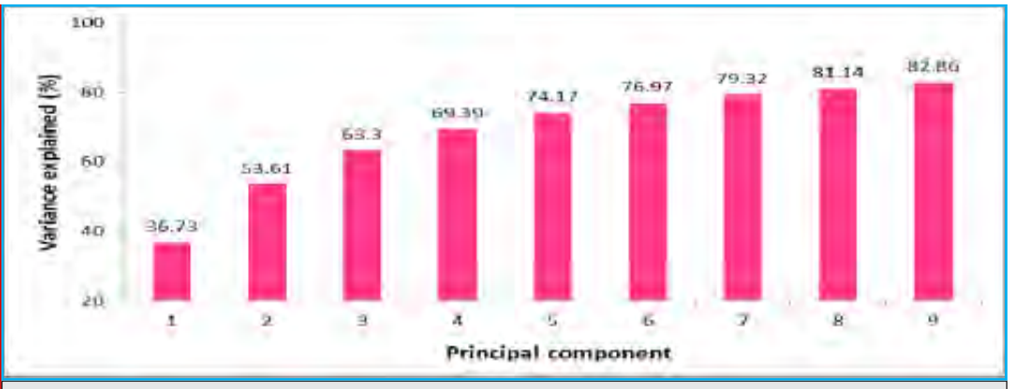

FIGURE 2. Principal components (PCs) and variances explained in the analysis of the 59 relative synonymous codon usage (RSCU) indices. The first 9 PC vectors are listed on with accumulated variance (%). The plot on the shows that the first five PCs have explained more than 70% of the variance of the original data.

since its deficit will enable virus to infect the host. (Dorn and Kippenberger, 2008; Krieg, 2003)

CORRELATION ANALYSIS BETWEEN ENC AND GC_3 VALUE

A plot of ENC versus GC_3 is widely used to study codon usage variation among different organisms. It is the most important part of investigation of codon usage pattern. The ENC values of each member of *Flaviviridae* family were plotted against its corresponding GC_3 values and all values lie below the expected curve as shown in [Fig. 1]. Therefore it can be hypothesized that the codon usage bias, in all these 114 viruses is principally influenced by the mutational pressure.

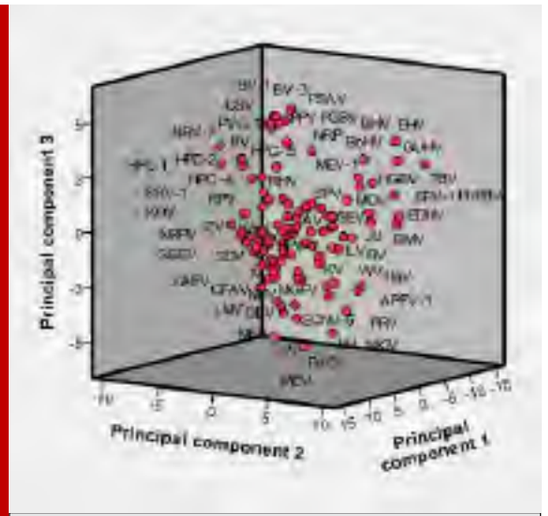

FIGURE 3. Principal component analysis (PCA) plot for analysis of the relative synonymous codon usage (RSCU) indices of 114 RNA viruses. The PCA scores of the 114 viruses were plotted in a three-dimensional coordinate system using the first three principal component vectors as axes.

IDENTIFICATION OF SIMILARITIES AND DIFFERENCES IN CODON USAGE PREFERENCES BY PCA

The identification of similarities and differences in codon usage preferences is an involved process that can be handled by using the Principal Component Analysis (PCA) approach. The PCA is a classical data analysis method that identifies patterns and focuses on similarities and differences in a multivariate data set. The exploration of codon usage pattern differences among these RNA viruses involves processing of the 114 × 59 RSCU matrix by PCA. This enables calculating the principal components (PCs) which in turn are employed to highlight the similarities and differences in codon usages. [Fig.2] shows the trend of the first 9 PCs. PCs with Eigen value greater than or equal to 1 are usually considered as being of statistical significance (the Kaiser criterion) as indicated in [Supplementary material Table 4] .The first PC is associated with 36.73% of the variance among the 59 RSCU indices. The first two PCs taken together account for 53.61% of the variance whereas the first three PCs combined together account for 63.30% of the variance in codon usage.

The variances of a total of 114 PCs generated from PCA are listed in [Supplementary Material Table 4]. Fig. 3 is the three-dimensional PCA plot using the first three PCs of these 114 viruses as axes [the corresponding PCA coordinates are listed in Supplementary Material Table 5]. The PCA score diagram shows that the all viruses can be broadly classified into four categories. This classification is essentially based on different hosts,

vectors and ecological niche. Genus *Flavivirus* display negative values on the second and third PC axes. Members of genus *Pegivirus* displayed positive values on the three PC axes. The genus *Hepacivirus* displays more positive values on first and third PC axes. The genus *Pestivirus* displays negative values on first and second PC axes whereas all positive values appear on the third axis. The unclassified members manifest a heterogeneous distribution of values and, consequently, do not represent a fifth category but get merged into the four categories.

CONCLUSION

Our analysis reveals that the overall codon usage bias in *Flaviviridae* family is slightly biased and mutation pressure is the main factor that affects codon usage variation in viruses. Other factors like Compositional constraint and natural selection also significantly influence codon usage variation. Results show RNA viruses with same vector choice share similar codon usage preferences. However, more detailed analysis is needed to understand the relationship of codon choices between viruses and hosts.

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Prevalence of the root canal treatment errors and its related factors in patients treated by undergraduate dental students

Dadresanfar B[1], Mohammadzadeh Akhlaghi N[2] and Z. Khodabakhsh[3]*

[1,2]Assistant Professor of Endodontic Department, Islamic Azad University, Dental Branch, Tehran, Iran
[3]Postgraduate Student of Operative Department, Islamic Azad University, Dental Branch, Tehran, Iran

ABSTRACT

Since errors of root canal treatment can result in tooth loss, it is important to study effective factors on incident of these errors. In recent years, aiming at reducing errors of root canal treatments and improving performance of dental department in Islamic Azad University, Tehran, facilities were provided which included apex locator, teaching Passive-step back in pre-clinic period and presence of professional assistants and professors who helped students. Studying and investigating errors during treatment by students and providing proper preventative solutions increases the chance of successful treatment of patients. According to the changes in methods of teaching root canal preparation and considering benefit that annual and biennale assessment of prevalence of errors during the treatment has for studying educational performance and future planning, in this study we studied prevalence of errors during root treatment by dental students in general dentistry major and its effective factors, in order to study whether these applied changes reduced errors or not? Firstly, it is hypothesized that effective factors on this errors include quality of radiography, numbers of radiographies, patients' age, type of tooth, type of jaw, canal curvature, periapical lesion, student's semester of the study, student's gender, and numbers of treatment sessions, first treatment of root and second treatment of root. In this research 840 record of patients who had received root canal treatment in public Endodontics sector were evaluated from 2010/9/23 to 2012/09/23. Firstly, two endodontists were calibrated in order to make sure that kappa coefficient is positive and they both agreed on that, and then endodontists, separately, completed data forms pertinent to errors during root treatment and related factors. Prevalence of errors was identified in the samples and then role of associated factors was evaluated by logistic regression as statistical test. And after first evaluation of records, sample of study consists of 613 teeth (1131 canals) which out of them 567 teeth received RTC and 46 teeth received re treatment (Redo). Results indicated that there is statistical difference in frequency of errors

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during first root treatment and re treatment. In addition, factors including jaw type, tooth type, canal curvature, periapical lesion, and student's semester of study have significant effect on Non homogene-Exact filling length 'Transportation 'Ledge' Over filling's error. However, numbers of treatment sessions was effective with low correlation. Comparing frequencies of errors during root treatment in this study and paper, it was clarified that adding tools such as apex locator, teaching Passive-step back in pre-clinic period and presence of professional assistants and professors who helped students reduces errors during root treatment.

KEY WORDS: DENTAL STUDENTS, RADIOGRAPHIC ENDODONTIC ERRORS, QUALITY OF ROOT CANAL TREATMENT

INTRODUCTION

Root treatment, aiming at preventing periapical periodontitis and improving periapical lesion, has been recognized as a complicated treatment in dentistry treatments. This treatment is conducted by removing necrosis pulp, infectious pulp and batteries and via irrigation and preparing canal, mechanical cleaning and also high quality obturation. Epidemiologic studies introduced errors in mentioned procedure as the main reason of treatment failures and imposing higher costs (Lynch 2006; Mozayeni 2006; Yousuf, 2015). It has been indicated that acceptable RCT refers to treatments in which root fillings terminate within 2mm of the radiographic apex and that are of adequate homogene density and without void from crown to apical area (Bramanten 1987; Kulic 2011). Applied method for studying quality of root treatment is assessing PA Radiographs that are provided before and after root treatments, radiographically (Kelbauskas 2009; Mosby Co. 2009). Literature review indicated that errors rate varies from 10 to 58% in different centers while prevalence of these errors rate in academic centers (25-45%) is significantly higher than professional centers (10-16%) (Cohen 1998; Er 2006; Estrela, 2017).

Estrela1 et al (2017) performed a research titled as "Common Operative Procedural Errors and Clinical Factors Associated with Root Canal Treatment". They concluded that in each phase of RCT, an operative error can have adverse implication on prognosis, and these errors show that risk factors lead to failure.Akbar (2015) performed radiographic study of the endodontic treatment's problems and failures. His results illustrated that compare to anterior and premolar teeth, endodontic problems and failures were most common in molars. The most frequently canals with endodontic problems and failures included Mesiobuccal, mesiolingual and distobuccal root canals. Finally, based on results he concluded that the most common cause of endodontic treatment failure was under filling followed by poor filling and over filling and first molar was the most frequently involved tooth with endodontic problems and failures. Yousuf, et al., (2015) studied endodontic procedural errors and showed that the most frequently treated tooth was right permanent mandibular first molar. The least commonly treated teeth were the permanent mandibular third molars. Bakhshi and Shahabi (2015) identified the least mistakes compared to obturation phase, with the most frequent errors including void, overfilling and imperfect cleaning.

A research was carried out by Kulic et al in 2011 in Serbia which indicated that 51.6% treatments were acceptable with accurate working length and homogeny density. In Fonseka et al (2015) study, it was reported that 74.3% treatments were acceptable. In addition, in a research in 2008 in Islamic Azad University, endodontics department, Tehran, it was reported that 51.5% errors happened during root canal treatment while 49.5% were error-free. Most of the studies illustrated that highest error incident was occurred in posterior teeth (Bramanten CM. 1987; Eleftheriadis GI. 2005; Khabbaz M.G. 2010) and factors including increasing instruction hours reduces errors (Yousuf, W. 2015). Study and consideration of students' errors during treatment and providing appropriate preventive ways increase treatment success in patients (Bramanten CM. 1987) Recent years, facilities including apex-locator, teaching Passive-step back method in pre-clinic, presence of professional assistants and professors who helped students were added into endodontics department of Islamci Azad University in order to reduce errors during treatment.

Based on literature review (Estrela, 2017; Lynch 2006; Yousuf, 2015), popular errors during endodontic treatments are as follow:

Last years equipment including teaching Passive-step back method in pre clinic and presence of professional assistants helping students were applied in order to reduce errors during treatment in endodontic sector. According to the changes made in methods of teaching root canal preparation and considering the benefits that annual and biennale assessment of prevalence of errors during the treatment has for studying educational performance and future planning, thus due to lack of information about the subject in endodotics sector of Islamic Azad University, Tehran, and also because of differences and lacks of previous researches (Er O. 2006 and Yousuf 2015), studying students' error during treatment and providing proper methods increase chance of successful treatment of patients. This study, therefor, investigates prevalence of errors during root trees and related factors in patient referring to endodontics sector of Islamic Azad

Table 1. popular errors during endodontic treatments are as follow

	Error type	Description
1	Under filling	Space between canal obturation and radiographic apex is more than 2 mm
2	Over filling	Radiographic beyond apex filling
3	Non-homogen	Lack of homogene density of filling material from coronal area to apical area
4	Ledge	Deviation from main path of canal and creating one step in some cases is cause of underfilling
5	Transportation	Deviation from main path of canal and finding new path in root
6	Zipping	Perforation of apical area which results in reverse cone and it disrupts apical seal
7	Strip perforation	Association of pulp space with periodontal space in root branching *region*
8	Cervical perforation	Association of pulp space with periodontal space in *cervical region of tooth*
9	Forcation perforation	Association of pulp space with periodontal space in *forcal region of tooth*
10	Broken Instrument	Broken instrument that is not extracted from canal
11	Gouging	Over Opening cavity more than required space due to not locating grinder in longitudinal axis of tooth or futile attempt for accessing to the pulp

University, Tehran, during 2010-2012 in order to find out whether these applied changes reduced error or not?

MATERIALS AND METHODS

All people who received root treatment from 2010/9/23 to 2012/09/23 in public Endodontics sector in Islamic Azad University; Tehran belonged to the population of the current study. Then, out of endodontics sector records' list that belonged to two years and was gathered statistically, accurate records were selected and studied. In this study, research method was performed by existing data which collected by observation and filing information forms. In addition, samples were selected statistically from all treated patients by dentistry students in general coarse. Out of 840 selected records, 227 records (27%) were eliminated due to poor radiography quality (182 records, 21.6%) and lack of sufficient radiography (45 records, 5.3%).

Records that lacked final radiography, or did not contain at least both *diagnostic* and final radiography and also records that, in spite of second recording, their radiographic quality was poor were eliminated from the study. Lack of radiography quality happens due to manual radiography developing and fixing by student in public sector of endo which results in over developing the films, insufficient fixing, and lack of PA Radiographs quality. Assessing quality of student's performance in root treatment procedure was conducted in two steps including preparation and root canal obturation based on recorded radiographic images in patients' records. Nevertheless, there are 2 dimension images instead of 3 dimension structures, this system has been applied in different studies (Cohen 1998; Guttman 1997).

Assessing images of all records was conducted by two experts from university endodontic sector, separately, by microscope with at times enlargements and desktop negatoscope. Before study, observers agreed on similar interpretation for radiographic PA Radiograph after performing an experiment. Evaluators were calibrated and Kappa coefficient was reported as 0.88 which proved there is perfect coefficient of agreement among evaluators. Due to perfect coefficient of agreement among evaluators, mentioned samples were assessed again in cases with no agreement among observers. This method was utilized by Khabbaz et al. (2010).

For PA Radiograph homogenization by observers, ray radiation direction considered mesial in all radiographies. Poor radiographies were fixed again and reevaluated. Records which lacked 2 diagnostic and final radiographies were eliminated. Two endodontists conducted diagnostic and final radiographies and used microscope at five times enlargement and one negatoscope. In addition, some rare errors mentioned in the record and they were not observable in radiography but they were mentioned in the record were studied. Firstly, errors' evaluators explained errors, then coefficient of concordance (Kappa) were identified and then records' assessment was started. Evaluators were calibrated in order to make sure that Kappa coefficient is positive and they are compatible. For preventing errors of answers, each endodontist filled data forms separately and then errors that endodontist were agreed upon considered as real error. In cases that observers did not agree on, due to perfect coefficient of agreement between evaluators, mentioned samples were reassessed and evaluators agreed on that.

In epidemiologic studies, there are different standards for categorizing root treatment quality. The most prevalent parameters of acceptable treatment categorization

are length of root canal filling, filling homogenization without void, and absence of iatrogenic errors (Cohen 2006; Haji-Hassani, 2015; Mozayeni 2006). In this study, standards of determining radiographic categorization of root treatment were based on length and density of filling in the absence of iatrogenic errors and they were categorized into two acceptable and unacceptable treatments.

The filling material ends 0-2 mm shorter than radiographic apex with no visible voids within the material or between the material and root canal walls. 2) Unacceptable treatments: A) the filling material ends more than 2mm from radiographic apex or beyond the radiographic apex. B) Visible voids within or between filling material and root canal walls

1. Acceptable treatment: under filling is 0-2 mm and density of filling material is homogene with no visible void within the material or between crown and apical area. Besides no observable iatrogenic error is in patient's record and in canals.
2. Unacceptable treatment

a. The filling material ends more than 2mm from radiographic apex or beyond the radiographic apex
b. Density of filling material in not homogene and there is void between crown to apical area.
c. Iatrogenic error is observed in canals and it is also reported in pateint's record.

In addition, in this study we assessed demographic information of students (gender, and the semester) and factors pertinent to patient and tooth including patient gender, age, tooth location, tooth root numbers, canal numbers, periapical radiolucencies, canal curvature and treatment sessions numbers.

RESULTS AND DISCUSSION

Results indicated that 61% first treatments (RCT) were acceptable and 39% had errors during root treatment. Acceptable treatment which consists of exact length of filling and proper density of filling and no incidence of iatrogenic errors, in anterior teeth was 75.6%, in premolar was 72% and in molars was 38.4%. Redo were acceptable for 100% cases and the most prevalent redo tooth was Mandibular second premolar. Previous short filling was identified as the most common cause of redo.

In table 2, there is information about frequency of errors during root treatment in patients who received treatment by dental students in general coarse in endodontics sector of Islamic Azad University, dentistry department, during 2010-2012. In addition, table 2 indicates frequency of errors during root treatment associated to relevant factors.

Based on table 2 and table 3 and results of logistic regression, it is concluded that:

1. 55% studied canals had curvature which it was more in molar teeth. 24.2% canals with curvature had errors during root treatment. 14.5% tooth that received RCT had periapical lesion which was more in mandibular first molar. 47% canals had periapical error during treatment.

Table 2. Prevalence of errors during root treatment

total		Molar		premolar		anterior		Error type / Tooth type	
Percentage	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	Frequency		
100	7	42.8	3	28.6	2	28.6	2	Broken instrument	
100	40	67.5	27	20	8	12.5	5	Homogen	Over filling Root canal
100	22	59	13	32	7	9	2	Nonhomogen	
100	35	45.7	16	25.7	9	28.6	10	Homogen	Under Filling Root canal
100	28	67.8	19	21.4	6	10.8	3	Nonhomogen	
100	102	60.8	62	23.5	24	15.7	16	Nonhomogen- Exact filling length Root canal	
0	0	0	0	0	0	0	0	Forcal Perforation	
0	0	0	0	0	0	0	0	Cervical Perforation	
100	5	100	5	0	0	0	0	Strip Perforation	
100	9	100	9	0	0	0	0	Strip preparation	
100	25	52	13	16	4	24	6	Ledge	
100	54	72.2	39	18.5	10	9.3	5	Transportation	
100	4	75	3	25	1	0	0	Zipping	
100	6	0	0	33.3	2	66.7	4	Gouging	
100	567	35.8	203	35.9	204	28.2	160		

Table 3. related factors to errors during treatment

Related factors Error	gender		*manual endo coarse*			Numbers of treatment sessions				
	male	Female	two	three	four	one	two	three	Four	Five
No error	148 61.7	197 60.3	192 71.9	96 60	59 41.1	112 70.4	185 63.8	45 44.1	4 28.6	1 50
With error	92 38.3	130 39.7	75 28.1	64 40	81 57.9	47 31.6	105 36.2	57 55.9	10 71.4	1 50
Total	240	327	267	160	140	159	290	102	14	2

(A)

Related factors Error	Canal numbers				Tooth type			Jaw type	
	one	two	three	four	*anterior*	Premolar	molar	lower	upper
No error	205 75.3	69 67.6	64 40	9 28.1	121 75.6	147 72	78 38.4	125 53.9	221 66
With error	68 24.7	23 32.4	96 60	23 71.9	39 24.4	57 28	125 61.6	107 46.1	114 34
Total	273	92	160	32	160	204	203	232	335

(B)

Related factors error	Canal curvature		lesion PA		Patient age		
	Direct canal	Curved canal	With error	No error	Under 30	Between 30-45	Older than 45
No error	412 84.1	451 75.8	44 53	819 81.7	143 60.3	123 57.5	80 69
With error	78 15.9	144 34	39 47	183 19.3	94 39.7	91 42.5	36 31
Total	490	335	83	1002	237	214	116

(C)

2. 39.7% treatments conducted by female students and 38.3% performed by male students had errors during the treatment. But there was no significant difference between students' gender and errors during treatment ($p>0.05$).
3. 71.4% four sessions treatment and 29.1% one session treatment had errors during the treatment. It means that there was significant difference between errors rate during treatment and treatment sessions number ($p<0.001$). However, considering correlation coefficient ($\varphi = 0.21$), this relationship was poor.
4. Considering root treatment error, there was significant difference between two jaws ($p=0.004$). Regarding that its incidence in mandibular teeth was significantly more than maxillary teeth (34%). Considering correlation coefficient, $\varphi = -0.12$, this error was more prevalent in mandibular than upper jaw.
5. Error incidence rate by students in manual endo coarse 2 was identified 28.1%, by manual endo coarse 3 was 40%, and by manual endo coarse 4 was 57.1%, which indicated that there is significant relationship between errors during treatment and students study in manual endo coarse ($p<0.001$) and considering correlation coefficient, $\varphi = 0.52$, this relationship was average.

In addition, results obtained from prevalence of errors during treatment indicated that:

a. Non homogen-Exact fillng length was identified in 13.4% canals (145 canal) as the most prevalent error in root filling procedure (filling with in adequate quality in exact length of function). This error was significantly more prevalent in molar teeth canal (60.8%) than premolar and anterior teeth ($p<0.001$) but considering correlation coefficient, $\varphi = 0.25$, this relationship was poor. This error was 35.3% in mandibular molars and 25.5% in maxillary molars. Highest prevalence was identified in mandibular molars and **mesiobacal** canal (18%) and then it was observed in mesiolingual (17.2%).
b. In field of errors during root canal preparation, the most common error was transportation which was observed in 9.5% teeth (54 teeth) highest rate of this error happened among molars which prevalence rate was 72.3% and highest prevalence happened in mesiobacal canal of mandib-

ular first molar. There was significant relation between transport and canal curvature ($p<0.05$). However, considering correlation coefficient, $\varphi = 0.1$, this relation was weak. There was significant difference in transport incidence among molar teeth (72.2%) and premolars (18.5%) and anteriors (9.3%) ($p<0.05$).

c. Second prevalent error during canal preparation was ledge which was observed 4.4% of total teeth. There was significant relation between canal ledge and curvature ($p<0.05$) however considering correlation coefficient, $\varphi = 0.06$, this relation was poor. In molar teeth, error prevalence was 52% which was more than pre molar and anterior teeth. In addition, ledge prevalence was significantly higher in mandibular (40%) than maxillary (12%) ($P<0.005$). Highest rate of error happened in first mandibular (32%) and mesial root and it is observed equally in mesiobacal canals (25.6%) and mesiolingual (25.6%).

d. Over filling has been studied in 6.8% canals and it was identified that 4.2% were over homogene and 2.6% were over non-homogene. the highest prevalence rate happened in mesiolingual canals of mandibular molars. Significant difference was observed in overfilling and teeth with priapical lesion ($p<0.05$) however considering correlation coefficient, $\varphi = 0.08$, this relation was very poor.

e. Foracal Perforation and Cervical Perforation were not observed. 6 Strip Perforation cases, 7 Broken Instrument cases, 4 zipping cases, 6 Gouging cases and 9 Strip Preparation cases were identified and there was not significant relationship with related factors.

In this study, maximum error happened in root filling step, Non homogen-Exact filling length (filling with inadequate quality in exact length of function) and the most prevalent error in field of errors during root canal preparation was transportation error. Second prevalent error during canal preparation was ledge. In this study, 613 teeth including 1131 canal were evaluated which consists of 567 teeth which received RCT and 46 teeth received retreatment. Acceptable treatment in anterior teeth was 75.6%, 72% in premolars and 38.2% in molars. 100% redo were identified as acceptable treatment and the most prevalent retreated tooth was mandibular second premolar and under-filling was identified as the most prevalent cause of retreat.

In this study, similar to Kulic et al (2011), multi root teeth which had error during treatment, even in one canals, was considered as unacceptable treatment. Unal et al (2011) reported highest prevalence of acceptable treatment in anterior teeth was 90.1% and the least prevalence was reported in 46.6% molars. In 71% anterior teeth, 61% premolars and 30% molars were reported as acceptable treatment prevalence by Khabbaz et al (2010). In current study, 61% treatments were acceptable and 39% treatment were unacceptable. Acceptable treatment in anterior teeth was 75.6%, in premolars was 72% and in molars was 38.2%.

Mozayeni et al (2006) reported that the most prevalent error during root canal preparation was transportation. In addition, Dadresanfar et al (2008) reported that transport prevalence was 27.5%. Statistical difference of these findings can be due to applying Passive-step back preparation method in pre clinic coarse, applying Gates-Gliden drills for coronal preparation of root which reduces coronal interferences from deviation of first path canal and also due to professional assistance and their help to students. High prevalence of transportation in molar teeth its significant difference with anterior and pre molar teeth can be due to complicated anatomy of these teeth, high numbers of canals and curvature of canals in these teeth. There was significant statistic relationship between root canal curvature and transportation which shows there is potential effect of root canal curvature on canal displacement. Lack of attention to canal curvature, not providing Pre curve to files during preparation of curved canals and lack of removing interferences of root canal Orifices can be reasons of high transportation rate in canals.

Second prevalent error during anal preparation was ledge. Al-Kahtani et al reported that ledge prevalence was 7.5% which is compatible with current study. Eleftheriadis et al reported that ledge prevalence in molars were 34.9% which is caused by step-back technic and curvature of molar canals. Less prevalent of ledge in this study can be due to using other canal preparation technics including Passive-step back and applying files with higher flexibility such as Flexo File by dentistry students.The most prevalent error during root filling was Non homogen-Exact fillng length. More errors in molars can be because of lack of adequate access of students to these teeth and in adequate canal flaring which is caused by their stress for bad incidents. Because inadequate canal flaring prevents suitable penetration of spreader, especially stainless spreaders, which will cause bad filling density.

Er et al (2006) reported that 48.8% filling had inadequate density. In addition, Khabbaz et al (2010) stated that 33.5% filling had inadequate density and Kulic L et al (2011) in 25% and Dadresanfar et al reported that 29.2% filling had inadequate density. Statistical difference of these results with mentioned studies can be stemmed from increase of assistants and professors'

numbers and continuous study of patients during treatment by professors via providing constancy control radiographies during obscuration which reduces prevalence of errors during canal filling.

Maximum error prevalence of over filling was find out in mesiolingual canal of mandibular molars and there was significant statistical difference between overfilling and teeth with periapical lesion, because periapical lesion root top resorption so students cannot deal with length control easily then it ends to overfilling. Kelbauskas et al (2009) reported that 5.42% had overfilling which was compatible with this study. Kulic et al (2011) reported3.3% overfilling which this low prevalence can be due to less numbers of their samples (306 canal). Er et al (2006) reported 13% overfilling cases and Khabbaz et al reported 22.6% overfilling. Statistical difference of current studies with those can be due to studying more canals in samples (1109 canals).

Highest rate of underfilling error happened in Mesiobacal canal of mandibular molars. It can be due to higher rate of transportation and ledge in molars which results in diversion from main canal path and interference in filling steps. In addition in Step-back technic there is possibility of debris and dentin debris packaging in apex top so there is no way for filling with exact length. Kelbauskas et al (2009) reported that in 10.5% cases, underifilling happened. They also reported that the main reason is ledge and debris packaging.

CONCLUSION

It seems that using tools such as apex locator, presence of professional assistants and attention of professors of the related sector and also applying Passive-Step back in curved canals by some students reduces preparation error rate and errors of root canal filing. However utilizing more flexible files, emphasizing on before-during-and after treatment radiographies, meticulous supervision of professors during treatment and presence of lower semester students with higher semester students and professional assistants in third and second year of experience could be considered as a guide for increasing quality of root treatments and reducing errors.

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16S metagenomic analysis and taxonomic distribution of enriched microbial consortia capable of simultaneous biodegradation of organochlorines by illumina platform

Saghee Madhu Raju[1,2] and Rajkumar Bidlan[2#]

[1]*Research Scholar, Rayalaseema University, Kurnool, India*
[2]*Dr. Bidlan's Research Institute, Hyderabad, India*
[#]*Present Address: Department of Biotechnology, Delhi Technological University, Shahbad Daulatpur, Delhi, India*

ABSTRACT

Organochlorine pesticides are ubiquitous group of recalcitrant molecules that accumulate in food chains and have inherent toxic effects and adverse health effects. To circumvent the problem, microbial communities are found to be promising candidates for degrading the organochlorine pesticide's and removal of residues. In this study, a novel microbial consortium isolated from Yamuna and Godavari rivers capable of simultaneous biodegradation of organochlorine pesticides (DDT and Lindane) was subjected to metagenomic sequencing. This consortia used was enriched by progressively increasing concentrations of Lindane and DDT (organochlorine pesticides) for months till a stable Lindane and DDT tolerant population was established, and found to be degrading mixture of organochlorine pesticides with concentrations up to 30 ppm of DDT and Lindane. Currently, in the realm of our knowledge very few metagenomic analysis were carried out to characterize the consortia and understand the biodiversity of microbial communities in the riverine ecosystems, that was found to be unique and highly efficient in bio-degradation of organochlorine pesticides. The study concluded biodiversity with a shannon alpha-diversity index of 3.0317 and identified 871 species with *Brevundimonas diminuta* (previously assigned to the genus *Pseudomanas*) having abundance ratio of 17.57 % followed by *Stenotrophomonas acidaminiphila* in the mixed consortium and deciphered the systematic and functional contexts within riverine metagenome.

KEY WORDS: MICROBIAL CONSORTIUM, BIOREMEDIATION, DICHLORODIPHENYLTRICHLOROETHANE, HEXACHLOROCYCLOHEXANE, LINDANE, METAGENOMICS, AMPLICON, ILLUMINA

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INTRODUCTION

Organochlorine pesticides (OCPs) were excessively used globally for pest control and agricultural purposes and public health control (Aktar *et al.,* 2009). OCPs are ubiquitous group of recalcitrant molecules that degrade slowly and accumulate through food chains (Amrita *et al.,* 2007) and produce a significant magnification at each tropic level. One of the major sinks for persistent organic pollutants discharged into environment is the water ecosystem i.e. rivers and lake beds. Organochlorine pesticides were detected in rivers where higher concentrations of Lindane, Endosulfan and DDT were found (Pandey *et al.,* 2011)and the residue presence was even detected in drinking and bottled water (Mutiyar *et al.,* 2011). It is highly essential and vital to remove these pollutants from the environment, from the sinks primarily water and soil ecosystems to finally eliminate their residues. Microorganisms are found to be potential degraders of organochlorine compounds, notably water and soil habitants belonging to genera *Bacillus*, *Pseudomonas*, *Arthrobacter, Klebsiella, Acinetobacter, Alcaligenes, Flavobacterium* and *Micrococcus* were found to be effective bio-degraders (Kafilzadeh *et al.,* 2014 Eric et al 2017).

In this paper, we present the findings of metagenomic analysis leveraging next-generation sequencing (NGS) performed using Hiseq 2500 system (Kumar *et al.,* 2015). The Metagenomics was carried on the defined microbial consortium identified from water ecosystems, Yamuna River (North India) and Godavari River (South India) capable of simultaneous degradation of organochlorine pesticides (Bidlan, 2003). The taxonomic distribution and biodiversity among the microbial consortium was established that comprised of interacting microbial populations (Oulas A *et al.,* 2015 Eric *et al*., 2017).

MATERIALS AND METHODS

Lindane γ-HCH (insecticidal isomer) was of 97% purity and obtained from Sigma- Aldrich, USA. DDT, 99.4% pure, was donated by Hindustan Insecticides Ltd, India. All other chemicals and reagents used in the study were of analytical grade and were purchased from standard manufacturers. The microbial consortium subjected to Metagenomic analysis was isolated from Yamuna (North India) and Godavari rivers (South India) and enriched by progressively increasing concentrations of Lindane and DDT (organochlorine pesticides) for months till a stable Lindane and DDT tolerant population was established in the flask (Bidlan 2003). DNA was isolated using Xcelgen Bacterial gDNA kit and quality of gDNA was checked on 0.8 % agarose gel (loaded 5 µl) for the single intact band. The gel was run at 110 V for 30 min. 1 µl of each sample was loaded in Nanodrop 8000 for determining A260/280 ratio. The DNA was quantified using QubitdsDNA HS Assay kit (Life Tech). 1 µl of each sample was used for determining concentration using Qubit® 2.0 Fluorometer (Ogata *et al.,* 1990).

The amplicon library was prepared using Nextera XT Index Kit (Illuminainc) as per the 16S Metagenomic Sequencing Library preparation (Eric J. *et al.,* 2017). Primers for the amplification of the V3-V4 hyper-variable region of 16S rDNA gene of bacteria and Archaea are used for this study (Table-1).

The amplicons with the Illumina adaptors were amplified by using i5 and i7 primers that add multiplexing index sequences as well as common adapters required for cluster generation (P5 and P7) as per the standard Illumina requirements (Esling *et al.,* 2015). The amplicon libraries were purified by 1X AMpureXP beads and checked on Agilent High Sensitivity (HS) chip on Bioanalyzer 2100 and quantified on fluorometer by QubitdsDNA HS Assay kit (Life Technologies).

After obtaining the Qubit concentration for the library and the mean peak size from Bioanalyser profile, library was loaded onto HiSeq 2500 at appropriate concentration (10-20 pM) for cluster generation and sequencing (Sharpton, 2014). Paired-End sequencing allows the template fragments to be sequenced in both the forward and reverse directions. Kit reagents were used in binding of samples to complementary adapter oligos on flow cell. The adapters were designed to allow selective cleavage of the forward strands after re-synthesis of the reverse strand during sequencing. The copied reverse strand was then used to sequence from the opposite end of the fragments (Blomquist *et al.,* 2013).

The libraries were prepared from sample after amplifying the V3-V4 region of the 16S segment. Size of library was 644 bp and the library was sequenced using the Illumina sequencing chemistry to generate ~150 Mb of data per sample. The next generation sequencing (NGS) for the sample was performed on the Illumina platform, HiSeq 2500 (Kumar *et al.,* 2015).

Paired end sequence stitching was carried out for sample using FLASH (Fast Length Adjustment of Short reads) with parameter minimum overlap of 10 bases to merge paired-end reads from next-generation sequencing experiments (Tanja *et al.,* 2011). QIIME (Quantitative Insight into Microbial Ecology) was used for analyzing 16S metagenome data from NGS platforms and, is implemented in python language (Kuczynski *et al.,* 2011). Chimeras composed of DNA from two or more microbial species which are artifacts made during the PCR process. They were filtered first, using usearch61 algorithm (de novo, abundance-based), from the Flashed/stitched data then taken for analysis. A total of 2,44,283 non chimeric sequences from sample were used for OTU pick. In the next step, the similar sequences were clustered,

FIGURE 1. QC of gDNA on 0.8% agarose gel

Table 1. Primers used in the Study

Oligo Name	Oligo Sequence (5' to 3')	Length of primer	Product size (Approx.)
Prokaryote V3-Forward	CCTACGGGNBGCASCAG	17	~ 460 bps
Prokaryote V4-Reverse	GACTACNVGGGTATCTAATCC	21	

i.e., sequences coming from the same genus, together into one representative taxonomic unit called as Operational Taxonomic Unit (OTU). The basis of this sequence clustering is 97% sequence similarity and implemented through UCLUST algorithm. OTU-picking identified highly similar sequences across the samples and provided a platform for comparisons of community structure. All the sequences from all the samples were clustered into Operational Taxonomic Units (OTUs) based on their sequence similarity.

A representative sequence was selected for each of these OTU's picked. As these OTU's made up of a group of sequences, they were represented through one sequence to assign a taxonomic name to the group. Thus repre-

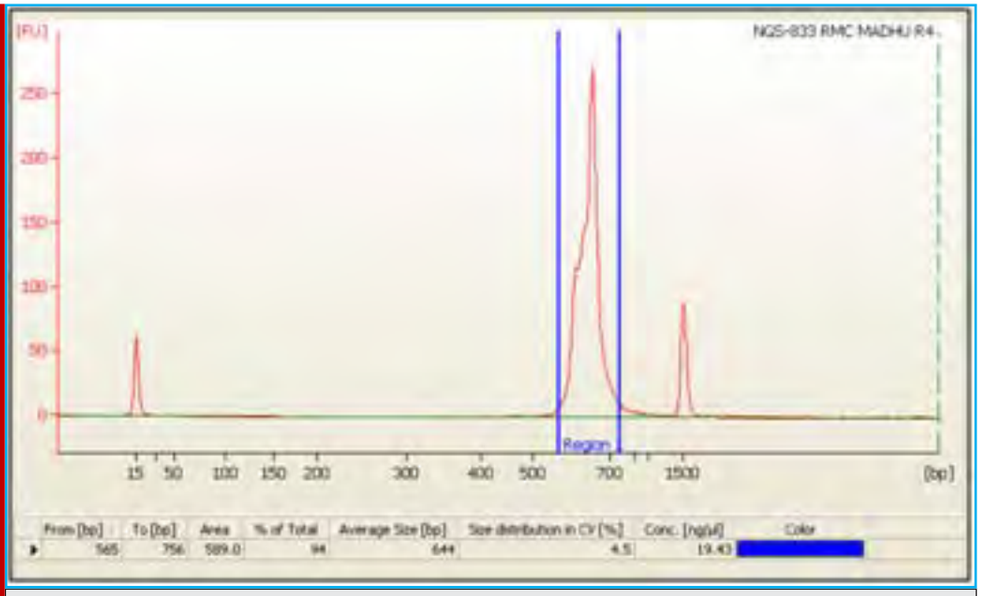

FIGURE 2. Bioanalyzer profile of the Consortium on DNA 1000 chip.

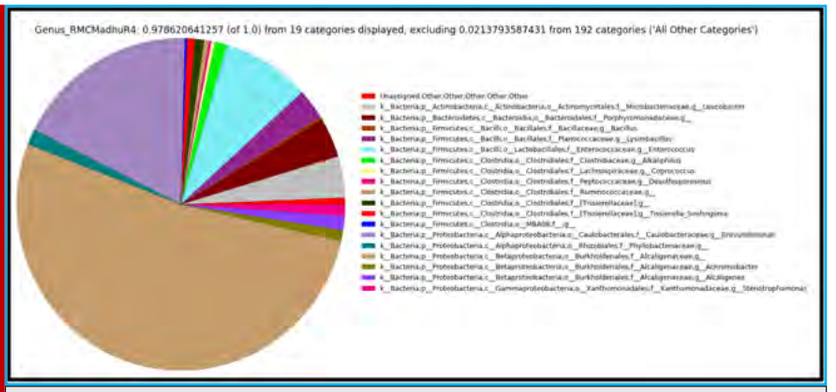

FIGURE 3. Taxonomic distribution of defined consortium at genus level showing the relative abundance of each genus within microbial community.

Table 2. Relative Abundance of the genus in the consortium

Taxonomy (Genus)	Abundance
Brevundimonas	17.60%
Enterococcus	8.50%
Leucobacter	3.90%
Lysinibacillus	2.90%
Alcaligenes	1.40%

Table 3. Alpha-diversity

Sample	shannon	Observed species	chao1
Consortiaenriched with pesticides	3.0317	871	871

sentative set of OTUs were prepared which consist of 2,911 sequences. With representative sequence in hand, the taxonomic names to these sequences were assigned at 90% sequence similarity. This is done using UCLUST algorithm, where query is representative sequences and subjects that are curated sequences at greengenes database.

RESULTS AND DISCUSSION

Diversity calculation for each sample was performed and compared the types of communities, using the taxonomic assignments.

α-DIVERSITY

α-Diversity or within-sample diversity is calculated using an OTU table which gives idea about species richness. Alpha diversity summarizes the diversity of organisms in a sample using different metrics in a habi-

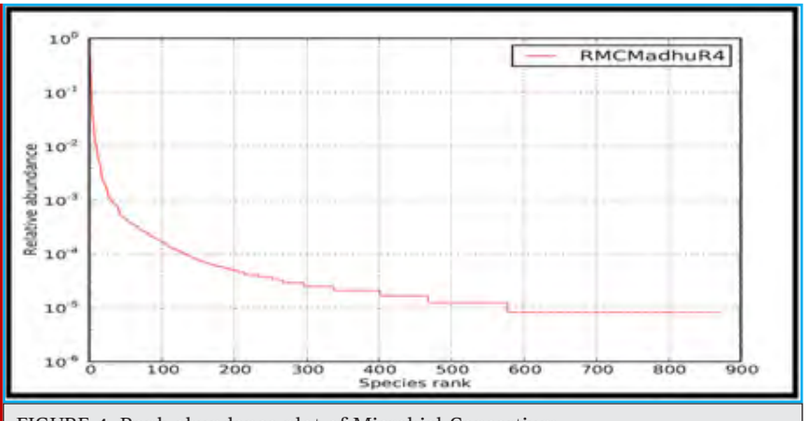

FIGURE 4. Rank abundance plot of Microbial Consortium

FIGURE 5. An OTU table heat map showing taxonomy assignment. The OTU heatmap displays raw OTU counts per sample, where the counts are colored based on the contribution of each OTU to the total OTU count present in that sample (blue: contributes low percentage of OTUs to sample; red: contributes high percentage of OTUs).

tat/sample. The below table summarizes the α-Diversity, where the columns correspond to alpha diversity metrics and the rows correspond to samples and their calculated diversity measurements (Lozupone, Catherine *et al.,* 2007).

The rank abundance curve representing species richness and species evenness is shown in Figure 4. Species richness can be viewed as the number of different species on the chart and species evenness is derived from the slope of the line that fits the graph.

The OTU table was developed to visualise as a heatmap where each row corresponds to an OTU and each column corresponds to a sample. The higher the relative abundance of an OTU in a sample, the more intense the color at the corresponding position in the heatmap.

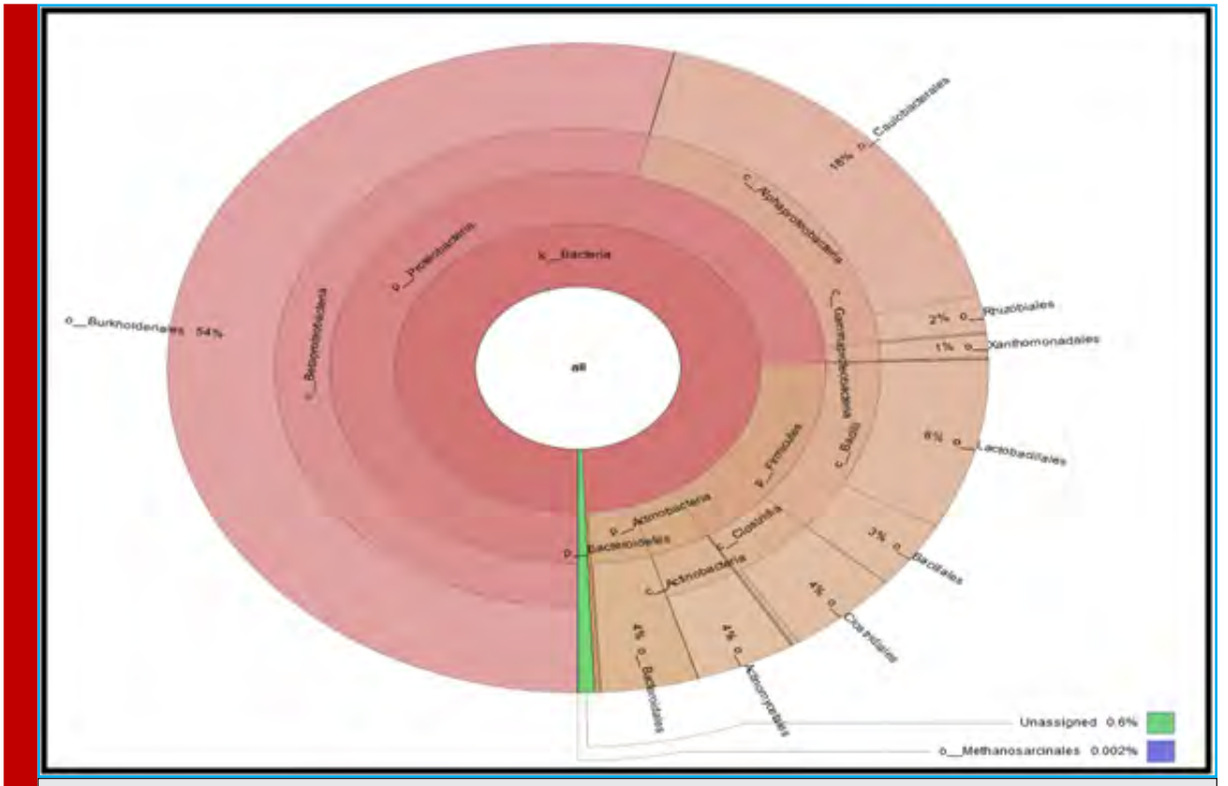

FIGURE 6. Krona graph for taxonomy assignment for Microbial Consortium at order level.

Table 4. Organisms Identified through Metagenomic Characterization by Hiseq2500, Illumina Platform, NGS	
Total Reads	**5,88,408**
Total number of stitched reads	2,81,957
Number of OTUs	2,911
Abundant phylum	Proteobacteria
Abundant class	Betaproteobacteria
Abundant order	Burkholderiales
Abundant family	Alcaligenaceae
Abundant genus	Brevundimonas
Abundant species	dimunita
shannon alpha-diversity	3.0317
Observed species	871

Krona graph tool was used to display abundance and hierarchy simultaneously using a radial space-filling display. The Krona chart features a red-green colour gradient signifying average value within each taxon (Ondov *et al.,* 2011)

CONCLUSION

The metagenomic sequencing comprehensively sampled all genes in all organisms present in microbial consortia and evaluated bacterial diversity and abundance of microbes (Table-4). This study also identified at genotypic level any unculturable microorganisms that are otherwise difficult or impossible to analyze (Handelsman J. *et al.,* 2004). The study concluded biodiversity with a shannon alpha-diversity of 3.0317 and identified 871 species genotypically, with *Brevundimonas diminuta* having abundance ratio of 17.57 % followed by *Stenotrophomonas acidaminiphila* in the mixed consortium. This consortia characterized was found to be degrading mixture of organochlorine pesticides with concentrations up to 30 ppm of DDT and Lindane confirmed by GC-MS/MS. Although research has been carried out using on single strain and single compound of organochlorines, the current study data provides an insight on how bacterial communities in mixed consortia are taxonomically distributed and their biodiversity. The metagenomic characterization identified the consortia in a definitive manner which acts as promising solution for bioremediation of organochlorine mixtures.

NCBI Sequence Accession Number: DNA sequences obtained have been deposited at National Center for Biotechnology Information (NCBI) Sequence Read Archive under the bioproject ID PRJNA420925 and accession codeSRX348847.

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Wettability alteration in enhanced oil recovery process using new amphoteric and cationic surfactants

Reza Qanbari Moqaddam Nouqabi[1], Ghasem Zargar[2]*, Mohammad Ali Takassi[3] and Siyamak Moradi[4]

[1]M.S. Student of Petroleum Exploration Engineering, Abadan Faculty of Petroleum Engineering, Petroleum University of Technology, Northern Bowarde, Abadan, Iran
[2]Department of Petroleum Exploration Engineering, Petroleum University of Technology, Northern Bowarde, Abadan, Iran
[3]The Department of Science, Petroleum University of Technology, Ahwaz, Iran
[4]Department of Petroleum Exploration Engineering, Petroleum University of Technology, Northern Bowarde, Abadan, Iran

ABSTRACT

Over time, production of hydrocarbons decreases due to sequential producing and nowadays using Enhanced oil recovery (EOR) methods is a necessity. One of the methods in order to improve the oil recovery is altering the rock wettability toward water-wet by using Surfactant flooding. Surfactants have a variety of applications in the petroleum industry due to their remarkable ability to lower the oil-water interfacial tension and alter wettability. In this study new cationic and amphoteric surfactants synthesis and investigation of wettability alteration in EOR process is described. The goal of this work is to compare the wettability of a carbonate rocks from oil (mixed)-wet towards water-wet. Changing the wettability to preferentially water-wet condition will reduce the residual oil saturation (Sor). Wettability alteration is measured based on the contact angle method.

KEY WORDS: EOR, PETROLEUM, WETTABILITY, CTAB, HABSA

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INTRODUCTION

Oil recovery from a reservoir can be divided into three steps which are primary recovery, secondary recovery and tertiary recovery. Further discussion will be well served by a brief review of the "primary", "secondary" and "tertiary" terms. These terms are generally understood and accepted (although a formal definition of these terms does not exist, either). They reflect and describe the natural progression of oil production from its inception to the point where economic production is no longer feasible. Production depends on the natural energy of the reservoir itself. The natural energy varies from pressure decline and the accompanying evolution of dissolved gas, to the expansion of gas cap, or the influx of water. The key element forces are "natural". When natural drive energy is depleted, or too small for economic oil recovery, energy must be added to the reservoir to permit additional oil recovery. That additional energy is usually in the form of injected water or gas. The process depends mainly on physical displacement to recover additional oil. It can be said that it mimics the natural process of water influx or gas expansion. The key element forces are not natural; rather they are physical, as opposed to thermal, chemical, solvent, interfacial tension, etc. One could think of these as being a physical augmentation of the natural drive mechanism, (Stosur et al 2003 and Ge and Wang, 2015).

When secondary recovery is no longer economic, supplemental energy of a different kind permits additional oil recovery. A critical distinction that should be noted is that this energy (ies) is (are) in addition to, or in lieu of the natural or physical displacement mechanisms of the primary or secondary methods. Enhanced fluid flow conditions within the reservoir are usually induced by addition of heat, chemical interaction between the injected fluid and the reservoir oil, mass transfer, and/or changing of oil properties in such a way that the process facilitates oil movement through the reservoir. Tertiary recovery processes generally include *thermal, chemical, gas miscible and microbial*. They are also often referred to as enhanced oil recovery (EOR) processes Almost half of the world's discovered oil reserves are located in carbonate fractured formations, which are mostly oil-wet . These oil reservoirs are good candidates for enhanced oil recovery if the wettability of the matrixes is altered more toward water-wetness. Sandstone reservoirs are more complex than carbonate reservoirs. The wettability of sandstone reservoirs may vary widely from strongly water-wet to strongly oil-wet states. Neutral or intermediate wettability is also common, (El Mofty 2012, Ge and Wang 2015 Mohammed and Babadagli 2015).

Buckley and Leverett (1942) published one of the first papers on the effect of wettability on oil recovery. Since then, studies have continuously debated the optimum wettability that provides maximum oil recovery. Recently, Enhanced Oil Recovery methods based on chemically-induced wettability alteration have gained a great deal of attention. Yong Zhu et al. (2012) investigated the adsorption of cationic-nonionic mixed surfactant (HDPB/TX100) onto bentonite and showed the cationic surfactant improved the adsorption of TX100 and total adsorbed amount significantly, indicating the good synergistic effect between HDPB and TX100. The co-adsorption of the cationic and nonionic surfactants increased the ordering conformation of the adsorbed surfactants on bentonite, but decreased the thermal stability of the organo bentonite system. The goal of this study is to describe the wettability of reservoirs and some surfactants, in addition to their measures and method. The motivation behind this approach is to keep the injection architecture similar to that of waterflood.

MAIN SUBJECTS

Wettability is defined as "the tendency of one fluid to spread on or adhere to a solid surface in the presence of other immiscible fluids.Types of wettability are divided into 3 classes: (1) Strong Wettability, (2) Neutral Wettability and (3) Fractional Wettability which is described below: *Strong Wettability*: this class is divided into 2 types as below: *Water-Wet:* When the rock is water-wet, there is a tendency for water to occupy the small pores and to contact the majority of the rock surface Anderson (1986). *Oil-Wet*: Similarly, in an oil-wet system, the rock is preferentially in contact with the oil; the location of the two fluids is reversed from the water-wet case, and oil will occupy the small pores and contact tie majority of the rock surface. *Neutral Wettability*: When the rock has no strong preference for either oil or water, the system is said to be of neutral (or intermediate) wettability. *Fractional Wettability*: Besides strong and neutral wettability, a third type is fractional wettability, where different areas of the core have different wetting preferences (Fall 2016).

Almost all minerals in a natural, clean state exhibit water-wet behavior. Certain components, primarily heavy asphaltene and the resin fractions of crude oil, can alter the wettability of the original water-wet rock. Components carrying a charged group, such as an acid or a base, significantly affect wettability during the formation of the reservoir. Additional significant components include oil and mineral composition, water solubility of polar oil components, capillary pressure and thin film forces. Mohammed and Babadagli (2012). Temperature, salinity, pressure and initial water saturation can affect the degree of wettability alteration as well.

Many different methods have been proposed for measuring the wettability of a system. They include

quantitative methods such as contact angles, imbibition and forced displacement (Amott), and USBM wettability method and qualitative methods such as imbibition rates, microscope examination, flotation, glass slide method, relative permeability curves, permeability/saturation relationships, capillary pressure curves, capillary metric method, displacement capillary pressure, reservoir logs, nuclear magnetic resonance, and dye adsorption. Although no single accepted method exists, three quantitative methods generally are used: (1) contact-angle measurement, (2) the Amott, and (3) the USBM method. The contact angle measures the wettability of a specific surface, while the Amott and USBM methods measure the average wettability of a core (Anderson 1986).

This is an imbibition-based method to measure the wettability of a core. The principle is that the wetting fluid will spontaneously imbibe into a core and displace the non-wetting fluid. The experiment begins with a restored state core sample at irreducible water saturation (Swirr) and high initial oil saturation. In this method drainage and imbibition capillary pressures are measured through centrifuge tests. The sample is saturated initially with water. The water is then displaced by oil to irreducible water saturation (Swi) using the centrifuge. Afterward, the sample which contains initial oil saturation and irreducible water saturation (Swi) is then centrifuged in water to residual oil saturation (Sor). Qualitative methods for wettability measurement are: imbibition rates, microscope examination, flotation, glass slide method, relative permeability curves, permeability/saturation relationships, capillary pressure curves, capillary metric method, displacement capillary pressure, reservoir logs, nuclear magnetic resonance and dye adsorption. In below explain some important qualitative methods for wettability measurement:

Wettability alteration

Changing the wetting state of materials is a growing field of research in many areas of engineering and science. In the oil industry, the term wettability alteration usually refers to the process of making the reservoir rock more water-wet. This is of particular importance in naturally hydrophobic carbonates, fractured formations, and heavy-oil systems. This shift in wettability enhances oil recovery in oil-wet and weakly water-wet reservoirs and eventually increases the ultimate oil recovery.Wettability alteration process in each reservoir is a unique process and requires the understanding of the mechanisms that caused a reservoir to be oil-wet.Wettability alteration may increase oil recovery by gravity or capillary imbibition, (Mohammed and Babadagli 2012).

Surfactants may be one of the best options to improve recovery from geologically challenging reservoirs. During recent years, depressed oil prices have limited surfactant consideration. However, surfactant recovery can be economically attractive for reservoirs where recovery is dominated by gravity and imbibition processes. Surfactant is an abbreviation for surface active agent, which literally means active at a surface Holmberg et al., (2002).

It is common practice to divide surfactants into the categories anionics, cationics, non-ionics and zwitterionics as following classification: Anionics are used in greater volume than any other surfactant class. Important facts about anionic surfactants: 1. They are by far the largest surfactant class. 2. They are generally not compatible with cationics (although there are important exceptions). 3. They are generally sensitive to hard water. Sensitivity decreases in the order carboxylate > phosphate > sulfate ~ sulfonate. 4. Sulfates are rapidly hydrolysed by acids in an autocatalytic process. The other types are stable unless extreme conditions are used Holmberg et al.,2002).

Nonionic surfactants come as a close second with about 45% of the overall industrial production. They do not ionize in aqueous solution, because their hydrophilic group is of a non-dissociable type, such as alcohol, phenol, ether, ester, or amide. Important facts about nonionic surfactants: 1. They are the second largest class of surfactant. 2. They are normally compatible with all other types of surfactants. 3. They are not sensitive to hard water. 4. Contrary to ionic surfactants, their physicochemical properties are not markedly affected by electrolytes. 5. The physicochemical properties of ethoxylates are very temperature dependent. Contrary to most organic compounds they become less water soluble – more hydrophobic – at higher temperatures (Holmberg et al.,2014).

Cationic Surfactants are dissociated in water into an amphiphilic cation and an anion, most often of the halogen type. A very large proportion of this class corresponds to nitrogen compounds such as fatty amine salts and quaternary ammoniums.Important facts about cationic surfactants: 1. They are the third largest surfactant class. 2. They are generally not compatible with anionics (although there are important exceptions). 3. Hydrolytically stable cationics show higher aquatic toxicity than most other classes of surfactants. 4. They adsorb strongly to most surfaces and their main uses are related to *in situ* surface modification (Holmberg et al., 2014).

Zwitterionic surfactants contain two charged groups of different sign. Whereas the positive charge is almost invariably ammonium, the source of negative charge may vary, although carboxylate is by far the most common. Zwitterionics are often referred to as *amphoterics* .Important facts about zwitterionic surfactants: 1. They are the smallest class of surfactant (partly due to high price). 2. They are normally compatible with all other types of surfactants. 3. They are not sensitive to hard

water. 4. They are generally stable in acids and bases. The betaines, in particular, retain their surface activity at high pH, which is unusual. 5. Most types show very low eye and skin irritation. They are, therefore, well suited for use in shampoos and other personal care products Holmberg et al., 2014),

MATERIALS AND METHODS

Two surfactants, one new amphoteric and one cationic surfactant are considered in this study. Initial surfactant is hexadecylaminobenzenesulfonic acid (HABSA) which recognized amphoteric surfactant. HABSA formulation is (C16H33C6H3NH2SO3H) that show, when it dissolves in water, it contains two charged groups of different sign at its head and a long alkyl tail. The second surfactant is Cetrimonium bromide ((C16H33)N(CH3)3Br, cetyltrimethyl ammonium bromide, hexadecyl trimethyl ammonium bromide, CTAB) which is one of the components of the topical antiseptic cetrimide. The cetrimonium cation is an effective antiseptic agent against bacteria and fungi. It is a cationic surfactant. Its uses include providing a buffer solution for the extraction of DNA. It has been widely used in synthesis of gold nanoparticles (e.g., spheres, rods, and bipyramids). It is also widely used in hair conditioning products. Because of Property soapy this is a good candidate for chemical oil recovery in world (Ito et al 2016).

Experimental procedures

Synthesis of new surfactants: Two different surfactants are considered in this study that synthesized in PUT lab in Ahwaz: New amphoteric surfactant (hexadecylaminobenzenesulfonic acid (C16H33C6H3NH2SO3H), HABSA) A cationic surfactant (hexadecyltrimethylammonium bromide ((C16H33)N(CH3)3Br), CTAB)

Synthesizing procedure of HABSA:

5 mL of concentrated hydrochloric acid and 54 mmol of ortho-sulfanilic acid are added to 250 mL beaker (A) with 125 mL water. It is stirred until a homogenous solution is obtained. 6.5 mL (69 mmol) of acetic anhydride is added to this mixture. To another 250 mL beaker (B), 5.6 g (69 mmol) of sodium acetate is dissolved in 35 mL of water. Then the content of beaker A is added to beaker B and the mixture is vigorously stirred in an ice bath. A white precipitate (compound 1) is obtained. It is collected by filtration and dried in vacuum oven at 80 °C. Anhydrous aluminum chloride (0.13 g, 1.0 mmol) is weighed into an aluminum weighing boat in the fume hood and quickly transferred to a clean dry 100 mL round bottom flask containing a magnetic stir bar. The flask is stoppered and brought to the bench where it is fitted with a Claisen adaptor, a dropping funnel, and a condenser vented to a gas trap. 3.46 g (15 mmol) of the aforementioned white product (1) and 20 mL of acetonitrile are added to the flask. While rapidly stirring the mixture, 15 mmol hexadecyl bromide is added slowly drop wise over a period of about 10 minutes. After the addition is completed, the stirring is continued at reflux temperature for an additional 24 h. Then the reaction mixture is cooled to room temperature. The resulting product (2) is collected by filtration and dried under reduced pressure at 80 °C. Into a 100 mL round-bottomed flask equipped with a condenser and a magnetic stirring bar, 7.6 mmol of compound 2 and 17 mL of a 5.0 M hydrochloric acid solution are added and refluxed. After 10 minutes, the reaction mixture is cooled to room temperature. On completion of the reaction, the solution is neutralized with 25% w/w sodium hydroxide solution, and a precipitate is formed slowly. (Yield = 80%, m.p. 283-284 °C).

Synthesizing procedure of CTAB:

10 ml of hexadecyl bromide (C16H33Br) is placed in a 250 mL round-bottomed flask, and 5 ml of tri Methylamine [(CH3)3N] and 100 ml of solvent acetonitrile (CH3CN) are added to the flask. A magnetic stirring rod is placed in the flask. The flask continent is heated under reflux and stirred using a magnetic stirrer for 24 hours. The solution is cooled to room temperature. The product is formed as a white precipitated. The product is collected ished with small amount of acetonitrile then air dried.

RESULTS AND DISCUSSION

There are many ways in which CMC could be determined. The CMC is the narrow concentration range over which amphiphilic or surfactant solutions show an abrupt change in a physical property such as electrical conductivity, surface tension, osmotic pressure, density, light scattering or refractive index Hoolmberg et al (2002). The conductance of a solution, can give important quantitative information regarding the ionic composition of a sample. Conductance is a measure of a sample's ability to pass a current and strongly depends on the concentration, mobility, and charge of ions in solution (Settle 2017).

The Jenway model 4510 Conductivity/temp meter with dual display and TDS range is easy to use with a flexibility that will enable it to meet the broadest range of applications. Set-up menu options include cell constant, temperature coefficient and reference temperature. With automatic range selection and endpoint detection, readings can be taken quickly and with minimum intervention. For applications where greater accuracy is required the 4510 has automatic conductivity standard recognition which can be overridden by entry of user

specific values. This setup includes following issues: ■ Auto ranging to give best resolution ■ Simultaneous display of conductivity or TDS and temperature ■ Calibration to cell constant or standard solutions ■ Auto Standard recognition with manual override ■ 32 location memory ■ Bi-directional RS232 link to printer or PC Technical specification, (Fall 2016).

When conductivity meter is used to find the CMC, conductivity of the solution increases linearly with total surfactant concentration. However, the slope of the lines has an inflection point that indicates the CMC.The pellets and plug are cleaned by Toluene with Soxhlet extractor. Two main reasons to clean core are: To remove all liquids from the core so that porosity, permeability, and fluid saturations can be measured. To clean the core as a first step in restoring the wettability of cores are altered. Distilled water is used as the aqueous phase for contact angle, flooding tests and solutions. One of the best wettability measurement methods when pure fluids and artificial cores are used is the contact angle.

In the sessile drop method the flat surface of pellet is suspended horizontally in the oil (kerosene) and placed a drop of water on the surface of the pellet. Then the contact angle between water drop, slice surface and oil is measured. When θ is between 0° and 60° to 75° in such a system, it is defined as water-wet. When θ is between 105° to 120° and 180° the system is defined as oil-wet. In the range of a 75° to 105° contact angle, the system is neutral-wet.

After preparing and cleaning the core sample, it is saturated with distilled water by vacuumed pump. Then the core is placed in the rubber sleeve in the core holder. This sleeve is used as a connection to exert overburden pressure on the rock. In these experiments the overburden pressure is provided by water (2500 psi). To reach Swi, injection of oil is continued until no water is detected at the outlet. Volume of discharged water is measured and the Swi is calculated by: S_{wi} = 1 – (Volume of produced water/Pore volume)Now to reach residual oil saturation Sor, distilled water is injected into the core plug at constant flow rate of 1 cc/min until no oil is produced at the out let. Difference between injected oil volume and produced oil divided to total pore volume indicates residual oil saturation, Sor. The core sample is flooded with two surfactants (at CMC). At this step, core holder is connected to another transfer vessel and surfactant solution is injected into the core with a constant rate. The oil produced would be measured to calculate the oil recovery.

CONCLUSION

This study was conducted to compare wettability alteration in EOR process, using new amphoteric and cationic surfactants. New cationic and amphoteric surfactants synthesis and investigation of wettability alteration in EOR process is described. The goal of this work is to compare the wettability of a carbonate rocks from oil (mixed)-wet towards water-wet. Changing the wettability to preferentially water-wet condition will reduce the residual oil saturation (Sor). Wettability alteration is measured based on the contact angle method. Cationic Surfactants are in general more expensive than anionic ones, because of the high pressure hydrogenation reaction to be carried out during their synthesis. As a consequence, they are only used in two cases in which there is no cheaper substitute.

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Diabetic Predecessors: A factor leading to diabetes in successors

Manju Dewan

Assistant Professor, Post Graduate Department of Zoology, DAV College, Sector 10, Chandigarh

ABSTRACT

Type 2 diabetes has several causes: genetics and lifestyle are the most important ones. A combination of these factors can cause insulin resistance. Diabetes is a complex condition. Several factors must come together to develop type 2 diabetes. In the total population of 2048 subjects, 558 subjects had the positive family history of diabetes. Out of 558 subjects 12 (2.15%) subjects were found to have impaired glucose level, 12 (2.15%) subjects have already developed diabetes and both have family history diabetes in first degree relatives (Father, Mother, Father Mother both). In the present study, 558 (27.3%) subjects have shown positive family history for diabetes.

KEY WORDS: TYPE 2 DIABETES, FAMILY HISTORY, FATHER, MOTHER

INTRODUCTION

Heredity plays an important role in determining the susceptibility to diabetes mellitus. Diabetes mellitus is multifactor in its etiology.A significantly greater frequency of diabetes has been found in close blood relatives of diabetic than in other population.Prevalence of overweight and obesityweremore in children with family history of diabetes and obesity.Environmental factors play a major role in the development of diabetes. Environmental risks factors known to influence the development of obesity and type 2 diabetes due to sedentary lifestyle, wrong eating habits and stress. Other nutritional factors and toxins may also play a crucial role (Adeghate et al, 2006). These environmental factors clearly play a major role in the development of diabetes, but they do not impact everyone in the same way (Omar 2013). Family history of diabetes (FHD) and lifestyle are associated with type 2 diabetes (Yanyan et al, 2017). Nina et al, 2016 also found that children had a strong family history of cardiovascular disease and diabetes. The estimated risk for the diagnosis of type 2 diabetes (T2DM) increases approximately by 2-4 times, when father, mother or both have this condition. Conversely, many T2DM patients have family members with DM (Papazafiropoulou et al, 2017). The modern generalization of

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sedentary life and caloric abundance has created new physiological conditions capable of changing the level of expression of a number of genes involved in fuel metabolism and body weight regulation.

MATERIALS AND METHODS

2048 children had undergone questionnaire and dietary survey and health examination. Out of these, 1017 were from urban population and 1031 from rural population Children and adolescent aged 10-19 years were selected randomly for questioning regarding the different aspects of epidemiology and their health examination was done. The permission from parents of the children, undergoing examination and questionnaire survey was also taken on the self designed consent form.The Centers for Disease Control and Prevention (CDC) suggests two levels of concern for children based on the BMI-for-age charts.

At the 85th percentile and above, children are "at risk for overweight". At the 95th percentile or above, they are "overweight". The cutoff for underweight of less than the 5th percentile is based on recommendations by the World Health Organization Expert Committee on Physical Status 1998. The diagnostic criteria for diabetes mellitus have been modified from those previously recommended by WHO (1985).Revised criteria from the report of the expert committee on the diagnosis and classification of Diabetes Mellitus (2003) was used for the diagnosis of diabetes. Same criterion was used by Holly et al, 2003, Jung-Nan et al, 2003 and Reinehr (2013)

RESULTS AND DISCUSSION

Type 2 diabetes has several causes: genetics and lifestyle are the most important ones. A combination of these factors can cause insulin resistance. Diabetes is a complex condition. Several factors must come together to develop type 2 diabetes. For example, obesity and a sedentary lifestyle play a role. Genetics can also influence obesity and type 2 diabetes. Type 2 diabetes is caused by both genetic and environmental factors. Obesity has been attributed to various factors including genetics, environment, metabolism, behavior, personal history of obesity, culture, and socioeconomic status .In the total population of 2048 subjects, 558 subjects had the positive family history of diabetes. Out of 558 subjects 12 (2.15%) subjects were found to have impaired glucose level, 12 (2.15%) subjects have already developed diabetes and both have family history diabetes in first degree relatives (Father, Mother, Father Mother both) Table 1 and graphs 1&2.

The lifetime risk of developing type 2 diabetes is 40% for individuals who have one parent with type 2 diabetes

Table 1. Prevalence of Family History of diabetes.

	Father	Mother	
All Data	106 69.28	47 30.72	N.A.:1895 (92.53%)
FBG Categories			
1. <110		102 70.83	42 29.17
2. 110-126	2 40.00	3 60.00	
3. >=126	2 50.00	2 50.00	Chi^2=2.88(df:2)
			C=0.14;
BMI Categories			
1. <18.5	195 54.78	161 45.22	
2. 18.5-22.9	69 52.67	62 47.33	
3. 23.0-24.9	15 51.72	14 48.28	
4. 25.0-29.9	15 45.45	18 54.55	
5. >30.0	3 33.33	6 66.67	Chi^2=2.62(df:4)
			C=0.07;
BMI Categories			
1. <18.5	195 54.78	161 45.22	
2. 18.5-22.9	69 52.67	62 47.33	
3. 23.0-27.5	28 50.91	27 49.09	
4. >27.5	5 31.25	11 68.75	Chi^2=3.58(df:3)
			C=0.08;
Percentile Based			
1.Under Wt.	76 55.88	60 44.12	
2.Healthy Wt.	186 53.14	164 46.86	
3.At Risk	24 60.00	16 40.00	
4.Over Wt.	11 34.38	21 65.62	Chi^2=5.69(df:3)
			C=0.10;
W/H based (All)			
1.Normal	255 52.80	228 47.20	
2.Over Wt.	42 56.00	33 44.00	Chi^2=0.27(df:1)
			C=0.02;

and 70% if both parents are affected. Type 2 diabetes are about 3 times more likely to develop the disease than individuals without a positive family history of the disease (Florez et al,2003). Type 2 diabetes itself is thought to be a polygenic disorder that develops due to complex interaction between multiple genes and environmental factors.For type 2 diabetes, risk for developing the disease is increased if a close family member (parent, sibling, or child) has type 2 diabetes or a medical condition being overweight or obese, having lipid abnormalities, or high blood pressure (Gibson, 2011).

In the present study, 558 (27.3%) subjects have shown positive family history for diabetes.The increased prevalence of type 2 diabetes in the relatives of affected subjects is likely to reflect genetic predisposition to hyperglcaemia with additional affects from shared environment

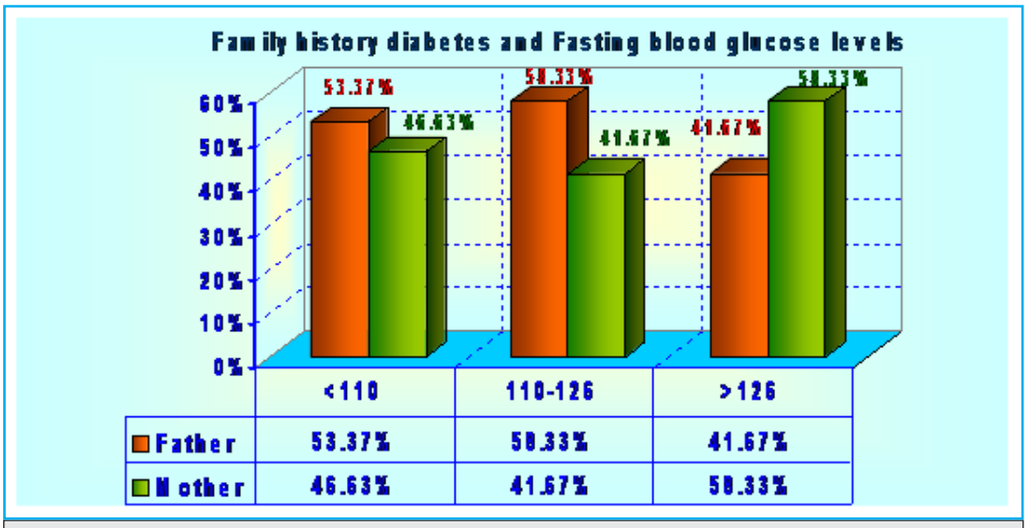

FIGURE 1.

and life style (Shaw et al., 1999). Type 2 diabetes is recognized to arise from a combination of insulin resistance and impaired beta cell function. There are several reasons to consider that this could be particularly important in the obesity-diabetes field.(Ganada and Soeldner, 1987).

The first reason is that evolutionary forces may have shaped the human genome according to mechanisms (fat storage and mobilization, insulin secretion and sensitivity, leptin signaling, weight and body composition regulation, availability of glucose to the brain, etc.) that are now directly involved in the pathophysiology of juvenile obesity and associated changes in insulin-fuel homeostasis. These physiological functions and traits were of major importance during the infancy, childhood, and puberty of ancestors for metabolism, development, and growth. It is likely that prehistoric metabolic genes welcomed new mutations, provided that they favored the storage of calories. The notion of the thrifty genotype (Neel, 1962) covers all kinds of genes that could help early humans adapt to their hostile environment, when food was scarce and rather unpredictable, but nevertheless crucial for fitness and reproduction. It is likely that gene alleles favoring fat accumulation have been selected by humans and are now turning their bad effects to modern subjects because of an unexpected caloric richness and sedentary environment. Similarly, it is possible that insulin sensitivity underwent evolutionary changes toward increased channeling of glucose to

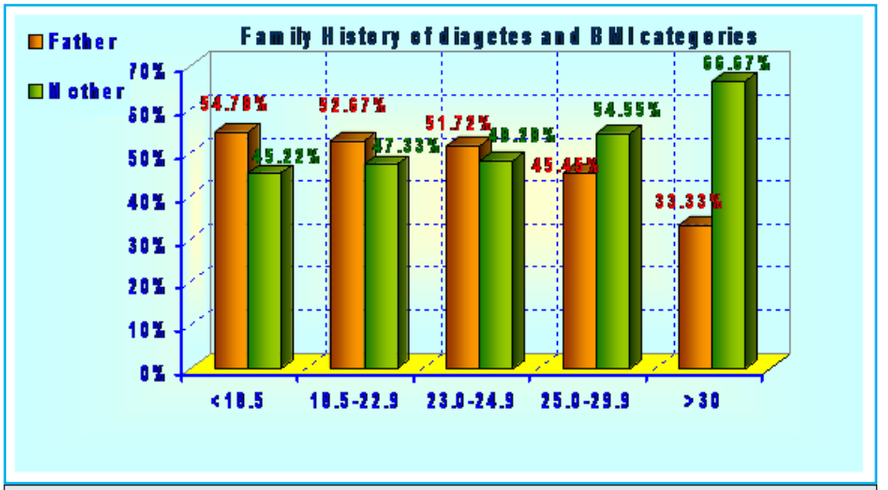

FIGURE 2.

the large human brain rather than to the insulin-sensitive muscle mass. Measuring these phenomena early in life rather than in adulthood may more closely reflect their evolutionary tendencies. In addition, the life span of early humans was limited and evolution has therefore mostly worked on the physiology of young people (Boyd-Eaton et al 1988and Ritenbaugh,1989)

The study of young individuals meets the goals of predictive genetic epidemiology because it allows the follow-up of genotyped patients through later phenotype evolution as well as clinical trials (Le Stunff et al, 2001). It has recently been put forward that several fat-derived cytokines, including the anti-inflammatory adiponectin, strongly modulate the risk of the metabolic syndrome and T2D associated with obesity (diabesity) (Lazar, 2006). Variation within the adiponectin gene is reported to modulate plasma levels of adiponectin and also to predict risk for diabesity and associated coronary heart diseases (Vasseur et al, 2003). Paradoxically, the adiponectin variant alleles that protect against the development of diabesity by maintaining high adiponectin concentrations also associate with obesity risk in both adults and obese children (Bouatia-Naji et al, 2003). Individuals with high adiponectin levels can be severely obese but seem to enjoy metabolic protection (Vasseur et al, 2005). In the general population, the same alleles, together with the type 2 diabetes protective PPAR-g 12Ala allele associates with a coronary heart disease protective risk factor pattern, elevated adiponectin and insulin sensitivity but also with a dramatic increase of 3 units of body mass index (Tanko et al, 2005).

People having a close relative with type 2 diabetes are at higher risk. There is also a strong inheritable genetic connection in type 2 diabetes: having relatives (especially first degree) with type 2 increases risks of developing this disease very substantially. In addition, there is also a mutation to the Islet Amyloid Polypeptide gene that results in an earlier onset, more severe, form of diabetes (Sakagashira, 1996). Developing type 2 diabetes is heavily influenced by environmental factors. Since our genetic code does not change significantly in one or two generations, the recent secular trend in diabetes must be due mostly to changes in the environment. Increased adiposity is the single most significant factor in the development of type 2 diabetes and the epidemics of obesity and type 2 diabetes largely parallel one another. The increasing prevalence of obesity is thought to be related primarily to changes in dietary habits and our increasingly sedentary lifestyle, though other factors (including toxins and infectious agents) may play a role. Genes may influence the risk of diabetes not only by directly altering insulin action or secretion, but also by altering how any given individual interacts with these environmental factors (Cho et al 2003).

However, environmental factors (almost certainly diet and weight) play a large part in the development of type 2 diabetes in addition to any genetic component. This can be seen from the adoption of the type 2 diabetes epidemiological pattern in those who have moved to a different environment as compared to the same genetic pool (Cotran and Collins, 1999).There is a stronger inheritance pattern for type 2 diabetes. Those with first-degree relatives with type 2 diabetes have a much higher risk of developing type 2 diabetes, increasing with the number of those relatives. Concordance among monozygotic twins is close to 100%, and about 25% of those with the disease have a family history of diabetes. Genes significantly associated with developing type 2 diabetes, include *TCF7L2*, *PPARG*, *FTO*, *KCNJ11*, *NOTCH2*, *WFS1*, *CDKAL1*, *IGF2BP2*, *SLC30A8*, *JAZF1*, and *HHEX* (Lyssenkoet al, 2008). *KCNJ11* (potassium inwardly rectifying channel, subfamily J, member 11), encodes the islet ATP-sensitive potassium channel Kir6.2, and *TCF7L2* (transcription factor 7–like 2) regulates proglucagon gene expression and thus the production of glucagon-like peptide-1 (Rother et al, 2007). Moreover, obesity (which is an independent risk factor for type 2 diabetes) is strongly inherited *(*National Diabetes Information-Clearinghouse (*NDIC),* 2008).

Various hereditary conditions may feature diabetes, for example myotonic dystrophy and Friedreich's Ataxia. Wolfram's syndrome is an autosomal recessive neurodegenerative disorder that first becomes evident in childhood. It consists of diabetes insipidus, diabetes mellitus, optic atrophy and deafness, hence the acronym DIDMOAD (Barrett 2001). A major risk factor of type 2 diabetes mellitus (T2DM) is a positive family history of diabetes. In the present study it was found that family history of obesity was more likely to have more prevalence of obesity and overweight than those having family history of diabetes. This indicates that children having family history of obesity are more likely to become obese or over weight and diabetes.In the present study, subject having impaired glucose levels and diabetes have the positive family history in first degree relatives 4.3% subjects have the 1st degree relatives in having impaired glucose level and diabetes. Children with type 2 diabetes usually have a family history of this disease. Of the patients, 74–100% have a first- or second-degree relative with type 2 diabetes (American Diabetes Association, 2000 and Arslanian, 2002). Of note, diabetes in the parent or other relative may not be recognized until the child is diagnosed. The high frequency of relatives with type 2 diabetes demonstrate the strong hereditary (likely multigenic) component to the disease (Kiess et al, 2003).

Papazafiropoulou et al, (2017) suggested that the likelihood of type 2 diabetes in the next generation is

higher in the event of a diabetic mother than father. Both genetic factors, such as mitochondrial DNA mutations, and environmental components such as intrauterine environment, have been implicated in the higher maternal transmission of type 2 diabetes. Despite the above findings, some studies in populations with high frequency of type 2 diabetes have not corroborated the predominantly maternal transmission. Such works have shown either an excess paternal or an equal transmission of type 2 diabetes.

Studies of (Patel et al 2013) and Srikanth(2015) also showed that family history of diabetes was highly prevalent among type 2 diabetic patients.First degree relatives (FDR) of patients with type 2 diadetes were more insulin resistant and are reported to had larger abdominal subcutaneous adipocytes than adults without a family history (Anthanont et al, 2017). Among the type 2 patients in the study from Tamiln Naidu, 68.8% had a positive family history of type 2 diabetes and 31.2% had a negative family history. Family history of the study participants with type 2 diabetes was enumerated. Among the participants with positive family history of diabetes, 25.1% of them had diabetic mother, 15.3% had diabetic father, 12.1% had both father and mother with diabetes, 47.4% of them had siblings with diabetes and 40% had family history of diabetes among second degree relatives such as grandparents, aunts and uncles (Geetha A et al. 2017)

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Effect of deficit and adequate irrigation and nitrogen fertilizer levels on physiological traits of maize in Kermansha province – Iran

Hossein Heydari Sharif Abad[1], Mohamad Javad Mirhadi[1], Ghorban Normohamadi[1] and Afshin Charabeh[2]*

[1]*Department of Agronomy, Science and Research Branch, Islamic Azad University, Tehran, Iran*
[2]*Ph.D. Student of Science and Research Branch, Islamic Azad University and Senior Expert of Agricultural Bank, Tehran, Iran*

ABSTRACT

In order to evaluation effects of drought stress and nitrogen levels on physiological traits of maize hybrid (Ksc703), a field study was conducted at Kermanshah province, Western Iran, during 2009 and 2010. Effects of irrigation (optimum, 85% and 60% of water requirement) and nitrogen (recommended, plus 25% and minus 25%) levels were studied by using a split plot model and 4 replications. The LAI, chlorophyll content and Chlorophyll fluorescence (Fv/Fm) were measured with a Sun Scan, chlorophyll meter (SPAD) and fluorimeter respectively. Chlorophyll content and Fv/Fm was found to decrease with diminishing of available water, hence increase of N resulted in increase of chlorophyll content and Fv/Fm in both normal and stress conditions. Drought stress reduced LAI in all N levels. The increase of N increased LAI in normal condition. In contrast LAI increased by increase of N usage, but in high level of N (more than recommended rate) LAI reduced. Drought stress reduced RWC, high level of N reduced RWC also.

INTRODUCTION

Maize (*Zea mays* L.) is an important crop that used as food, feed and industrial products. Maize is the third most important cereal after wheat and rice all over the world and the world's largest grain crop in term of total production on a MT basis. Maize planting area is about 184 million hectares in 125 countries and is the most important crop in 75 countries (FAOSTAT, 2015). Maize is one of the most important crops in the western part of Iran there is a shortfall in the production of animal feeds. Kermanshah is located in western Iran and Maize is the most important crop after wheat, grown on an area of 45,000 ha with the production of 382,500 tones

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with 8500 kg ha-1 average grain yield; the third and first place of Iran for area harvested and mean yield, respectively. The mean annual precipitation in Iran is 240 ml and it is seen as a dry or semidry country. Maize is an irrigated crop in Iran and recent drought periods in Iran imposed pressure on groundwater resources. The groundwater is the primary source of irrigation of maize production in province and in recent years, the groundwater levels has gradually decreased in this region mainly because of increasing annual irrigation and the dry climate (Agricultural Department of Kermanshah, 2015).

The dearth of water is one of the major factors challenging maize production. Among agro-meteorological hazards, drought has the greatest effect on yield stability (Vinocur and Altman, 2005). It can seriously affect the grain quality and grain output reducing average yields by 50% or even more (Wang et al., 2003). Water shortage due to decreasing annual precipitation and the dry climate, as well as low fertility and low percentage of organic matter in soil is major problems of maize production in Iran. For this reason, overuse of chemical fertilizers in Iran is rising, leading to environmental pollution and soil degradation. Yield losses include more than two- thirds of the total damage of abiotic stresses due to drought, salinity and other factors. Maize is highly sensitive to drought stress, specifically in flowering stage (Tollenaar and Lee, 2011).

Drought is a major problem for the production of the world's five principal cereals: maize, wheat, rice, pearl millet, and sorghum. Water stress reduced yield in crop, but interactive effects of water and nitrogen deficits on physiological traits and on physiological changes associated with leaf aging have received little attention (Valliyodan and Nguyen 2006). Soil water deficit reduces yield of maize (*Zea mays* L.) and other grain crops by three main mechanisms. First, whole canopy absorption of incident Photosynthesis Active Radiation (PAR) may be reduced, either by drought induced limitation of leaf area expansion, by temporary leaf wilting or rolling during periods of severe stress, or by early leaf senescence. Second, drought stress reduces the efficiency with which absorbed PAR is used by the crop to produce new dry matter. Third, drought stress may limit grain yield of maize by reducing the harvest index (Earl and Davis, 2003).

Mihailovic et al (1992) demonstrated one of the factors influencing physiological responses of plants to water stress is mineral nutrition. A significant role of nitrogen in regulating plant responses to water stress was established in a number of plant species. According to estimates of CIMMYT (International Maize and Wheat Improvement Centre) regarding abiotic stresses, the most significant causes of yield loss on farmers fields are low fertility (predominantly N deficiency) followed by drought and, less important, by plant competition related to low planting densities, weeds and intercrops (Ribaut and Poland, 1999). Some of the effects of drought stress on physiological traits of plants are suitable. Photosynthetic rate, leaf surface area and photosynthetic capacity enhanced with increase in nitrogen levels (Gungula and et al, 2005). Leaf area and LAI increase with increase in nitrogen levels (Oscar and Tollennar, 2006).

The chlorophyll meter (or SPAD meter) is a simple, portable diagnostic tool that measures the greenness or the relative chlorophyll concentration of leaves. Compared with the traditional destructive methods, this equipment might provide a substantial saving in time, space and resources. The Minolta Soil Plant Analysis Development (SPAD-502) chlorophyll meter is one tool that enables researchers to determine chlorophyll content by measuring leaf greenness (Peterson et al., 1993). The SPAD uses a silicon photodiode to derive the ratio of transmittance through the leaf tissue at 650 nm compared with transmittance at 940 nm, and a value is given based on that ratio. SPAD measures relative chlorophyll content in plant leaves. Because chlorophyll content is closely related to N supply (Pandey et al., 2000), SPAD is also used to diagnose maize N status and predict maize grain yield potential (Vetsch and Randall, 2004). Janos (2010) reported a close correlation between N fertilization and SPAD readings. Increasing N application increased N content and chlorophyll content in maize (Rambo et al., 2010). Factors affecting SPAD values include radiation differences between seasons, variety and species differences, plant and soil nutrient status (including N and other nutrients), and biotic and abiotic stresses (Peterson et al., 1993).

Atteya (2003) showed exposure of plants to drought lead to noticeable decrease in leaf water potential and relative water content (RWC) and Water stress changed the relation between leaf water potential and relative water content of maize so that stressed plants had lower water potentials than control at the same leaf RWC. The RWC measurement characterizes the internal water status of plant tissues and is also a convenient method for following changes in tissue water content without errors caused by continually changing tissue dry weight (Erickson et al., 1991). On the other hand using the chlorophyll fluorescence technique, is useful possible to estimate the parameters of actual photosynthetic efficiency of leaf, under various conditions at various times, and also the potential maximum of the quantum efficiency (Fv/Fm). Fv/Fm is the measurement of quantum yield potential of photosynthesis, or maximal photochemical efficiency of PSII. The Fv/Fm ratio has been shown to be a reliable

indicator of stress (Duraes and et al, 2001). Photosynthesis, as a significant physiological process to yield is sensitive to water stress. The photosynthetic rate keeps decreasing while the intension of stress increases, which is the main reason for the reduction of yield by drought, Moreover, it is possible to determine if there is damage to light reaction systems in photosynthetic machinery during drought (Liu et al., 2012). Measuring chlorophyll fluorescence has become a very useful technique in obtaining rapid qualitative and quantitative information on photosynthesis (Rohácek, 1999), and it can provide information on the relationship between structure and function of photosystem II (PSII) reaction center (Rosenqvist and Van Kooten, 2003). Chlorophyll fluorescence provides useful information about leaf photosynthetic performance of many plants under drought stress (Baker and Rosenqvist, 2004).

Schlemmer et al (2005) reported a very strong relationship between the Minolta SPAD-502 chlorophyll meter readings and direct measurements of chlorophyll content in maize and soybean leaves. Since chlorophyll content is usually strongly related to N concentration, these meters can be used as indicators of need for agricultural N application. So, present research conducted in Kermansh, a drought stress prone province, to study of physiological aspects of water deficit and nitrogen levels on growth and development of maize hybrids.

MATERIAL AND METHODS

In order to evaluation effects of drought stress and nitrogen levels on physiological traits of maize hybrids, a field study conducted in Kermanshah province, western Iran, at 2016 and 2017 at the agricultural research farm, Agricultural and Natural Resources Research Centre in Kermanshah, Iran. This farm is located at 34.08 N, 46.26 E, 1345 m altitude, silty clay soil, pH=7.5-8, 450 mm precipitation Mediterranean climate.. The KSC703 (late maturating group) maize hybrid is dominate commercial cultivar of Kermansha province. Experiment plots were seeded with 75 cm row to row distance and plant density was 75000 plant/ha (conventional plant density). Seeds were sown 7 cm deep. Maize was planted in May and by experimental planter. Irrigation (optimum, 85% and 60% of water requirement) and nitrogen levels (recommended, plus 25% and minus 25%) arranged as main and sub plots respectively using a complete randomized block and 4 replication were used. LAI was measured with a Sun Scan canopy analysis system (Delta-T Devices, Cambridge, UK) and in stages V6, V10 and R1. Chlorophyll meter (SPAD-502, Minolta) readings were taken in all plots. SPAD reading were taken on the midpoint of the youngest fully expanded leaf.

Chlorophyll fluorescence (potential maximum of the quantum efficiency (Fv/Fm)) in leaves of non-stressed and water stressed plants was measured in R1 stage with chlorophyll fluorimeter (Pocket PEA). The RWC was measured using flag leaves after imposing drought conditions. Leaves were sealed within plastic bags and quickly transferred to the lab. Fresh weight (FW) was determined within 2 h after excision. Turgid weight (TW) was obtained after soaking leaves in distilled water in test tubes for 16 to 18 h at room temperature. After soaking, leaves were quickly and carefully blotted dry with tissue paper in preparation for determining turgid weight. Dry weight (DW) was obtained after oven drying the leaf samples for 72 h at 70°C. RWC was calculated from the formula:

RWC (%) = [fresh weight- dry weight/ turgid weight – dry weight] × 100

Where FW, TW and DW are fresh weight (g), turgid weight (g) and dry weight (g) respectively.

RESULTS AND DISCUSSION

Analysis of data showed that chlorophyll content at tasseling decreased by diminish of available water, hence increase of N, resulted in increase of chlorophyll content in both normal and stress condition. A direct close relationship of nitrogen levels with SPAD (Chlorophyll Meter Readings) was reported in maize (Schlemmer and et al 2005). Ciganda et al (2008) had similar results and reported that chlorophyll content is among the most important crop biophysical characteristics. Chlorophyll can be related to photosynthetic capacity, thus, productivity, developmental stage, and canopy stresses, also Munne-Bosch and Alegre (2000) reported the chlorophyll content was decreased with decreasing the irrigation water and this decrease was correlated with relative water content in leaves. Chlorophyll loss is a negative consequence of water stress.

Drought stress reduced LAI in all N levels. The increase of N increased LAI in normal condition. In contrast LAI increased by increase of N use, but in high level of N (more than recommended rate) LAI reduced. Similarly in accordance with our results, LAI was positively correlated with nitrogen application in normal condition (Oscar and Tollennar, 2006). For most plant species, the shortage of nitrogen or water causes a reduction in leaf area development, changes in leaf tissue composition, leaf cell structure and plant water content (Casa, 2003) and also in maize, drought reduces leaf area, leaf chlorophyll contents, photosynthesis and ultimately lowers the grain yield (Athar and Ashraf, 2005). Stone et al. (2001) reported that water deficit reduces crop growth

Table 1. AOVA table of data (2009 and 2010).

Source of variation	df	Mean squares (MS)			
		CH C	LAI	RWC	Fv/Fm
Replication	3	10.9	0.594	3.203	0.002
Year	1	398.5	2.584	54.028	0.002
Year* replication	3	3.358	4.23	2.425	0.001
Water levels	2	293.136**	4.871**	1312.09**	0.006**
Year* water	2	70.996	0.43	9.915	0.001
Error	12	6.156	0.07	24.556	0.001
Nitrogen levels	2	325.969**	0.56**	41.383**	0.001
Year* Nitrogen	2	8.211 ns	0.32 ns	0.156 ns	0.0010 ns
Water* Nitrogen	4	2.091 ns	1.71**	19.202*	0.001 ns
Water * Nitrogen * Year	4	0.976 ns	0.076	1.977	0.001
Error	36	5.108	0.54	7.065	0.001
C.V.		6.9	9.16	4.71	5.78

Table 2. The effect of Water levels on Chlorophyll content (Ch C), LAI, Relative water content (RWC), and Fv/Fm.

Water levels	CH C	LAI	RWC	Fv/Fm
Optimum (100%)	41.44	3.03	79.15	0.81
80% requirement	38.96	2.60	71.66	0.789
60% requirement	34.54	2.13	64.36	0.781

Table 3. The effect of Nitrogen levels on Chlorophyll content (Ch C), LAI, Relative water content (RWC), and Fv/Fm.

Nitrogen levels	CH C	LAI	RWC	Fv/Fm
Recommended - 25%	34.45	2.49	73.02	0.78
Recommended	38.45	2.51	71.76	0.79
Recommended + 25%	41.8	2.06	70.39	0.80

and morphological characteristics of maize plant. In maize, reproductive growth after the silking and flowering stages is the critical period for yield, and chlorophyll content and intact chloroplast structure are key factors for accumulation of dry matter and high yields (Yu et al., 2010).

The RWC measurement characterizes the internal water status of plant tissues and is also a convenient method for following changes in tissue water content without errors caused by continually changing tissue dry weight. Drought stress reduced RWC. The RWC was 80 to 64 for normal and drought stress condition respectively. High level of N reduced RWC also. Jabasingh and Saravana Babu (2014) had similar results and reported that the relative water content in leaves of different maize cultivars decreased significantly and with drought stress, the membrane permeability of the leaf cell markedly increased. Also Higher RWC indicates better growth

Table 4. The effect of Nitrogen and water levels on Chlorophyll content (Ch C), LAI, Relative water content (RWC), and Fv/Fm.

Water levels	Nitrogen levels	CH C	LAI	RWC	Fv/Fm
Optimum (100%)	Recommended - 25%	37.6	2.69	78.8	0.80
	Recommended	41.83	2.9	79	0.81
	Recommended + 25%	44.9	3.7	79.5	0.82
80% requirement	Recommended - 25%	34.9	2.4	74	0.78
	Recommended	38.9	2.7	72.1	0.79
	Recommended + 25%	43.03	2.5	68.1	0.79
60% requirement	Recommended - 25%	30.84	2.3	66	0.76
	Recommended	35.33	2.1	64.1	0.76
	Recommended + 25%	37.46	1.9	62.8	0.77

and development, which in turn depends on leaf area (Sivakumar, 2014).

The results showed that with reduction of water availability, the quantum efficiency (Fv/Fm) decreased. On the other hand with increase of nitrogen level, Fv/Fm increased but not statistically significant. Duraes et al (2001) reported that the Fv/Fm will reduce by drought stress in maize hybrids. Photochemical chlorophyll fluorescence quenching, photosystem II quantum yield and electron transport rate and more heat dissipation as compared to controls (Dias and Bruggemann, 2010). Light energy absorbed by chlorophyll molecules in a leaf can undergo one of three fates: it can be used to drive photosynthesis (photochemistry), excessive energy can be dissipated as heat or it can be re-emitted as light-chlorophyll fluorescence. These three processes occur in competition that is any increase in the efficiency of one will result in a decrease in the yield of the other two (Maxwell and Johnson, 2000).

CONCLUSION

Results showed that under drought stress RWC, chlorophyll content, LAI and the quantum efficiency (Fv/Fm) decreased. Therefore, reducing of RWC, LAI and Chlorophyll content or quantum efficiency (Fv/Fm) could be indicative of water stress. On the other hand increase of nitrogen, resulted in increase of chlorophyll content and Fv/Fm in both normal and stress conditions and with increasing amounts of nitrogen up to 2nd level, LAI increased, but RWC decreased.

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Isolation and identification of pharmaceutically active lipase producing *Bacillus* spp. from mangrove sediments against Methicillin Resistant *Staphylococcus aureus* isolated from wound of patients

M.I. Farheena, A. Chithira and A. Mohankumar

Division of Microbial Technology, PG and Research Department of Zoology, Chikkanna Govt. Arts College, Tirupur, Tamilnadu, India

ABSTRACT

Ninety two clinical wound samples were collected, among, 50 positive *Staphylococcus aureus* were isolated. These isolates were characterized morphologically and biochemically. Twenty three antibiotics were used to determine susceptibility patterns by disc diffusion method. The multiple antibiotic resistances (MAR) index were calculated according to the MAR index formula ranged from 0.21 to 0.78. All the 50 isolates of MRSA were showed MAR index in between these ranges. Among these MAR index the pathogenic isolates (100%) were resistant of Penicillin, while trimethoprim showed resistance (86%), cephoxitin (80%), kanamycin (78%), vancomycin and cepfodoxamine (72%), moxalactam and quinupristin (70%), cotrimoxazole (66%), methicillin (64%), novobiocin (62%) and erythromycin (56%) and there was no resistance found to chloramphenicol, and rifampicin. More than 65% resistance MRSA isolates were selected for plasmid isolation. Natural products are boundless source for important novel compounds having antagonistic activity against pathogenic organisms. Marine environment covers almost 70% of the earth surfaces. Organisms present in these environments are extremely rich sources of bioactive compounds. The ocean remains as an unexploited source of many drugs and pharmacologically active substances. Microbial enzymes have many advantages over the animal and plant enzyme, firstly; they are economical and can be produced on large scale within the limited space and time. Secondly, they are capable of producing a wide variety of enzymes; they can grow a wide range of environmental condition. Enzymes have many roles in the pharmaceutical and diagnostic industries. The bacteria *Bacillus* spp. was isolated from the mangrove sediment, for the lipase production. These enzymes may inhibit the growth of MRSA. Recently with the advent of biotechnology, there has been a growing interest and demand for enzymes with the novel properties.

KEY WORDS: PUS, *STAPHYLOCOCCUS AUREUS*, ANTIBIOTIC SUSCEPTIBILITY TESTING, ANTIMICROBIAL RESISTANCE PLASMID DNA, LIPASE

ARTICLE INFORMATION:

**Corresponding Author: farheena1980@gmail.com*

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INTRODUCTION

Wound infection is one of the major health problems resulting from colonization of wounds by pathogenic organisms Recurrently the predominant *Staphylococcus aureus* is one of the most important opportunistic pathogen among *Staphylococci* belonging to Micrococaceae family causing significant infections under appropriate conditions (Prescott et al., 2002). About 20%–30% of the general populations are carriers of *S. aureus*. The anterior nasal cavity is the main site of *S. aureus* carriage. Among nasal *S. aureus* carriers, approximately one-half also carry the organism on their skin. Recent studies have established that *S. aureus* is often found at nonnasal sites, particularly the pharynx and the gastrointestinal tract, with some carriers having colonization confined to these sites (Mertz et al., 2009; Acton et al., 2009, Fagbomedo and Femi-Ola, 2017).

Staphylococcus aureus is one of the most versatile nosocomial (i.e. acquired in hospital) and dangerous human pathogen since publication of its role in sepsis by Ogston in 1880 and 1882 (Lowy, 1998). At present, Staphylococcal resistance to antibiotic has been associated with resistant plasmids (R-plasmid) that have the ability to mediate the production of drug inactivated enzymes such as β-lactamase.The spread of resistance to antimicrobial agents in *S. aureus* is largely due to the acquisition of plasmids and or transposons (Lyon and Skurray, 1987Adeleke and Odelola, 1997; Adeleke et al., 2002 and other functions (King et al., 2006 and Diep et al., 2008).

The un-hygienic hand swab showed presence of MRSA and improvement in hand hygiene, coinciding with a reduction of nosocomial infections and MRSA transmission (Pittet et al., 2000). Treatment options for patients with serious invasive infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) are limited (Drew, 2007).Increasing resistance of MRSA in recent years has had a significant impact on several aspects of patient care and infection control. Antibiotic policies need to be updated regularly, along with comprehensive monitoring of antibiotic prescribing and antibiotic consumption in healthcare settings. These facts clearly highlight the need of a characterization of MRSA strains at a regular basis at all levels. With the increasing incidence of MDR, recourse to new antibiotics has become necessary.Microbial enzymes have many advantages over the animal and plant enzyme, firstly; they are economical and can be produced on large scale within the limited space and time. Secondly, they are capable of producing a wide variety of enzymes; they can grow a wide range of environmental condition. Enzymes have many roles in the pharmaceutical and diagnostic industries. Therefore the main objective of this study is to isolate lipase enzyme from mangrove sediment bacteria to treat against MRSA isolates.

MATERIAL AND METHODS

In the present study, 90 pus samples were collected from various Hospitals in and around Tirupur District from November 2012 to February 2013. The samples were processed in the laboratory of PG and Research Department of Zoology, CGAC. First step done was to isolate the organisms from pus samples and then studied the culture susceptibility of *Staphylococcus aureus*. All pus samples were directly streaked on Mannitol Salt Agar plates and incubated aerobically at 37°C for 24 hours. The isolates were identified with standard tests used to identify *S. aureus* such as Gram stain, IMViC, Nitrate reduction, Oxidase, catalase, slide and tube coagulase tests. (Forbes et al., 2007).

Antibiotic sensitivity testing (AST) was done only for confirmed *S.aureus* strains. Antibiotic sensitivity was performed by Disc Diffusion Method (Bauer et al., 1966). Overnight cultures of *S.aureus* in nutrient broth were flooded over the surface of Mueller Hinton agar plates. The Mueller Hinton agar plates were allowed to dry before applying antibiotic disc (Baddour et al., 2006). Commercially available antibiotic discs were obtained from (Himedia Labs, Mumbai, India) were gently and firmly placed on the agar plates, which were then left at room temperature for 1 hour to allow diffusion of the antibiotics into the agar medium. The plates were then incubated at 37°C for 24 hours. If an antimicrobial activity was present on the plates, it was indicated by an inhibition zone. The diameter of the inhibition zones was measured in millimeter after 24 hours using a standard chart and detected various pattern of antibiotics as sensitive, intermediate and resistant to MRSA.

Multiple drug resistant strains of *S. aureus* which showed 65% resistance to the antibiotics were selected for plasmid extraction. Isolation of plasmids was performed by Alkaline Lysis Method (Jegadeesh Babu and Rajamanickam, 1998). The plasmids were observed in 0.7% Agarose gel electrophoresis.Extra cellular enzyme such as lipase was detected from the marine bacteria isolated from mangrove soil sediment. Using plate precipitation test with their specific media screening of lipolytic activity was done by using Rhodamine-B agar plate (Kouker and Jaegar, 1987). The antibacterial activity of the lipase was performed by using well diffusion method. So, hence in view to the significance of mangrove ecosystem which provide a rich source of novel lipase against MRSA isolates.

RESULTS AND DISCUSSION

Among the 92 clinical pus sample (Plate: 1) isolates of *S. aureus*, 50 positive *Staphylococcus aureus* (Plate: 2) were identified as methicillin resistant *Staphylococcus aureus* (MRSA) by disc diffusion method (Plate: 3). Electrophoretic analysis of the plasmid DNA prepared was carried out by agarose gel electrophoresis on 0.7%. Five MRSA isolates (FA21, FA24, FA48, FA49 and FA50) which was showed more than 65% resistance against tested antibiotics were selected for plasmid isolation (Plate: 4).Strain no FA49 harbored a single plasmid DNA on basis of electrophoretic mobility on agarose gel. Other four strains (FA21, FA42, FA48 and FA50) harbored a double plasmid DNA. The molecular size of the plasmid DNA was calculated to be 1500bp and 1000bp respectively. 100bp DNA ladder (Medox biotech, Chennai) was used as marker DNA.

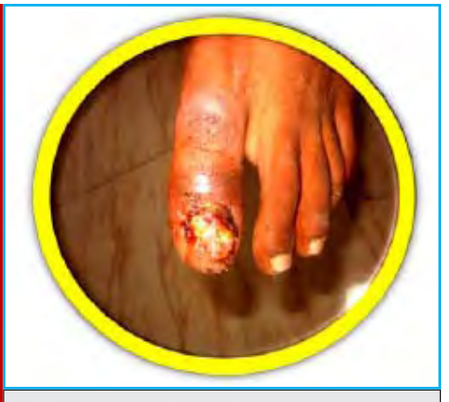

PLATE 1. Wound Sample

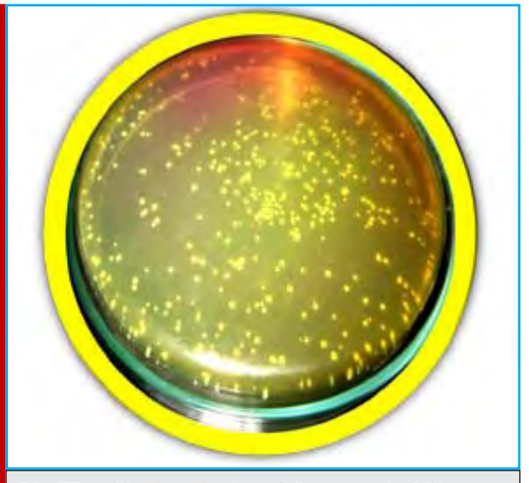

PLATE 2. Single colonies of *S. aureus* in MSA agar plate

Marine bacterium *Bacillus* spp. was isolated from marine sediment collected from Pitchavaram mangrove. This bacterium was identified by biochemical characters. This bacterium was identified by using ZoBell medi a and lipase activity screening by Rhodamine B agar media (Plate: 5) followed by the antimicrobial activity of lipase enzyme was done by well diffusion assay (Plate: 6).

In Rhodamine B agar the isolates *Bacillus* spp. showed halos around the bacterial colonies under ultra violet irradiation. Kouker and Jaeger (1987) detected a plate assay for microbial lipase in a medium containing triacylglycerol with addition of fluorescent dye rhodamine B. Substrate hydrolyses causes the formation of orange fluorescence halos around bacterial colonies visible upon UV irradiation. The isolated strains were screened for lipase production.

Staphylococcus aureus is recognized as an important bacterial pathogen contributing towards hospital infection, globally. Despite the use of potent antibiotic still high mortality exist in case of *Staphylococcus aureus* infection. In the present study, antibiotic susceptibility pattern was assessed for Gram-positive cocci from pus and a high resistance was recorded against antibiotics tested. Despite the numerous studies found that advent of antibiotics, it was though that warrant for the treatment of the *S. aureus* related infection got issued but due to the development of antibiotic resistant gene in the plasmid of *S. aureus* could depend itself in a much secured manner. So, these facts clearly highlight the need of a characterization of MRSA strains at a regular basis at all levels. With the increasing incidence of MDR, recourse to new antibiotics has become necessary. In recent year the microbial enzymes have many roles in the pharmaceutical and diagnostic industries. Therefore the main objective of this study is to isolate lipase enzyme from mangrove sediment bacteria to treat against MRSA isolates.

Adame et al (2010) collected 250 samples from healthy humans, cattle, sheep and goats for the isolation of *S. aureus* and they reported that the antimicrobial susceptibility test showed highly susceptible to Ciproxcin (91.1%), Norfloxacin (90.2%), Rifampicin (73.2%), Streptomycin (72.3%), Erythromycin (71.4%), Norbactin (64.3%), but the isolates showed resistant to Ceftazimide (7.1%), Cefotaxime (14.3%), and Ampiclox (31.3%). Totally 92 wound samples were collected from infected human, among 50 *S. aureus* were isolated, Penicillin showed 100% resistant against the isolates, Amikacin and tetracycline showed 6% and 4% resistance respectively in the present study.

Susmita Bhattacharya et al (2013) have isolated 280 MRSA strains from 714 *Staphylococcus aureus* of various clinical samples. Among 280 MRSA, 21 strains were

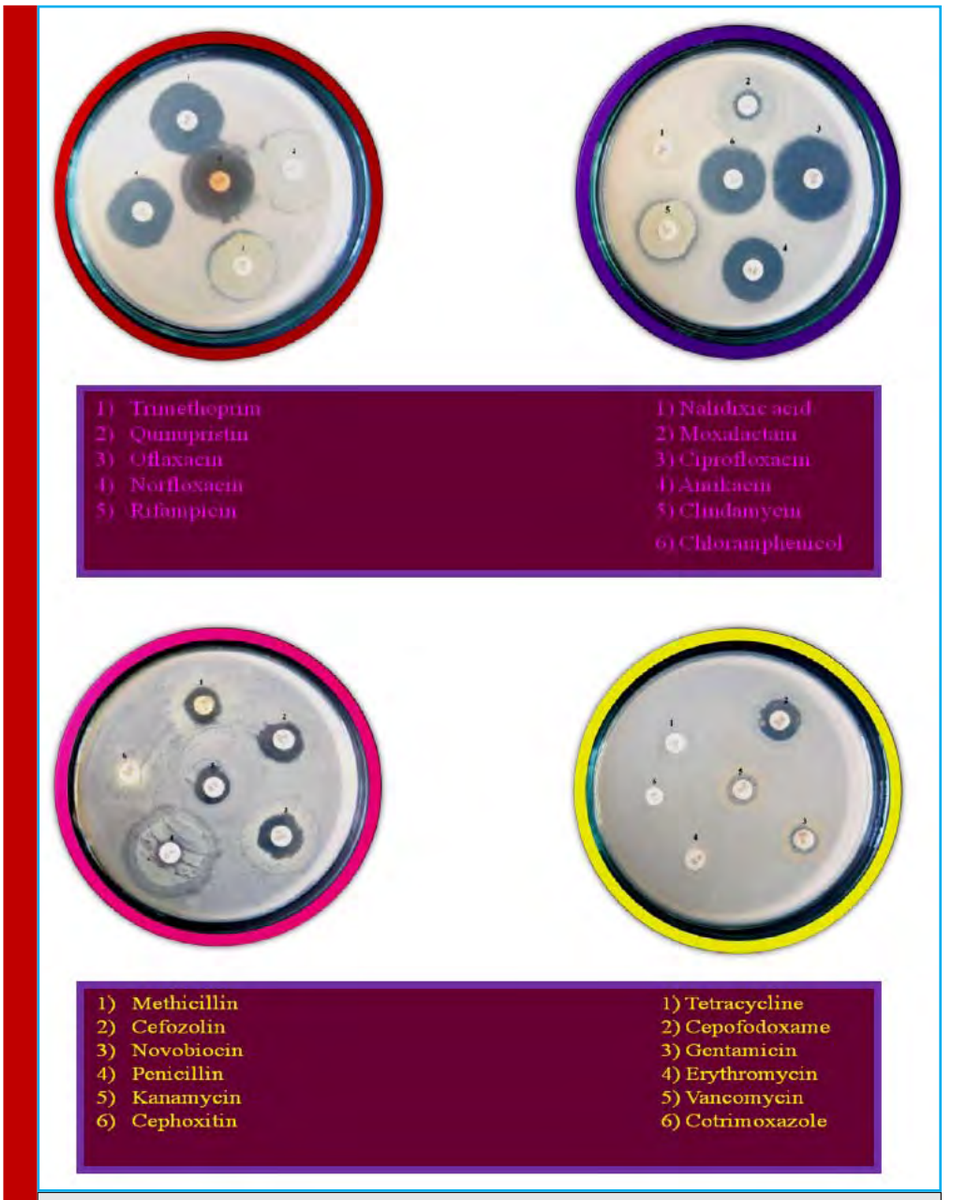

PLATE 3. Antibiotic susceptibility test of *S. aureus*

found to be resistant to Vancomycin by disc diffusion test. Similar method was used in the present investigation for antimicrobial susceptibility test. Among 50 MRSA strains, 36 strains were found to be resistant to Vancomycin.High rates of resistance to Penicillin among *S.aureus* have been observed since 1959, when this frequency was recorded at 80% which have been extended to amoxicillin and to ampicillin. Giarola et al (2012) observed that the 90% resistance to Penicillin, 14% to rifampicin and 59% to azithromycin. Similarly high rates of resistance was observed against Penicillin (100%) but none of the isolates showed resistance against rifampicin in this study.

All the isolates of *S.aureus* were multidrug resistant and one isolate was pane resistant for all the tested drugs. 50 % resistance was observed in penicillin, methicillin, polymyxin – B and Chloramphenicol 95.5%, 77.3%, 68.2% and 51.5% respectively (Alebachew et al 2009). Similarly Penicillin and methicillin showed 100% and 64% resistance respectively, whereas none of the isolates

PLATE 4. Plasmid Profile of *S. aureus*

showed resistance against chloramphenicol. Totally 23 antibiotic drugs were tested, among 23 antibiotics, 12 antibiotics were showed were than 50% resistance.

Twenty four *Staphylococcus aureus* isolates were tested *invitro* to determine their antibiotic susceptibility patterns by antibiotic disc diffusion method. All the isolates showed multiple antibiotic resistances to the antibiotics tested. All the isolates were 100% resistant to Ampicillin, Amoxicillin Nalidixic acid (87.5%), Ceftriaxone (75%), Streptomycin (54.16%), Erythromycin, tetracycline, Kanamycin and Neomycin (25%), Oxacillin (41.6%), Tobramycin and there was no resistance found to Chloramphenicol, Vancomycin, Norfloxacin, Ciprofloxacin and Rifampicin (AI-Hamdani and Hamad, 2012). Totally 50 *Staphylococcus aureus* isolates were tested *invitro* by similar method. Here also all the isolates were showed multiple antibiotic resistance to the drugs tested. But Chloramphenicol and Rifampicin were showed 100% resistant.

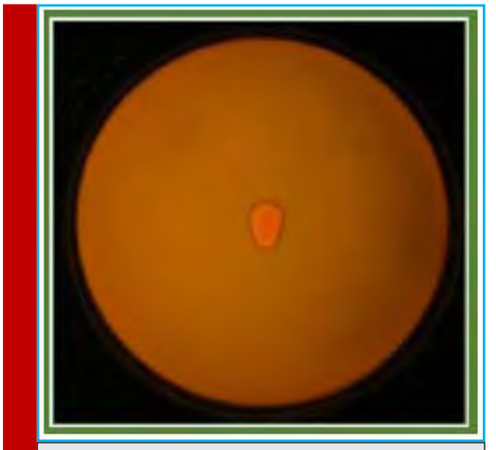

PLATE 5. Screening of Lipase enzyme from *Bacillus* spp.

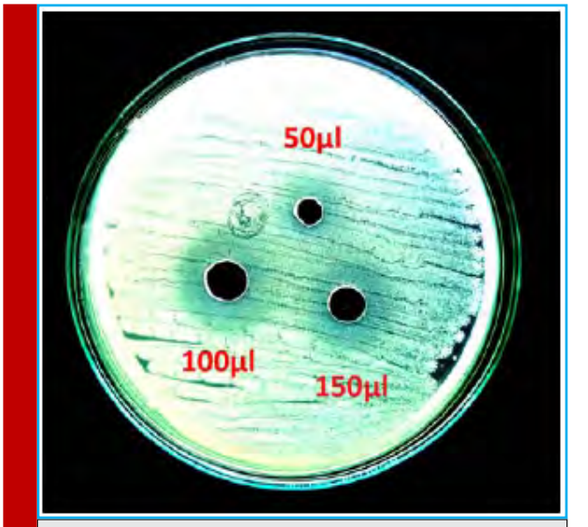

PLATE 6. Antimicrobial activity of lipase against *S. aureus*

Udo and Sarlchoo (2010) have characterized four MRSA isolates , during plasmid analysis, four isolates had showed three plasmid patterns, Isolate K6482 contained two plasmids (28 and 26 Kb), K6531 and K6533 each contained three plasmids (28, 21 and41 Kb) and K6552 contained two plasmids (41 and 41 Kb). In this present study five strains which showed more than 65% resistance against all drug tested were taken for plasmid isolation, Isolate FA49 contained one plasmid (1.5Kb) and the isolates FA21, FA24, FA48 and FA50 each contained two plasmids (1.5kb and 1Kb).Herari et al (2008) studied that the lipase production in an indigenous lipolytic *Bacillus* spp. in media containing tributyrin, tween 80 and rhodomine B- Olive oil. The statistical model was used to predict the optimum experimental conditions for bacterial growth and lipase production. Similarly in the present study *Bacillus* spp. was used for lipase production, it was isolated from the sediment of Pichavaram mangrove, lipase production was confirmed by Rhodomine B-olive oil plate.Mohankumar and Tamilselvi (2012) have isolated marine bacteria includes *Bacillus* spp. *Pseudomonas* spp. *Staphylococcus* spp. and *Vibrio* spp. for lipase production. Amount of lipase was

estimated by plate assay method and titration. Similar method was followed in the present study that the *Bacillus* spp. was isolated from mangrove sediment samples and the lipase production was estimated only by plate assay method.

CONCLUSION

The present study has indicated that MRSA strains were found to be develop resistance day by day to the currently used antibiotics. This situation needs some novel therapeutic drugs from marine environment for an alternative therapy for treatment of wound causing MRSA isolates.

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Effect of vibration on pain during Injection of local anesthesia: A split-mouth randomized clinical trial

Gholam Hossain Ramezani[1], Mohammad Tajjedin[2], Nasser Valaee[3] and Hooman Ebrahimi[4*]

[1]*Department of Pediatric Dentistry, Dental Branch, Islamic Azad University, Tehran, Iran*
[2]*Specialist in Pediatric Dentistry*
[3]*Epidemiologist, Dental Branch, Islamic Azad University, Tehran, Iran*
[4]*Department of Oral Medicine, Dental Branch, Islamic Azad University, Tehran, Iran*

ABSTRACT

Vibration can reduce pain. This study was conducted to use this effect while injecting dental anesthesia. This split-mouth randomized clinical trial was performed on 40 injection sites from 20 patients. In the experimental sides, the turned-on device would be positioned in contact with the injection area for 5 seconds; then the anesthesia would be administered, while the device was in place; finally, the device would remain in place for 5 seconds after removing the needle. In the control sides, the device would be placed on the mucosa in a similar fashion but turned off. Immediately after removing the tip of Dental Vibe, patients were asked to rate their pain using Wong Baker method. Pain scores were compared statistically. Average pain levels in the experimental and control sides were 1.95 ± 1.57 (95% CI: 1.22 to 2.68) and 0.65 ± 0.81 (95% CI: 0.27 to 1.03), respectively. Their difference was significant according to Wilcoxon test ($P < 0.001$). Age ($P = 0.670$), injection type ($P = 0.175$), and sex ($P = 0.160$) did not affect the response to the Dental Vibe significantly, according to chi-square test. Dental Vibe is a useful and effective device in reducing pain while injecting local anesthesia.

INTRODUCTION

Most common anxiety provoking and fearful experience for children in dental operatory is administration of local anesthesia. Pain management when injecting local anesthesia (LA) is one of the most critical stages in performing dental treatments. Since painful dental treatments typically begin with LA, pain control at this step is essential. Pain is a deterrent of dental treatment and many patients avoid or cancel their treatments because of this factor because of being afraid of pain, which is usually caused by previous painful experiences during dental procedures. Therefore, reducing dental pain is important and researchers have been seeking methods

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to reduce dental pain, including pain of injecting local anesthesia Milgrom et al., 1997, Yenisey 2009 Bonjar 2011, Ungor et al 2014, Rakshan and Rakshan 2015, Agarwal et al 2017).

Mechanisms for pain due to local anesthesia are mainly quick expansion of the tissues and their tension, followed by mechanical trauma by the needle puncture to the region of the injection. Various methods have been proposed to reduce or prevent pain while administering local anesthesia, including the application of topical anesthetics, suggestion, slow injection , transcutaneous electrical nerve stimulation (TENS) computer-assisted local anesthesia (such as Wand), and vibration (Hersh et al. 1996 Peretz et al., 2004 and Primosch 2003).

According to the "gate control" theory, providing non-noxious stimuli might interrupt nociceptive signals reduce the perceived pain . Thus, it is hypothesized that stimulating larger-diameter A-beta fibers with vibration and pressure might reduce pain sensation (Saijo et al., 2005 Nanitsos et al., 2009 and Rakshan and Rakshan 2015).

The Dental Vibe device (BING Innovations, Florida, USA) is a new portable system that transmits pressure-rotational pulses to the injection area, without any need to changing the routine protocols of injection. We hypothesized that vibration concurrent with injection might decrease perceived pain in children.

MATERIALS AND METHODS

This split-mouth randomized clinical trial was performed on 40 injection sites from 20 patients (13 boys and 7 girls, with an average age of 5.7 years old) referred to the Department of Pediatric Dentistry at Islamic Azad University during 2014-2015. The protocol ethics were approved by the Ethical Committee of the university, and informed consents were taken from patients' parents. Inclusion criteria were being systemically healthy, being aged between 5 and 7 years old, a cooperation level of 3 or 4 (according to Frankel scale) determined by a pediatric dentist, ability to determine pain levels according to Wong Baker scale (8thesis), and clinical need for bilateral local anesthetic injection in the mandible or maxilla.

Randomization, Local anesthesia administration, and Pain evaluation

Randomization was performed by picking out a card from a box, for each patient. Patients with "vibration on" cards would receive the injection together with the DentalVibe application while the device was on. They would receive the second injection on the contralateral side, with the vibration off. The patients with "vibration off" cards would first receive the placebo (the device touch without vibration) first, and the treatment in the second session.

In the experimental sides, the turned-on device would be positioned in contact with the injection area for 5 seconds; then the anesthesia would be administered, while the device was in place; finally, the device would remain in place for 5 seconds after removing the needle. In the control sides, the device would be placed on the mucosa in a similar fashion but turned off. The inferior alveolar nerve (IAN) blocks were administered using a carpul of 2% lidocaine and 1:80000 epinephrine (Darupakhsh, Tehran, Iran). The infiltration blocks were administered for the maxilla, using a carpul of the same anesthetic solution. Of the 40 injections, 22 were IAN blocks while 18 were maxillary infiltration injections.

Immediately after removing the tip of Dental Vibe, patients were asked to rate their pain using Wong Baker method. All injections were performed by the same person (a resident of pediatric dentistry).After data collection, the treatment would be started in its routine fashion; the patient would receive as many carpules as needed/wanted after the data collection.

After summarizing the descriptive statistics and confidence intervals (CI), Wilcoxon test and chi-square tests used to compare control and treatment groups.

RESULTS AND DISCUSSION

Of the 20 participants, 65% reported lower pain levels when using the device; 25 % rated the pain on both sides similarly; 10% reported greater pain levels on the control sides. Average pain levels in the experimental and control sides were 1.95 ± 1.57 (95% CI: 1.22 to 2.68) and 0.65 ± 0.81 (95% CI: 0.27 to 1.03), respectively. Their difference was significant according to Wilcoxon test ($P < 0.001$).

Age ($P = 0.670$), injection type ($P = 0.175$), and sex ($P = 0.160$) did not affect the response to the Dental Vibe significantly, according to chi-square test (Table 1).

Table 1. Results pertaining to experimental sides in which the device was turned on (n = 20, control sides are not used or shown).

Factor		Painless	Painful	RR	AR
Gender	Boy	9	4	3.6	37
	Girl	2	5		
Age	5 yr old	5	6	2.4	21
	> 5 yr old	3	6		
Injection	IAN block	8	3	3.5	30
	Infiltration	3	6		

RR, relative risk; AR, attributable risk.

Dental anxiety and fear are the most frequent reasons preventing patients from dental visits, and are usually a byproduct of local anesthesia injections. Hence, pain and anxiety control during local anesthetic injections is of significant clinical importance, (Ungor et al., 2014, Bonjar 2011 Berggren and Meynert 1984). Topical anesthetics numb the injection surface and provide pain relief on needle insertion, although there are other factors which should be controlled (such as the clinician's expertise and amount, type, and dose of the injected medicine) for a complete pain control. Although this method reduces the pain during needle insertion, total elimination of injection pain relies on causes like the amount, type, and injection speed of anesthesia plus the experience of clinician. In addition, local anesthetics have narrow potential to enter deep into tissue. These might reduce the discomfort during insertion of needle through the surface however, they are not as effective when needle passes through deeper layers, (Singh and Roberts 1994, Meechan et al., 1998 Ungor et al 2014).

Hence, methods such as Wand and TENS are introduced to solve this. TENS triggers large-diameter nerves that are more sensitive to electrical stimuli than do smaller-diameter nerves. The result is closure of central gating mechanism to signals coming through nerves with smaller diameters. The same mechanism of gate control works for the vibration, which has impulses that are transmitted very fast (75 meters per second) through myelinated, thick, A-beta nerves. On the other hand, sense of pain travels at a 2 meters per second speed through unmyelinated and thin C fibers, (Ungor et al., 2014, Nanitsos et al., 2009, Hall and Guyton 2015).

Simultaneous transmission of vibration signals through thick A-beta fibers versus pain signals through C fibers will make the sensory area of the brain release inhibitory neurotransmitters these and inhibit the activation of projection neurons within dorsal horn of spinal cord, leading to gate closure over pain stimuli. This is the reason vibration is used to reduce pain during many painful medical and dental procedures (Reed 2001, Ungor et al., 2014). Another factor that contributes to perception of pain is psychological status of person, particularly his or her fear or anxiety of pain. Dental fear can prolong and intensify the pain (Peretz et al 2004).

Our results are similar to those of Ungor et al. (2014) and Nanitsos et al. (2009) although in the latter study, source of vibration was extra-oral, which might decrease the efficacy of expected gate control mechanism because of pain and vibrated sites being distant. Another study done by Saijo et al. (1995) examined injection pain together with vibration of the site using VibraJect. They could not find significant differences between control and treatment groups. Difference could be due to different devices and methods.

CONCLUSION

This study has found DentalVibe as a useful and effective device in reducing pain while injecting local anesthesia.

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Genetical Communication

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Red cell enzyme gene polymorphism in Gond tribe of Madhya Pradesh

Deepak Bharti*[1], Ruchira Chaudhary[2] and SMS Chahal[3]

[1]*Department of Zoology, Govt. M.V.M., Affiliated to Barkatullah University Bhopal, M.P.*
[2]*Department of Zoology, Sarojini Naidu Govt. Girls Post Graduate College, Bhopal*
[3]*Department of Human Biology, Punjabi University, Patiala Punjab*

ABSTRACT

The present biochemical study is planned primarily to characterize genetically the Gond Tribe of Madhya Pradesh. The blood samples for the present study were collected at random from a total of 200 apparently healthy and not closely related individuals of either sex, of the Gond of Hoshangabad, Betul and Sehore districts of Madhya Pradesh. The samples were analyzed for phenotypes of A1A2BO and Rh (D) blood groups by standard tube method and for Red Cell Enzymes electrophoretic method.Typed Red Cell Enzymes were Adenosine Deaminase, Acid Phosphatase locus 1, Phosphoglucomutase locus 1, Esterase D, Adenylate kinase locus 1 and Glucosephosphate isomerase. A rare allele ACP*C was recorded in Gond tribe despite the fact that it was totally absent not only in Keer of Betul, but also in Korku of Pachmarhi Hoshangabad and Bhils of Jhabua . In case of GPI, rare variant (GPI*1-7) was recorded in Gond Tribe of Hoshangabad of the State. There was great heterogeneity (h) values over the loci in the Gond material, varying from as low as 0.0304 at GPI locus to as high as 0.6244 at A1A2BO locus. Analysis of heterozygosity revealed that in the Gond tribe GPI was the least variable locus and A1A2BO was the most variable locus. Genetic relationships among the present Gond tribe and earlier studied Tribal and Caste Populations of Neighboring States of Gujarat and Rajasthan shows that the Hindu and Muslim separated out from the Tribal population of neighboring States from earlier stage of evolution. Rajasthan Bhil shows distant single line subcluster, the latter tribes were placed together in an another subcluster. In addition all the Tribes of Gujarat and Madhya Pradesh shows close genetic affinities.

KEY WORDS: BIOCHEMICAL MARKERS, GENETIC POLYMORPHISM, GOND TRIBE, RED CELL ENZYMES

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INTRODUCTION

The Scheduled Tribe population of the State is 15,310,000 as per 2011 Census, this constitutes 20.1 percent of the total population (72,620,000) of the State. Madhya Pradesh holds first rank among all the other states in terms of Scheduled Tribe population. The State has a total of forty six (46) Scheduled Tribes (Census of India, 2011). The Great tribal community mostly found in dense forests of the central India is Gond, They are widely spread in the Chhindwara, Betul and others district of Madhya Pradesh, Bastar district of Chhattisgarh and also in the parts of Maharashtra, Andhra Pradesh, and Orissa. Gonds are one of the largest tribal group in the world. The main dialect of Gonds is Gondi boli which is related to Telugu and the other Dravidian languages. In the northern parts, Gonds are often seen speaking Hindi and Marathi while in the southern parts Parsi or Persian is the frequently used language. Gonds are mainly divided into four tribes namely - Raj Gonds, Madia Gonds, Dhurve Gonds, Khatulwar Gonds. Gonds have been largely influenced by the Hindus and for the long time have been practicing the Hindu culture and traditions.

A molecular study (Chaubey et al., 2017) based on allele frequency and haplotype revealed that the Gond share genetic ancestry with the Indian Austroasiatic (ie, Munda) groups, rather than with different Dravidian groups to whom they are most closely related linguistically and The haplotype based analysis (Chaubey et al., 2015) suggested the genome sharing Gond with among Bhil, Kol and with other ethnic groups of South Asian descent.

Although, recently studies (Thakur and Singh 2017; Sharma 2017) were done on Gond tribes but in present investigation we have selected different most Gond tribe populated districts of the state. The present biochemical study was planned to characterize genetically the Gond tribe. They are endogamous population groups, having their own unique culture and language. Although serological studies have been done on some tribes but biochemical genetic markers are still to be examined. There is no previous report on the extensive study of blood genetic markers in Gond of Hoshangabad district of Madhya Pradesh.

MATERIAL AND METHODS

The present genetic study was based on Biochemical and Serological markers for which the blood samples were collected at random from a total of 200 apparently healthy and not closely related individuals of either sex, of the Gonds of Hoshangabad, Betul and Sehore districts of Madhya Pradesh. About 0.5 ml of blood samples were collected by finger prick method into EDTA-K_2 vials, kept in thermo cool icebox and transported to the laboratory within 3-4 days. The samples were analyzed for phenotypes of A_1A_2BO and Rh (D) blood groups by standard tube method and for red cell enzymes by electrophoresis (Bhasin M.K. and Chahal, S.M.S.,1996). For this purpose haemolysates were prepared using freezing and thawing method and stored at -20°C in the freezer. Prepared haemolysates were used for isoenzyme typing by biochemical technique of electrophoresis and specific staining protocols. Typed red cell enzymes were Adenosine Deaminase (ADA), Acid Phosphatase locus 1 (ACP1), Phosphoglucomutase locus 1 (PGM1), Esterase D (ESD), Adenylate kinase locus 1 (AK1) and Glucosephosphate isomerase (GPI).

RESULTS AND DISCUSSION

The distribution of two blood groups and four biochemical markers in Gond tribe are presented in table 1. In A_1A_2BO, the frequency of A_1 allele in Gond is found to be 20.41% while a low value (7.05%) has been recorded in Keer tribe (Bharti et al., 2007) but on the other hand showed partial variation with the Gond tribe (17%) of Anuppur and Dindori districts of MP (Thakur S. and Singh H.S., 2017), whereas Bharti et al., (2007) reported high incidence of allele B in Keer (33.68%) and low in Gond (28.11%) were observed The allele frequency of A_1 in Gond is similar to that of Bhil (21.9%) and Gond (20.1%) of Ambikapur district (Bhatia et al.,1986). On comparison with the caste population, it revealed that, the A_1 allele frequency of Gond tribe is greater than Hindu (15.91%) and Muslim (18.39%) of Indore (Roberts et al., 1974). The percentage frequency of A_2 allele in Gond has been found to be 1.0 %, which is the lowest recorded incidence in any tribal population, but it is noteworthy that it is totally absent in Keer (Bharti et al., 2007) while recorded high (2.0%) in Gond tribe of Anuppur and Dindori districts of Madhya Pradesh (Thakur and Singh 2017). The distribution of allele frequencies of allele O in Gond shows approximate similarity with earlier data of the tribes. In case of Rh blood group, only one case has been found to be Rh negative in Keer of neighboring district Sehore while in Gond tribe four cases has been recorded . In contrast to the caste population of the State these frequencies are lower than those recorded in Sunni (25.2%), Shia (27.7%), Bohra (28.28%) and Brahmin (18.26%), endogamous group of Hoshangabad district (Khan et al., 1985).

The allele frequencies obtained after the electrophoretic typing of the biochemical marker viz, Adenosine Deaminase (ADA), Acid Phosphatase locus 1 (ACP1), Phosphoglucomutase locus 1(PGM1), Esterase D (ESD), Adenylate Kinase locus 1 (AK1) and Glucosephosphate isomerase (GPI), are given in table 2, which shows great

Table 1. Distribution of A1A2BO blood groups in various Scheduled Tribe and Non Tribal populations of Madhya Pradesh and Neighboring States.

S. No.	NAME OF POPULATION	N	ALLELE FREQENCIES OF DIFFERENT BIOCHEMICAL GENES						REFERENCES
			A1	A2	B	O	RHD	RHd	
MADHYA PRADESH									
1.	GOND	200	0.2041	0.01	0.2811	0.5048	0.8586	0.1414	Present study (n=200)
2.	HINDU	175	0.167	0.0294	0.2503	0.5533	0.7732	0.2268	Roberts *et al.*, 1974
3.	MUSLIM	168	0.1869	0.033	0.2587	0.5214	0.8457	0.1543	Roberts *et al.*, 1974
4.	BHIL MP	145	0.219	0.025	0.221	0.535	0.8339	0.1661	Papiha *et al.*, 1978
5.	KEER	131	0.0705	0	0.3368	0.5926	0.9126	0.0874	Bharti *et al.*, 2007
GUJARAT									
6.	VANIA SONI	267	0.1791	0.0138	0.2478	0.5593	0.703	0.297	Undevia *et al.*, 1978
7.	GHANCHI	58	0.255	0.051	0.254	0.44	0.7726	0.2274	Papiha *et al.*, 1981
8.	KUNBI	116	0.146	0.016	0.249	0.589	0.7543	0.2457	Papiha *et al.*, 1981
9.	ANAVIL	50	0.189	0.013	0.213	0.585	0.6536	0.3464	Papiha *et al.*, 1981
10	MUSLIM GUJRAT	65	0.19	0.01	0.269	0.531	0.6962	0.3038	Papiha *et al.*, 1981
11.	VASAVA	71	0.22	0.013	0.185	0.582	0.7626	0.2374	Bhasin *et al.*, 1985
12.	KOTWALIA	102	0.158	0.019	0.315	0.508	0.7797	0.2203	Bhasin *et al.*, 1985
13.	GAMIT	261	0.282	0.031	0.227	0.46	0.7297	0.2703	Bhasin *et al.*, 1985
RAJASTHAN									
14.	BHIL	92	0.14	0.027	0.231	0.602	0	0.2085	Kumar *et al.*, 1999

Table 2. Distribution of Red cell Enzymes polymorphism in the Gond Tribe of Madhya Pradesh.

S. No.	Gene	n	Phenotypes	Number Observed	Allele	Allele frequencies	$\chi 2$ (H.-W.)
1.	ADA	145	1	126	*ADA*1*	0.9345	0.003
			1,2	19	*ADA*2*	0.0655	
			2	0			
2.	AK1	189	1	167	*AK1*1*	0.9418	0.003
			1,2	22	*AK2*2*	0.0582	
			2	0			
3.	ESD	196	1	79	*ESD*1*	0.5995	1.040
			1,2	77	*ESD*2*	0.4005	
			2	40			
4.	PGM1	196	1	94	*PGM1*1*	0.6856	0.087
			1,2	78	*PGM2*2*	0.3144	
			2	22			
5.	ACP1	182	A	13	*ACP1*A*	0.2390	0.413
			A,B	60			
			B	106	*ACP1*B*	0.7527	
			A,C	1	*ACP1*C*	0.0083	
			B,C	2			
6.	GPI	195	1	189	*GPI*1*	0.9846	-
			1,3	5	*GPI*3*	0.0128	
			1,7	1	*GPI*7*	0.0026	

Table 3. Distribution of Allele frequencies of Red Cell Isozymes in various tribal and non tribal populations of Madhya Pradesh and neighboring states.

S. No.	NAME OF POPULATION	ALLELE FREQENCIES OF DIFFERENT BIOCHEMICAL GENES									REFERENCES
		AK1	AK2	ESD1	ESD2	PGM1	PGM2	ACPA	ACPB	ACPC	
1	GOND	0.9418	0.0582	0.5995	0.4005	0.6856	0.3144	0.239	0.7527	0.0083	Present study
2	HINDU	0.901	0.099	0	0	0.7241	0.273	0.3103	0.6868	0.0029	Roberts *et al*., 1974
3	MUSLIM	0.902	0.098	0	0	0.7256	0.2744	0.3282	0.6687	0.0031	Roberts *et al*., 1974
4	BHIL MP	0.9586	0.0414	0.793	0.207	0.7042	0.2958	0.1993	0.8007	0	Papiha *et al*., 1978
5	KEER	0.8931	0.1069	0.8931	0.1069	0.8511	0.1489	0.3228	0.6712	0	Bharti *et al*., 2007
6	VANIASONI	0.8774	0.1226	0.8452	0.1548	0.6502	0.3498	0.2595	0.7405	0	Undevia *et al*., 1978
7	GHANCHI	0.956	0.044	0.873	0.127	0.634	0.357	0.364	0.646	0	Papiha *et al*., 1981
8	KUNBI	0.918	0.082	0.811	0.189	0.665	0.326	0.274	0.713	0.013	Papiha *et al*., 1981
91	ANAVIL	0.928	0.072	0.84	0.16	0.714	0.286	0.357	0.643	0	Papiha *et al*., 1981
10	MUSLIM GUJRAT	0.918	0.082	0.815	0.185	0.667	0.325	0.254	0.738	0.008	Papiha *et al*., 1981
11	VASAVA	0.923	0.077	0.773	0.227	0.758	0.242	0.103	0.862	0.035	Bhasin *et al*., 1985
12	KOTWALIA	0.977	0.023	0.908	0.092	0.82	0.18	0.244	0.722	0.033	Bhasin *et al*., 1985
13	GAMIT	0.965	0.035	0.866	0.134	0.673	0.327	0.265	0.721	0	Bhasin *et al*., 1985
14	BHIL RAJ.	0.889	0.111	0.793	0.207	0.759	0.241	0.259	0.741	0	Kumar *et al*., 1999

variations. From the six enzymes studied, four enzyme systems (ADA, PGM1, ESD and AK1) showed common phenotypes, while in ACP1 a rare allele ACP*C was recorded in Gond tribe despite the fact that it was totally absent not only in Keer of Betul, but also in Korku of Pachmarhi Hoshangabad (Saha et al., 1987) and Bhils of Jhabua (Papiha et al., 1978). In case of GPI, rare variant (GPI*1-7) was recorded in Gond Tribe of Hoshangabad of the state. The comparison of allele frequencies of Gond tribe with other caste and tribal populations of the state is shown in table 3. The allele frequencies of ADA*1 (0.9345), PGM1*1(0.6856), ACP1*A (0.239) and AK1*1 (0.9417) in Gond are approximately similar from Bhil of Jhabua {ADA*1 (0.921), PGM1*1(0.704), ACP1*A (0.201) and AK1*1 (0.959)} (Papiha et al., 1978).

HETEROZYGOSITY (H)

Table 4 shows the locus–wise and population–wise estimates of heterozygosity (h) in the Gond and erlier studied caste and tribal populations of Madhya Pradesh. There was great heterogeneity in h values over the loci

Table 4. Heterozygosity (h) estimates in the present tribe and earlier studied Caste populations of Madhya Pradesh.

Locus	Caste		Scheduled Tribe			Locus-wise Average heterozygosity (H)
	Muslim	Hindu	Gond	Bhil	Keer	
A1A2BO	0.6297	0.6028	0.6244	0.6163	0.5304	0.6007
RH(D)	0.2610	0.3507	02428	0.2770	0.1595	0.2582
ADA	-	-	0.1224	-	0.1909	-
AK1	0.1768	0.1784	0.1096	0.0793	0.1909	0.1470
ESD	-	-	0.4802	0.3283	0.3986	-
PGM1	0.3982	0.4012	0.4311	0.4166	0.2535	0.3801
ACP1	0.4451	0.4319	0.3763	0.3192	0.4410	0.4027
GPI	-	-	0.0304	-	0.0076	0.0127

Table 5. Genetic differentiation in the present studied Gond tribe and earlier studied Tribal and Castes population of Madhya Pradesh – estimates by Nei's GST.

Genetic locus	Gene diversity in total population (H_T)	Intra-subpopulational gene diversity (H_S)	Inter-subpopulational gene diversity (D_{ST})	Coefficient of gene differentiation (G_{ST})
A1A2BO	0.6041727	0.5997652	0.0044075	0.0072951
RH(D)	0.2622226	0.2582126	0.00401	0.01529235
AK1	0.1483750	0.1446447	0.0037303	0.025141027
PGM1	0.3869012	0.3805439	0.0063573	0.01643132
ACP1	0.40892743	0.4035656	0.00536183	0.013111935
Average	0.362119786	0.3573464	0.004773386	0.013181787

in the Gond material, varying from as low as 0.0304 at GPI locus to as high as 0.6244 at A1A2BO locus. Thus, analysis of heterozygosity, a measure of genic variation, revealed that in the present Scheduled Tribe material GPI was the least variable locus and A1A2BO was the most variable locus.

NEI'S GENE DIVERSITY ANALYSIS

Estimates of the various measures of gene diversity (Nei, 1973) viz., H_T, H_S and G_{ST} among (Based on five loci) the present Tribe and earlier studied populations of Madhya Pradesh are set out in Table 5. The table shows that the intra–subpopulational gene diversity (H_S) varied widely (range 0.1446447 at AK1 locus to 0.5997652 at A1A2BO locus). As for the inter–subpopulational gene diversity (D_{ST}), it ranged from a low of 0.0037303 at AK1 locus to a high of 0.0063573 at PGM1 locus. Thus, it is clear that the gene diversity between populations (0.004773386) was much lower than the gene diversity within them (0.357364). As for the coefficient of gene differentiation (G_{ST}), the values were quite variable over loci (range 0.0072951 – 0.02514102), A1A2BO being the least differentiated locus and AK1 being the most differentiated locus among the Tribal and Caste populations of Madhya Pradesh (Table 5).

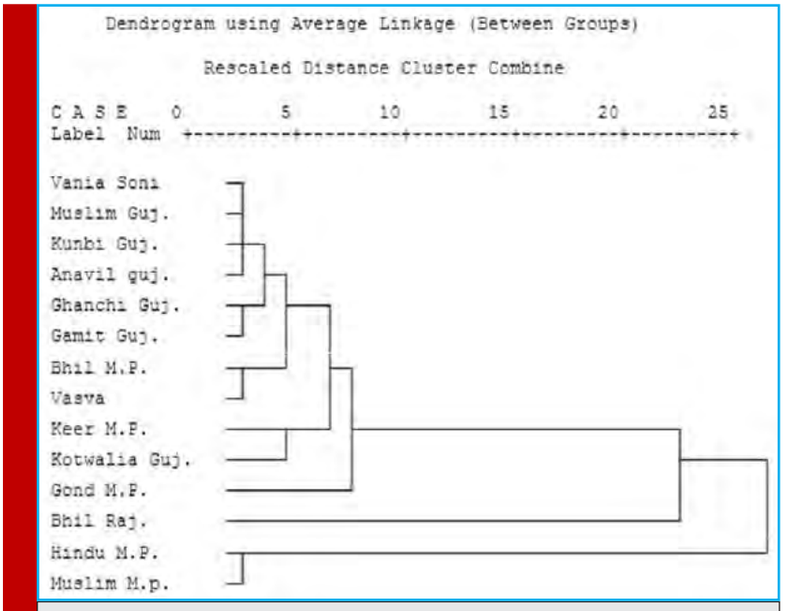

FIGURE 1. Dendrogram showing genetic relationships among the present Tribe Gond of Madhya Pradesh and various Scheduled Tribe and Non tribal populations of the state and neighboring states of Gujarat and Rajasthan based.

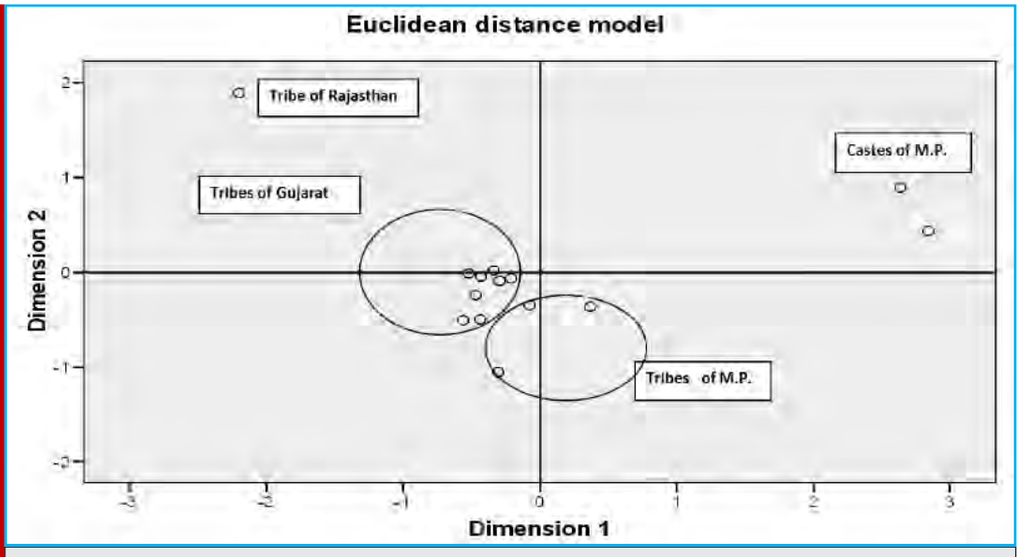

FIGURE 2. The Euclidean distance Tribes of Madhya Pradesh and various Scheduled Tribe and non tribal populations of the neighboring states of Gujarat an Rajasthan based on 6 common markers (A1A2BO, RH(D), AK1, ESD, PGM1, ACP1).

Nei's genetic distance (D) and Eulclidean model analysis

Genetic relationships among the present studied Gond tribe and earlier studied tribal and Caste Populations of Neighboring States of Gujarat (Papiha et al., 1981; Bhasin et al.,1985 and Undevia et al., 1978) and Rajasthan (Kumar et al.,1999) presented in figure 1 and 2 (Based on Six common Genetic Markers). These figures show that the Hindu and Muslim separated out from the Tribal population of all the studied States. Rajasthan Bhil showed a single line subcluster, the latter tribes were placed together in an another subcluster. In edition all the Tribes of Gujrat and Madhya Pradesh showed close genetic affinities. The chi square (χ^2) test depicted that serological and biochemical (ADA, PGM1, ACP and AK1) markers are in genetic equilibrium as the differences between observed and expected frequencies are statistically not significant, except for ESD where the (χ^2) value is statistically significant.

CONCLUSION

The present serological markers study concludes with the low incidence of A_1 allele and high of B and presence of A_2 allele in few cases of Gond of Hoshangabad District. On the other hand Rh negative allele was found higher in Gond. In Biochemical trait, presence of less common phenotype in ACP i.e. ACP*C and GPI (GPI*7) were recorded The allele frequencies of ADA, PGM1, ACP1 and AK1 indicate closeness between Gonds of Hoshangabad, Bhil and Korku of Hoshangabad district of M.P. (Pachmarhi) but different from Keer of neighboring district. Gond tribe has genetic affinities with tribes of M.P. and Gujarat but great distance showed against Caste population of M.P. and tribal population of Rajasthan. The present study will help to characterize genetically the Gond tribe of Madhya Pradesh.

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CONFLICT OF INTEREST

None, all authors equally contributed to this research work

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Associations of physical activity and sedentary behaviors with dietary behaviors among mid-adolescent female students in the southeast of Iran

Fariba Shahraki-Sanavi[1], Fatemeh Rakhshani[2], Alireza Ansari-Moghaddam[3]* and Mahdi Mohammadi[4]

[1]*PhD Student Health Education & Promotion, Health Promotion Research Center, Public Health Department, Zahedan University of Medical Science, Zahedan, Iran*
[2]*Professor Health Education, Safety Promotion and Injury Prevention Research Center (SPIPRC), Shahid Beheshti University of Medical Sciences, School of Public Health, Tehran, Iran*
[3]*Professor Epidemiology,Health Promotion Research Center, Epidemiology & Biostatistics Department, Zahedan University of Medical Sciences, Zahedan, Iran*
[4]*Associate Professor in Statistic, Health Promotion Research Center, Epidemiology & Biostatistics Department, Zahedan University of Medical Sciences, Zahedan, Iran*

ABSTRACT

Given the complexity of dietary behaviors and physical activity of adolescents and its relationship to the chronic diseases later on, the present study aimed to investigate the relationship between the physical activity and sedentary behaviors with dietary behaviors of female high school students in the southeast of Iran. This cross-sectional study was conducted in four public high schools in the southeast of Iran (Zahedan) on a sample of 457 female students of Tenth Grade. Information collected using a self-reported 38-item questionnaire with the content validity ratio (CVR) of 0.79, the content validity index (CVI) of 0.88 and the reliability (α) of 0.70. The data were analyzed by SPSS15 Software by linear and logistic regression models. Physical activity and socio-economic class of students were predictors of their healthy dietary behavior. Students who had spent much time in sedentary behaviors were more likely to consume fast foods.It is recommended to implement some combined interventions including training, consultation and environmental supports in schools along with the focus on family intervention so as to strengthen and lengthen the effects of interventions.

KEY WORDS: PHYSICAL FITNESS, NUTRITION, TEENS

ARTICLE INFORMATION:

**Corresponding Author: ansaryalireza@yahoo.com*
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INTRODUCTION

Despite major plans of public health to increase the public awareness regarding the promotion of healthy dietary behaviors and physical activity of healthy people by 2020, these issues are still considered as major concerns of the public health. (America's Health Literacy: 2008) Nowadays, non-communicable diseases have been prevalent in both developed and developing countries. It has been well proven that poor dietary habits are directly associatedwith the outbreak of some serious health problems such as diabetes, cardiovascular diseases and probably some types of cancers in the future life ofthe adolescents. (Ogden et al 2012, Rao 2008).

In comparison, increased physical activity is related to the lower blood pressure, body fitness and blood fat regulation. It also reduces the depression and anxiety and increases self-esteem and quality of life. On the other hand, a decrease in the physical activity and increase in the sedentary activitiesare significantly associated with the risk of obesity, (Singh et al., Vuori 2010; Hills et al., 2006; Andersen et al., 2011; Kantomaa et al., 2008; Motl et al., 2004) cardiovascular health defects, (Ankoski et al. 2011; Healy et al 2011; Henson et al. 2013) and mortality. (León-Muñoz et al., 2013; Matthews et al. 2012).

Key indicator of bodily activity includes physical activity and activities with low mobility (sedentary activities), while dietary behavior includes the diet and the number of meals. (Jiménez-Pavón et al. 2011-Rodríguez et al., 2011).

The ideal diet for teenagers includes supplying needed calories by eating nutrient foods such as fruits, vegetables, dairy, whole grains, and low-fat protein and limiting the consumption of high-fat foods and beverages. Then, it is recommended for the teenagers to have healthy diet with eating at least five portions of fruits and vegetables a day and limiting fat intake to less than thirty percent of their energy needs, (Nelson et al., 2005) as well as having at least sixty minutes of moderate to severe physical activity most of the days of the week and participation of two hours or less per day in sedentary activities, (Sanchez et al., 2007).

However, some studies reported that only 30-40% of teenagers have at least sixty minutes of physical activity five times or more per week (Larsen et al. 2004) and the level of sedentary activities increases in their older age. (Driskell et al., 2008).

In addition, the nutritional trends suggest that most teenagers don't adhere to the recommended diet of servings five portions of fruits and vegetables a day, consuming dairy products two or more times a day, and having grains six times a daywhereas they may not eat breakfast regularly.In comparison, they may eat fast food two or more times a week. (Larson et al., 2008) On the other hand, nearly a quarter of their daily calorie is supplied by having sugary drinks, (Harrington 2008).

Data suggest that in recent years, there has not only been a decrease in the level of Iranian teenagers' physical activities, but their food tastes have also had a tendency towards the high-calorie foods and the foods lacking of nutritional value (Kelishadi et al. 2005).

However, healthy nutrition and regular physical activities which are parts of the health promoting behaviors can preventwasting of costs, causingmorbidity, and mortality (Lee and Loke 2005). Given the importance of healthy nutrition and physical activity to maintain and improve the public health, the currents study aimed to investigate the relationship between the physical activity and sedentary behaviors, and dietary behaviors of female high school students in the southeast of Iran (Zahedan) so as to assess the current status of the adolescent behaviors; then, the results can be used for planning of health promotion interventions in schools.

MATERIAL AND METHODS

The present cross-sectional school-based study was carried out in the South East of Iran (Zahedan) in 2015. First, 4 female state high schools were randomly selected; then, all students in Grade 10 (n = 457) wereenrolled in the study through a census. The objectives of the study and the way of answering the questionnaire were explained to the participants. The subjects were entered into the study with observing the ethical codes and voluntary. Data collected via a self-reported questionnaire including demographic questions (11 items), healthy dietary behaviors (14 items), physical activity and sedentary behaviors (7 items), thestatus of the school and description of the weight (6 items). The questionnaire has content validity ratio (CVR) of 0.79, the content validity index (CVI) of 0.88 and the reliability (α) of 0.70 that examines the students' nutritional status and physical activity during the week before the study. The minimum and maximum scores for nutrition were 14 and 64, respectively; for the physical activity dimension,the minimum, and maximum scores were 7 and 47, respectively. The questionnaires did not include any identifying information and the students were explained that the information was solely analyzed in thegroup. The allocated time to complete the questionnaire was 15-20 minutes. After collecting the data, they were analyzedby SPSS15 Softwareusing the linear and logistic regression models.

RESULTS

The study includeda total of 457 teenage girls of 10th Grade in the 14-17 age range and majority of them were at the birth rank of 1-3. Half of their fathers were employees and their education levels were around diploma. Most of their mothers were housewives and majority of them had the high school diploma as well. Table 1 dem-

Table 1. The prevalence of dietary behaviors, Physical/sedentary activities and weightrelated

Dietary behaviors	Percent (95% CI)
Student who did not eat fruit drink 100% fruit juices	53.08(48.47-57.69)
drank 100% fruit juices one to three day/week	24.45(20.48-28.41)
drank 100% fruit juices four to six day/week	6.17(3.94-8.38)
drank 100% fruit juices one times/day	12.11(9.10-15.12)
drank 100% fruit juices two or more times/day	4.19(2.33-6.03)
Student who did not drink milk	35.90(31.47-40.33)
drank milk one to three day/week	35.24(30.83-39.65)
drank milk four to six day/week	9.47(6.76-12.17)
drank milk one times/day	14.54(11.28-17.79)
drank milk two or more times/day	4.85(2.86-6.82)
Student who did not drink a can or glass of cola or other	47.80(43.18-52.40)
drank a can or glass of cola or other one to three day/week	10.79(7.92-13.65)
drank a can or glass of cola or other four to six day/week	6.83(4.49-9.15)
drank a can or glass of cola or other one times/day	29.07(24.88-33.26)
drank a can or glass of cola or other two or more times/day	5.51(3.40-7.61)
Student who did not drink a can or glass of energy/Sports drank	90.52(87.82-93.23)
drank a can or glass of energy/Sports drank one to three day/week	5.50(3.40-7.61)
drank a can or glass of energy/Sports drank four to six day/week	0.66(0.08-1.40)
drank a can or glass of energy/Sports drank one times/day	2.20(0.84-3.55)
drank a can or glass of energy/Sports drank two or more times/day	1.10(0.13-2.06)
Student who did not eat fruit	3.74(1.99-5.49)
ate fruit one or two day/week	18.72(15.12-22.32)
ate fruit three to six day/week	28.85(24.67-33.03)
ate fruit five to seven day/week	48.68(44.06-53.29)
Student who did not eat vegetables	13.44(10.28-16.58)
ate vegetables one or two day/week	40.31(35.77-44.83)
ate vegetables three to six day/week	28.41(24.24-32.57)
ate vegetables five to seven day/week	17.84(14.30-21.37)
Student who did not eat beef/lamb	10.57(7.73-13.41)
ate beef/lamb one or two day/week	29.52(25.30-33.72)
ate beef/lamb three to six day/week	36.12(31.68-40.55)
ate beef/lamb five to seven day/week	23.79(19.85-27.72)
Student who did not eat chicken/fish	7.93(5.43-10.42)
ate chicken/fish one or two day/week	34.80(30.40-39.20)
ate chicken/fish three to six day/week	34.14(29.76-38.51)
ate chicken/fish five to seven day/week	23.13(19.23-27.02)
Student who did not eat dairy products	5.95(3.76-8.13)
ate dairy products one or two day/week	29.74(25.51-33.95)
ate dairy products three to six day/week	25.77(21.73-29.80)
ate dairy products five to seven day/week	38.55(34.05-43.04)
Student who did not eat sweets/chocolate	10.35(7.53-13.16)
ate sweets/chocolate one or two day/week	24.67(20.68-28.65)
ate sweets/chocolate three to six day/week	46.26(20.68-28.65)
ate sweets/chocolate five to seven day/week	18.72(15.12-22.32)
Student who did not eat nuts	18.94(15.32-22.56)
ate nuts one or two day/week	39.21(34.69-43.71)
ate nuts three to six day/week	24.01(20.06-27.95)
ate nuts five to seven day/week	17.84(14.30-21.37)
Student who did not eat goody	23.79(19.85-27.72)
ate goody one or two days/week	18.72(15.12-22.32)
ate goody three to six day/week	39.87(35.34-44.38)
ate goody five to seven day/week	17.62(14.10-21.13)

Student who did not eat fast food ate fast food one or two days/week ate fast food three to six day/week ate fast food five to seven day/week	40.31(35.77-44.83) 9.47(6.76-12.17) 42.73(38.16-47.29) 7.49(5.05-9.91)
Students who did not eat breakfast ate breakfast on all 7 days	11.45(8.51-14.39) 53.74(49.14-58.34)
Mean of Dietary behaviors (SD)	**43.69(6.20)**
Physical/sedentary activities	**Percent (95% CI)**
Student who did not participate in at least 60 minutes of physical activity participated in physically active at least 60 minutes/day on 1 or 2 days/week participated in physically active at least 60 minutes/day on 3 or 4 days/week participated in physically active at least 60 minutes/day on 5 or 6 days/week participated in physically active at least 60 minutes/day on all 7 days	46.56(32.11-41.01) 41.85(37.29-46.40) 13.44(10.28-16.58) 3.96(2.16-5.76) 4.19(2.33-6.03)
Student who did not participate in muscle-strengthening activities participated in muscle-strengthening activities on 1 or 2 days/week participated in muscle-strengthening activities on 3 or 4 days/week participated in muscle-strengthening activities on 5 or 6 days/week participated in muscle strengthening activities on all 7 days	50.00(45.38-54.61) 40.97(36.42-45.50) 4.41(2.51-6.30) 1.54(0.40-2.67) 3.08(1.48-4.67)
Student who did not go to jam went jam on 1 or 2 days/week went jam on 3 or 4 days/week went jam on 5 days/week	70.26(66.04-74.48) 21.59(17.78-25.38) 6.61(4.31-8.90) 1.54(0.40-2.67)
Student who did not play video or computer games or used a computer played video or computer games or used a computer for 1 to 2 hours/day played video or computer games or used a computer for 3 or more hours/day	49.34(44.72-53.95) 44.05(39.46-48.63) 6.61(4.31-8.90)
Student who did not watch television Student who watched 1 to 2 hours/day of television Student who watched 3 or more hours/day of television	9.03(6.38-11.67) 55.95(51.36-60.53) 35.02(30.61-39.42)
A student who did not use mobile (whats up, Viber, email, ...) used mobile (whats up, Viber, email, ...) 1 to 2 hours/day used mobile (whats up, Viber, email, ...) 3 or more hours/day	38.99(34.48-43.49) 37.00(32.54-41.46) 24.01(20.06-27.95)
Mean of Physical/sedentary activities (SD)	**22.49(4.84)**
Weight-related	
Student who described themselves as Obese	4.41(3.76-8.13)
Student who described themselves as Overweight	22.25(11.08-17.55)
Student who described themselves as Normal weight	53.08(48.47-57.69)
Student who described themselves as low weight	14.32(18.40-26.08)
Student who described themselves as Underweight	5.95(2.51-6.30)

onstrated the prevalence of dietary behaviors, Physical/sedentary activities and weight related variables. As can be seen, teenagers who do not have all-natural juice and milk twice a day or more were about 4 and 5 %, respectively. Nearly half of them had fruits every day, 18% vegetables, 15% milk and dairy products, and 18% nuts. In addition, nearly half of the surveyed adolescents reported the least level of daily physical activity and stretching exercises. The percentage of adolescents who spend more than two hours a day for sedentary activities including working with the computer, watching TV, using cell phones and social networks were about 35.7 and 24 %, respectively. Moreover, about 4% of students reported to be overweight and 6% reported severe weight loss.

The physical activity and socio-economic classeswere predictors of the healthy nutritional behavior (Table 2).

The results (Table 3) also showed that, there is a positive significant relationship between the consumption of fast foods and behaviors related to the physical activity in the studied high school students.

DISCUSSION

The findings of the present study illustrated that the majority of the studied adolescents do not have daily all-natural juiceand less than a quarter of them do not eat, milk. Almost half of them have daily breakfast, fruits and less than a quarter of them eatonce or more

Table 2. Linear Regression Test for the Prediction of Healthy Dietary Behaviors of the Studied Adolescents

Dietary Independent Variable	Unstandardized Coefficients		Standardized Coefficients	t	P value
	B	Std.Error	Beta		
Physical/sedentary activities	0.33	0.05	0.26	5.84	0.001
Family Economy	0.99	0.38	0.11	2.56	0.01

Table 3. Binary Logistic Regression for ate fast food with Sedentary-related behaviors

Sedentary Behavior	Fast food OR (95% CI)
Watched TV ≥2 hours per day No Yes	Reference 0.63(0.43-0.94)*
Used mobile (whats up, Viber, email, ...) ≥2 hours per day No Yes	Reference 0.55(0.36-0.84)**
Played video or computer games or used a computer for something other than school work≥2 hours on an average school day No Yes	Reference 0.54(0.28-1.03)*
Ate at least one meal or snack at a fast food restaurant ≥1 day/week *p<0.05 **p<0.01	

fizzy sugary drinks daily. Half of them do the least level of physical activity and stretching exercises and almost a third of them are engaged in sedentary activities. A quarter of them were overweight or obese. A statistical significant relationship was observed between the unhealthy nutrition and physical activity behavior and socio-economic classes of the student and between the fast food consumption and sedentary behaviors.

The results indicate that the removal of breakfast meal among the students ranged from 1.7 to 30% and having daily breakfast reported to be 31-33%.The results also suggest the high level of not having breakfast in female students and its growing prevalence rate with the low socio-economic status and higher ages. Students who eat breakfast regularly, have healthier food choices and adolescents who go to school without having breakfast, their typical foods are chips, popcorn and sugary drinks. In this regard, the results of the present study are consistent with the previous ones.

Regarding the consumption of milk and dairy products at least 1-3 times a day, the consumption level has been reported to be 2-25.1% and lack of consumption of the same group reported to be 14.8 – 22.4 % ; however, it has been reported that 98% of Swedish adolescents use milk and its products at least once a day. Similar to previous studies, the subjects in the present study, despite the daily distribution of milk in schools, use milk and dairy products at the lower level.

With respect to the consumption of fruits and vegetables, the results of previous studies indicate that 11.5 – 68.2 % of adolescents in different countries have daily natural juices and fruits, and 11.6 – 58% of them eat fruits and vegetables at least once or more a day. In a study in Iran, the mean frequency of the consumption of fruits and vegetables among the students were 1.2 and 1.1 times a day, respectively. In the present study, the consumption of natural juices was at the lower level, but due to the availability of fruits in Iran, almost half of the students have daily fruit consumption. However, like the previous studies, the consumption level of vegetables was at the lower level.

In the present study, the consumption of red and white meat was observed only in one quarter of the students. While the consumption level of red meat among the adolescents of Syrian and Swedish has been reported equal to 62% and 82 % a day, respectively.According to other studies, high-protein diets containing red meat would also be resulted in weight control.

The results of studies reflect lower consumption of fruits, vegetables, fish, dairy products and eggs among adolescents; they also get more unhealthy foods like French fries, burgers, sugary drinks and a variety of jellies. Due to containing high sugar and energy, sodas cause to overweight and reduced consumption level of fruits, vegetables and dairy products. However, this relationship has not been seen in some studies. In the pre-

sent study, half of the students had fizzy sugary drinks more than twice a day.

The prevalence of watching TV more than 2 hours a day among the American students showed a significant reduction from 43% in 1999 to 32% in 2013 and an increase to 43.3% in 2015. There has also been an increase in the time allocated to using computer and playing video games from 22% in 2003 to 43% in 2015. In comparison, Iranian female adolescents were similar to American teenagers in this regard; however, the prevalence of reduction in the physical activity and sedentary behaviors in Jordanian adolescents was reported to be at higher level; and in European adolescents, the results showed that only 18% of them have consistently healthy behaviors and 21% of them had consistently unhealthy behaviors.The percentage of watching TV in the studied adolescents was similar to the results of above-mentioned studies, but the time spent on computer games was lower in this population.

The results indicate that nearly one- third of adolescents had daily fast food and nearly a quarter of them eat fast food more than three times a week; in the families with high income and residents of the North of the city, the level of fast food consumption was high. The results indicate that one of the factors influencing the food choices in adolescents is their socio-economic status. On the other hand, consumption of fast foods and a sedentary life style are among the factors of overweight in adolescents.Like the previous studies, fast consumption level was high. Furthermore, a significant relationship was observed between the socio-economic status of students and their orientation to unhealthy nutrition. In general, the role of the family's economic status on food choices can be justified due to the increased purchasing power and access to a variety of foods.

The prevalence of overweight in female Asian teens was reported 6.2 – 18.9 % and the obesity 1.8 – 4.7 %; the overweight in adolescents of Spain, Mexico, Ecuador, Jordan, Taiwan, the U.S, and New Zealand has been reported to be 8.3 – 26.6 % and the obesity 3.9 – 18%. The results showed that the prevalence of overweight or obesity among the Filipino teens has had a reduction from 11.4 % in 2003 to 9.4% in 2011. Similar description of weight with Asian teens has been reported in the present study by our participants.

The results demonstrated a relationship between the sedentary behaviors and less healthy diet. The adolescents who watch TV more than 5 hours a day and don't exercise are more likely to consume fast foods.The present study also showedthat adolescents with more sedentary activities have more chances to use fast foods. Said the reasons for this might be the fact that adolescents who watch TV more and have less physical activity are more inclined to the advertised foods and they normally eat more food while watching TV.

According to the results and since the health promotion of this age range can be effective in the prevention of non-communicable diseases in the developing countries, it's recommended to establish the necessary measures to comply with the principles of healthy lifestyle habits, because subsequent attempts to change the established habits will be very difficult and perhaps impossible. On the other hand, given the importance of the family in Iran and due to the emotional and financial dependency of adolescents to families in this age range, the strong impact of family support on physical activity and dietary behaviors of students is logical. Therefore, designing training programs with an emphasis on the central role of the family can be effective in the promotion of healthy dietary behaviors and doing physical activities in this age group. Therefore, it's also necessary to design and implement interventions based on behavior change for adolescents in the schools.

LIMITATION

Students participated in this study were limited to the public schools and grade 10, therefore, are not representative of all adolescents in this age group. In addition, the data collected are based on a self-administered questionnaire.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS CONTRIBUTIONS

The overall implementation of study design, data management and analysis and manuscript preparation were the results of join efforts by multiple individuals who are listed as coauthors of this paper. All authors read and approved the final manuscript.

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Investigating the role of modern leadership styles and thinking style with productivity

Farah Shahraki-Sanavi[1], Mahmod Ghorbani[2]* and Fariba Shahraki-Sanavi[3]

[1]*Ph.D Student Educational Management, Zahedan University of Medical Science, Zahedan, Iran*
[2]*Associate Professor, Department of Management, Mashhad Unit, Islamic Azad University, Mashhad, Iran*
[3]*Ph.D Student Health Education & Promotion, Health Promotion Research Center, Public Health Department, Zahedan University of Medical Science, Zahedan, Iran*

ABSTRACT

Productivity is one of the most important indicators in the organization process and a variety of programs designed to maximize it. This indicator has a direct relationship with organizational success and profitability. The factors and components that are related to employee productivity and performance are leadership styles and thinking styles. Therefore, this study was designed to examine the role of leadership styles (modern leadership) and thinking style with productivity. The statistical population of this study consisted of all staff and officials of Kerman education and training organization. Out of 291 subjects, 165 subjects were selected according to Morgan table using convenient sampling method and completed modern leadership style, thought style and productivity questionnaires. The results of the research showed a significant positive and significant relationship between the thinking style and its components and modern leadership style and its components. Modern leadership style and thinking style are a strong and appropriate predictor of productivity. According to the results obtained, in order to increase productivity and efficiency, it is necessary to pay attention to the leadership styles and its methods, and the style of thinking and strengthening it according to organizational needs.

KEY WORDS: PRODUCTIVITY, MODERN LEADERSHIP STYLE, THINKING STYLE, TRANSFORMATIONAL LEADERSHIP

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**Corresponding Author: faribasanavi@gmail.com*
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INTRODUCTION

Applying efficient manpower and their capabilities to fit the needs of the organization and company is one of the most important organizational and productive challenges. The human resources are the basis of the plans and the specific program and the core of planning in achieving different policies. Hamdi et al. (2014). Organizational success depends on human resources, and in all production and service organizations, these are the human resources that are the core of the executive and the main supplier of the organization's interests Cogin, et al. (2016).

The importance of human resources in achieving organizational goals is crucial in this field. In fact, solutions and programs that improve the performance and efficiency of workforce and organization are one of the ultimate goals of a system. Productivity is a qualitative and quantitative component in relation to maximizing the performance and functionality of each domain, and its purpose is to manpower, exploitation to the optimum possible extent of the talents and abilities of the workforce and management to achieve the designated program. Achieving productivity and proper utilization of the factors in its place requires the proper management in certain areas Bloom et al. (2011).

Therefore, management and leadership are a key factor in this regard. Leadership and organizational management refers to the formulation of policies and lines of specific administrative and commercial frameworks that the organization and company members meet in accordance with that movement and the basic needs of the organization Downe et al. (2016). What kind of leadership is most effective and what determines the leadership style results from the style of thinking and its related programs. Bierema (2016). Thinking style addresses the conceptual framework and indicators of individual assessment of the environment and conditions that make decisions or conclusions in line with it, Goldman et al. (2015).

In other words, the thinking style is an indicator that plans, evaluates and concludes the basic and editorial principles of a person and organization, a thinking style that includes a variety of varieties, the product of the educational environment, the scope of knowledge and knowledge, experience, developmental structures, and ... and it is the basis of decision making in different categories. Bouhali et al. (2015).

Therefore, thinking style and its related factors develops the leadership and management style of an organization or institution. Various researches have shown that there are certain relationships between managerial styles and leadership, and the efficiency and various management components of employees and subordinates.

Various researches have shown a direct and specific relationship between management styles with organizational commitment and loyalty to the organization (Yahaya and Fawzy (2016) with employee motivation and performance El-Zayaty (2016) with self-esteem and self-efficacy Owoseni (2014), etc. For example, Bambale et al. (2016) have shown that senior management styles and directors of each unit directly predict employee behavior and their behavior, and type of behavior and accountability of employees is also identified and indexed for management and its related factors. As mentioned earlier, there is a clear relationship between leadership style and thinking with productivity and performance, but recognizing the best management style and thinking is an obscure problem that still has not been definitive.

The modern leadership, which has concerned the employees and intervention, and the style of dealing with them is the foundation for successful leadership on the balance of intervention, evaluation, and type of relationship Khan et al. (2016). For example, transformational leadership structures can facilitate the performance of its followers as a result of the leader's permeability, in which the overall framework shows that raising awareness of the evolutionary leadership attributes and its importance and value in developing its characteristics from the clan, the idealistic intrusive behavior, motivation Inspirational, intellectual motivation, individual considerations and an ideal influence on his followers to raise personal ambitions for a single collective goal in the organization, mission or vision of the organization, Blackwell (2006).

The pragmatic leadership, which refers to the correct intervention and the fulfillment of the requirements of the job and the subsequent reasonable demands, or the unconstrained leadership that guides the basis of its process without direct involvement in the work, all refer to the existence of different leadership styles and implement it in order to increase the revenue of the organization and participation. Considering the importance of the mentioned categories, this research studies the relationship between modern leadership styles and thinking style with productivity.

MODEL OF STUDY

The conceptual model based on the hypotheses of this research shows that in this research, we investigate the relationship between thinking style, executive, judiciary and legislative thinking, and modern leadership styles, including transformational, pragmatic and Laissez fair leadership, and its effects on productivity.

RESEARCH METHODOLOGY

This research describes current and existing conditions and examines existing relationships. Therefore, the

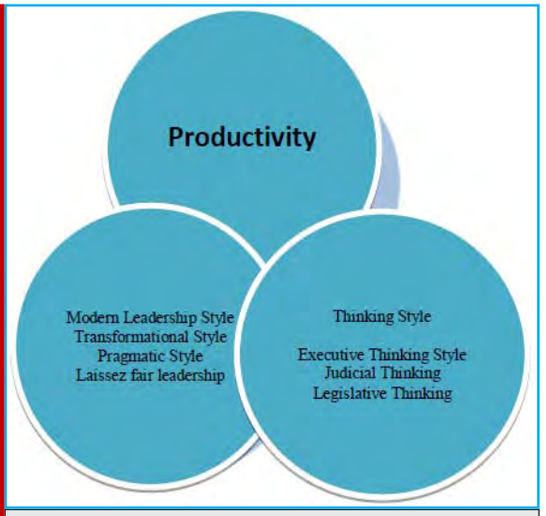

FIGURE 1. Conceptual Model of Study

nature of this descriptive research is also a correlation and applied.

In this research, two field and library methods were used to collect information and complete the questionnaire. The library method has been used to collect and complete theoretical foundations of the research. In order to analyze the inference and the relationships between research hypotheses and data collection, the field method has been used for statistical analysis and the questionnaire has been completed.

SOCIETY AND SAMPLE

In this research, the society consisted of all employees at Kerman education and training organization. They were 291 subjects. It should be noted that due to the nature of the research design and existing researcher-made questionnaire, to fill the questionnaire from both the personnel and employees as well as managers and leaders can be used. According to the form of work and dispersion of sample members and according to Morgan table, 165 of them were selected as sample of research using convenient sampling.

RESEARCH TOOL

THINKING STYLES QUESTIONNAIRE

The Sternberg and Wagner thinking styles questionnaire, which has 24 questions, measures levels of executive, judicial, and legislative thinking styles. The similarity of this test in Iranian sample according to the theoretical Nazari Far et al. (2010) was 0.75.

A Researcher-Made Questionnaire Was Used to Measure Modern Leadership Styles. The questionnaire has 18 questions, its content validity has been confirmed by three faculty members and experts in this field. The internal consistency of females for the whole test performed by Cronbach's alpha of 0.905, and for the subscale of pragmatic and transformational leadership and Laissez fair leadership respectively, is 0.856, 0.894 and 0.794, which is due to the fact that Cronbach's alpha for the entire questionnaire and subscales is more than 0.7. This questionnaire is reliable and an appropriate tool for measuring modern leadership style.

The Human Resources Productivity Questionnaire was presented by Hersey and Goldsmith in the 1980s based on the Achilles model. This questionnaire aims to assess the level of human resource productivity in the organization of dimensions. The questionnaire has a 5-point Likert scale, and Daniali, Deh et al. (2013) has an acceptable content validity and a reliability of 0.831, which indicates the proper internal consistency of the test subjects.

RESULTS AND DISCUSSION

89 percent of the members of the research were married and 86 percent had university degrees and high levels, of which 78 percent were men. At first, the results of the Kolmogorov-Smirnov test are examined to determine the normality of the community and the possibility of performing a parametric test.

Given that the critical size in all of the studied components is greater than the significance level of 0.05, null hypothesis is rejected and opposite hypothesis is confirmed and a parametric test is possible. In the following, the correlations of the research variables are investigated.

The results obtained from the statistical analysis of the correlation matrix of all the components of the research examined have been shown to be as follows: There is a positive and significant relationship between productivity and components of executive thinking,

Table 1. Kolmogorov-Smirnov Test

Significance	Number	Variables
0.25	165	Executive Thinking Style
0.14	165	Judicial Thinking
0.22	165	Legislative Thinking
0.38	165	Transformational Leadership Style
0.27	165	Pragmatic Leadership Style
0.28	165	Laissez Fair Leadership Style
0.39	165	Efficiency

Table 2. Matrix of Correlation Between Research Variables

7	6	5	4	3	2	1	Research Variables	Row
						1	Executive Thinking Style	1
					1	0.26*	Judicial Thinking	2
				1	0.26*	0.42**	Legislative Thinking	3
			1	0.36**	0.08	0.41**	Transformational Leadership Style	4
		1	0.58**	0.41**	0.20*	0.52**	Pragmatic Leadership Style	5
	1	0.36**	0.38**	0.39**	0.09	0.39**	Laissez Fair Leadership	6
1	0.31*	0.51**	0.52**	0.43*	0.24*	0.49**	Productivity	7

**P<01 *P<05

legislative thinking, transformational leadership style, and pragmatic leadership at the level of 0.01. There is a significant correlation between productivity and judicial thinking and Laissez fair leadership at the level of 0.05. In our relationship between the components of the research with each other, except for the relationship between judicial thinking; with transformational leadership and Laissez fair leadership, no significant relationship was observed. There was a significant relationship between judicial thinking with executive and legislative thinking and pragmatic leadership at the level of 0.05. The rest of the relationships was positive and significant at the level of 0.01.

After analyzing the correlation, the regression test was used to evaluate the distribution of the dispersion and the differences between the dependent variable and the independent variable. The linear regression test has some hypotheses, which is referred to below:

Given the fact that the distribution of the scores is normal and the type of scale of the variables is of a distance type, a linear regression test can be used. Another of the hypotheses about the use of independence regression are errors from one another (the difference between the actual values and the predicted values by the regression equation.) Durbin-Watson Test is used to check the independence of errors. The value of the test statistic is from one to four variables, and if the range of this statistic is from 1.5 to 2.5, the assumption of independence between errors is accepted. Durbin-Watson statistics in this study are for independent variables of thinking style and modern leadership style. The order is equal to 2.05 and 1.89, which indicates a lack of correlation between errors and the possibility of linear regression in this study.

The results of regression analysis of variance were used to verify the validity of the linear relation in the entire regression model, since the significance is less than 0.01, null hypothesis is rejected and opposite hypothesis is confirmed. The linear regression model is valid in both variables.

As seen in Table 4, the value of the multiplicity correlation coefficient between the three predictive variables entered in each component of the thinking style and the leadership style to the model and criterion variables

Table 3. Analysis of Variance Regression

Significant Level	F	Square Average	Freedom Degree	Total Square	Model
0.000	16.37	827.056	2	1654.112	Thinking Style
0.000	18.41	892.692	2	1785.384	Modern Leadership Style

Table 4. Regression Coefficient of Thinking Style and Modern Leadership Style on Productivity

Beta Coefficient	Standard Error	Determination Coefficient	Regression Correlation Coefficient	Model
0.42	0.59	23.42	0.484	Thinking Style
0.40	0.66	27.77	0.527	Leadership Style

Table 5. T Test of Independent Sample

	t	Freedom Degree	Significant Value	Difference Average
Productivity	8.41	164	0.001	0.752

are respectively 0.48 and 0.33. Value The coefficient of explanation is equal to 0.23 and 0.28, that is, 23% of the variation of the criterion variable, which is productivity, is explained by the three variables related to the thinking style and 28% by the components of the modern leadership style, they explain the rest of the variation of the criterion with other variables that the researcher did not consider and did not enter into the model. The mean of a statistical society was used to identify the status of the research variables as appropriate or not.

The null hypothesis: Productivity of the organization is not a good situation.

The opposite hypothesis: Productivity in the organization is in good condition.

Considering that the significance obtained is lower from the critical value of the table, the community average is appropriate in terms of productivity, thus the null hypothesis is rejected and the productivity variable is in good condition.

Adjusting, directing, and employing reasonable human resources is the most important goal in the organizational management and major part of the program in achieving the goals set in its different domains. Schuler et al. (2014). In 1913, Munsterberg argued that some employees are more suitable than others for some work. Gholipour et al. (2011). This is rooted in this topic. Organizations that are principally engaged in the optimum exploitation of their organizational elements, including human resources, should be given the highest precision in putting anyone in their proper place. Chaudhary et al. (2014).

In other words, the correct use of resources depends on the ability to apply correctly, and the correct use of resources and manpower depends on the correct management and leadership of the organization Bell (2013). The importance of this topic is to the extent that the productivity is result of quality and the proper use of resources in this area, the leadership style and organizational leadership is first of all the type of thinking and leadership style and management, and this is the specific routine of the program.

Due to the importance of the topic mentioned in this study, the role of thinking styles and modern leadership styles on productivity has been investigated. Considering the desired bases and indicators, as well as statistical analysis of the research findings, both the thinking style and the leadership style (New) affects organizational productivity and achievement of predetermined indicators, according to researches such as Keskes (2014), which showed that organizational productivity is distinctly affected by leadership style and the intellectual model of organization management.

Propeli et al. (2016) researched on the intellectual model and organization management as an important factor in achieving optimal performance and productivity, and a positive and positive relationship between these components and productivity was observed. In other words, thinking styles is motivating path and movement of each person undoubtedly lead to this style of thinking and cognition, the way of management and leadership of a person, and therefore, there is a certain relationship between thinking style and leadership style.

On the other hand, organizational leadership style and how to deal with employees and the type of structural relations governing the organization and the company, which determines the interactions and connections of the members of the organization and leadership in general indicators and affairs, due to the importance of these relationships in the organization's executive process. The productivity and performance of the company are predicted and analyzed. Therefore, there is a clear and meaningful relationship between thinking styles and leadership with productivity.

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Pharmacological activity of different solvent extracts of *Tribulus terrestris* against multi drug resistant *Staphylococcus aureus* isolated from post-operative wound patients

M.S. Uma Maheshwari, R. Rajendran and S. Vijayalakshmi

[1]*Associate Professor, Department of Microbiology, Tiruppur Kumaran College for Women, Tiruppur, India*
[2]*Associate Professor, Department of Microbiology, PSG College of Arts and Science, Coimbatore, India*
[3]*Research Scholar, PG and Research Department of Zoology, Division of Microbial Technology, Chikkanna Govt. Arts College, Tirupur, India*

ABSTRACT

Despite advances in infection control surgical site infections formerly called surgical wound infections, remain a substantial cause of morbidity and mortality among hospitalized patients. The post-operative surgical site infection is the third most commonly reported nosocomial infections, accounting for a quarter of all such infections. The early stages of invasive infection caused by *Staphylococcus aureus*, also play major role in this process. In this present study a total of 50 pus samples were collected from patients having postoperative wound infections from the different surgical departments in the PSG Institute of Medical Sciences and Reasearch (PSG IMSR), Coimbatore during Dec 2016 to July 2017. Identification of bacterial isolates was done by standard microbiological techniques. Further, the antimicrobial susceptibilities were done against following antibiotics, Penicillin (10 units), Chloramphenicol (30mcg), Vancomycin (30mcg), Streptomycin (10mcg), Neomycin (30mcg), Ampicillin (10mcg), Amoxicillin (10mcg), Amikacin (10mcg), Gentamycin (10mcg) and Ciprofloxacin (10mcg). Among these strains 100%, 30%, 90%, 50%, 40%, 100%, 50%, 20%, 30% and 60% were found to be exhibiting a significant degree of resistance to antibiotics tested. Currently, the development of bacterial resistance has necessitated the search for new antibacterial agents to combat the infectious disease using bio-medically active different solvent extract of *Tribulus terrestris* for antimicrobial activity. In this present study the maximum inhibition was observed in Ethyl acetate, moderate inhibition in Ethanol, minimum inhibition in Acetone, Chloroform, Methanol and no inhibition in aqueous and Petroleum ether. The results confirm that, the plant appears to contain substances that exhibit broad antimicrobial activity against wound pathogens.

KEY WORDS: *STAPHYLOCOCCUS AUREUS, ANTIBIOTIC RESISTANCE, ANTIMICROBIAL ACTIVITY OF TRIBULUS TERRESTRIS*, POSTOPERATIVE WOUND INFECTION

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*Corresponding Author: maheswariuma938@gmail.com
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INTRODUCTION

Recurrently, the post operative hospital acquired infections constitutes a major problem in surgical patients contributing to morbidity, mortality and increased resource utilization and health care costs. Patients in whose surgical site infection develop have an increased number of associated complications, the high risk of requiring a stay in ICU have two to three times higher risk of mortality. Their hospital stay is increased by 7 to 12 days and they are five times more likely to require readmission.The post-operative wound infections can be caused by different groups of microorganisms like bacteria, fungi and protozoa. However, different kind of microorganisms can exist in polymicrobial communities, especially in the margins of wounds and in chronic wounds (Percevil and Bowler, 2004, Anaya and Dellinger, 2006 and Jain *et al.* 2014) .

The risk of invasive burn wound infection is influenced by the extent and depth of the burn injury, various host factors, and the quantity and virulence of the microbial flora colonizing the wound (Church *et al.* 2006). The common burn wound pathogens are *Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella* spp., which produce a number of virulence factors that are important in the pathogenesis of invasive infection (Tredget *et al.* 2004) are frequently associated with post-operative wound infection. One of the major problems worldwide is the increase in antibiotic-resistant strains of bacteria, mainly in hospitals, that poses constrain for their control without considerable resources and expenditure (Ayliffe *et al.* 2000). It has been well documented that most of the clinical isolates of *Staphylococcus aureus* are multiple-drug resistant currently used antibiotics. The problem of microbial resistance is increasingly alarming and the outlook for the use of antimicrobial drugs in the future is still uncertain (Nascimento *et al.* 2000 Naaz 2017).

Due to this treatment of postoperative wound infection with antibiotics is becoming a challenge for the surgeon as multidrug resistance is reported to be high. It is therefore, important to have knowledge regarding the prevalent microorganism in the surgical units and their susceptibility patterns to antibiotics so that proper treatment can be started earlier. It is essential to take appropriate steps to curtail the spread of infection within the unit (Tahir, 1995). One of the measures to combat this increasing rate of resistance is to have continuous investigations into new, safe and effective antimicrobials as alternative agents to substitute with less effective ones. Plants have been traditionally proved to be a rich source of novel drug compounds, as the herbal mixtures have made large contributions to human health and well-being (Ergin and Mutlu, 1999). A wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, quinones and flavonoids are endowed with antimicrobial properties (Lewis and Ausubel, 2006 Mohammad et al 2015).

Currently, the research is being carried out to investigate ethno-botanical uses of plants prevailing among native people (Sibanda and Okoh, 2007). There are numerous reports evidencing the antibacterial activity of plants against microorganisms (Sundharameshwari and Radhika, 2007). Thus, it is very much necessary to analyze the potential of the plants in combating the antibiotic resistant organisms, (Al Maofari 2013).

Recurrently, the *Tribulus terrestris* is a strong herbal remedy which is used for various purposes in folk and modern medicine and sport, as well. It has been used as a tonic, aphrodisiac, astringent, analgesic, stomachic, anti-hypertensive, antibacterial, antifungal, skin infection and urinary anti-septic (Al-Bayati and Al-Mola, 2008). *T. terrestris* has been commonly used as a diuretic as well as treatment for hypertension, hypercholesterolemia and colic pains. (Wang *et al.*, 1990).The leaves of *Tribulus terrestris* are used traditionally for the treatment of various kinds of wound. *T. terrestris* is found to be a rich source of calcium (Bourke *et al.*, 1992). Extract from *T. terrestris* has immune stimulatory and antimicrobial effect (Sengul *et al.*, 2009, Al Maofari 2013) against pathogens. Recently Naz *et al.*, (2017) stated that the leaf extracts of plants with a history of traditional use should be tested using modern methods for activities against multidrug resistant human pathogens, with the aim of discovering potential new drugs. So, hence the present study has made an attempt to point out the different solvent extraction of *Tribulus terrestris* against multidrug resistant *Staphylococcus aureus* isolated from wound of post-operative patients.

MATERIALS AND METHODS

A total of 50 pus samples was collected from patients aseptically with a sterile cotton swab suffering from post operative wound infection at the PSG Institute of Medical Sciences and Reasearch (PSG IMSR), Coimbatore for the period of Dec 2016 – July 2017. For collection, the wound sample was washed thoroughly with normal saline and it was placed in the ice box. After reaching to the laboratory of PG and Research Department of Microbiology, PSG College of Arts and Science, Coimbatore. Finally the samples were incubated at 37°C for 24 hours in isolation of wound pathogens.

All the strains isolated from wound samples were serially diluted, plated onto Mannitol Salt Agar, it was incubated at 37°C for 48hours. The colonies with characteristic growth were subjected to routine biochemical test according to the Bergey's manual of systematic bacteriology.

FIGURE 1. *Tribulus terrestris*

The standard Kirby Bauer disk diffusion method was used to determine the antimicrobial profile of wound isolates against 10 antimicrobial agents such as Penicillin (10 units), Chloramphenicol (30mcg), Vancomycin (30mcg), Streptomycin (10mcg), Neomycin (30mcg), Ampicillin (10mcg), Amoxycillin (10mcg), Amikacin (10mcg), Gentamycin (10mcg) and Ciprofloxacin (10mcg). The diameters of the inhibition zone were measured using a ruler under a colony counter apparatus. The results were expressed as sensitive (S), marginally susceptible (I), and resistant (R).

The plants of *Tribulus terrestris* were collected from the dry lands of Coimbatore and Tirupur regions and identified. The identification was authenticated by the Botanical Survey of India ,Tamilnadu Agricultural University Campus (TNAU),Coimbatore. The plant extracts were prepared as per standard procedures. The leaf of *T. Terrestris* plant (Fig. 1) were washed with sterile distilled water to remove dirt, dried under shade and were ground to powder using household electric blender. The 20g of dry powdered *Terrestris* was weighed and transferred to a conical flask containing 100 ml of 80% of Ethanol, methanol, Chloroform, acetone, Ethyl acetate and Petroleum ether (Fig. 2) respectively and allowed to soak at ambient temperature for 72 hours. The extract was than filtered using Whatman no 1 filter paper and the filtrates were concentrated in vaccum at 40°C using a rotary evaporator. Residues of the extracts made into suspensions using sterile distilled water and sterile dimethyl sulphoxide at a concentration of 500 mg/ml of *Tribulus* extracts respectively.

The leaf of the purified *Tribulus* extract was tested for antibacterial activity by standard agar well diffusion method against pathogenic bacteria *S.aureus*. The pure culture of bacterial pathogen was sub cultured on nutrient agar. 20ml of nutrient agar were poured into petriplates. The well of 6mm diameter were made on nutrient agar using gel puncture 100µl of fresh over night grown culture of the respective bacteria were spread on nutrient agar medium containing petriplates. The culture was

FIGURE 2. Extraction of Solvent from bioactive leaf of *Tribulus terrestris* a) Petroleum ether, b) Acetone, c) Ethanol, d) Aqueous, e) Ethyl acetate, f) Methanol, g) Chloroform

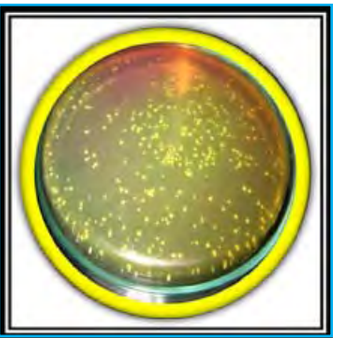


FIGURE 3. Colony of *Staphylococcus aureus* on Mannitol Salt agar

Table 1. MIC of different strains of Petroleum ether extract of *Tribulus terrestris by* broth dilution method

Test microorganism	Plant Extract (Petroleum ether) Concentration (500mg/ml)											
	100 (µl)	50 (µl)	25 (µl)	12.5 (µl)	6.25 (µl)	3.12 (µl)	1.56 (µl)	0.78 (µl)	0.39 (µl)	0.19 (µl)	0.09 (µl)	0.04 (µl)
MTUM02	-	-	+	+	+	+	+	+	+	+	+	+
MTUM03	-	-	+	+	+	+	+	+	+	+	+	+
MTUM05	-	-	+	+	+	+	+	+	+	+	+	+
MTUM06	-	-	+	+	+	+	+	+	+	+	+	+
MTUM07	-	-	+	+	+	+	+	+	+	+	+	+
MTUM09	-	-	+	+	+	+	+	+	+	+	+	+
(-) indicates absence of growth and (+) indicates presence of growth of microorganisms.												

swabbed uniformly using a sterile cotton swab and then 50µl of the plant extract solution was loaded into the well. After incubation at 37°c for 24 hours the different zone of inhibition was measured.

Minimum inhibition concentration was made by the lowest cost of the extract of *T. Terrestris* leaves where it can show the bactericidal and bacteriostatic effect. The test was performed in 96 well micro titer plates. Microtiter plate wells from each column in row 1 were marked and 100µl (500mg/ml) of stock (aqueous and solvent extract) was added. 50µl of sterile distilled water was added to rows 2-12. Two fold serial dilutions were performed by transferring 50µl of solution from row 1 to 2, using a multichannel pipette. This was repeated down the row 2 to 12. 40µl of double strength nutrient broth and 10µl of bacterial culture was added to all the wells in a separate column, so the final concentration of the inoculum in all the wells. Finally, to prevent dehydration, the plates were covered with a plastic cover and then incubated at 37°C for overnight. The bacterial growth was determined after adding of 40µl of 2, 3, 5 Tri Phenyl Tetrazolium Chloride Red (0.02mg/ml). The Minimum inhibitory concentration (MIC) of the isolates was taken as the lowest concentration of the antibiotic of which the bacteria tested did not show visible growth (Table: 1).

RESULTS AND DISCUSSION

Totally 50 clinical wound samples, 10 positive *Staphylococcus aureus* were isolated from different wound sites of patients admitted in surgical ward in the PSG Institute of Medical Sciences and Research (PSG IMSR) (Fig. 2), Coimbatore. All the wound samples were tested on Mannitol Salt Agar (MSA) for the isolation of *S.aureus*. On MSA, *S.aureus* colonies were appeared yellow colour which is containing mannitol to detect mannitol fermentation. In the routine microbiological laboratory, prompt identification of the *S. aureus* was done by grams staining, catalase, oxidase, coagulase, IMViC, nitrate reduction test, triple sugar iron agar test. After performing, all these tests was confirmed as *S.aureus*.

All the 10 *S.aureus* isolates were tested *invitro* to determine their antibiotic susceptibility patterns by antibiotic disc diffusion method and the following antibiotic discs were used for this assay are Penicillin (10 units), Chloramphenicol (30mcg), Vancomycin (30mcg), Streptomycin (10mcg), Neomycin (30mcg), Ampicillin (10mcg), Amoxycillin (10mcg), Amikacin (10mcg), Gentamycin (10mcg) and Ciprofloxacin (10mcg).. Totally 10 antibiotic discs were used to identify vancomycin resistant *Staphylococcus aureus*. All the isolates were shown multiple antibiotic resistances to the antibiotic tested none of the isolates showed 100% resistant to antibiotics tested. The maximum resistant pattern percentage (80%) was recorded in strain no MBUM02, MBUM06 and MBUM07 followed by minimum resistant pattern percentage (30%) was recorded in strain no MBUM01 and MBUM08.

Among 10 strains, 2 isolates were shown 30% resistant against all the antibiotics tested with strain No. MBUM01 and MBUM08 both were showed equally with the following antibiogram: VAN- AMP-P followed by the strain no. MBUM10, MTUM04 were showed 40% with antibiogram of STR-CHL-AMP-P, STR-CHL-AMP-CIP. Two strains MBUM03 and MTUM05 were showed 50% against the antibiotics tested with antibiogram of VAN-AMX-NEO-AMP-P followed by one strain no. MBUM09 was exhibit 70% resistant to antibiotics tested: VAN-AMX-AMP-P-GEN-AK-CIP, these antibiogram was recorded in the isolate. Finally, three strains MBUM02, MBUM06 and MBUM07 was showed the highest resistant percentage of 80% with the antibio-

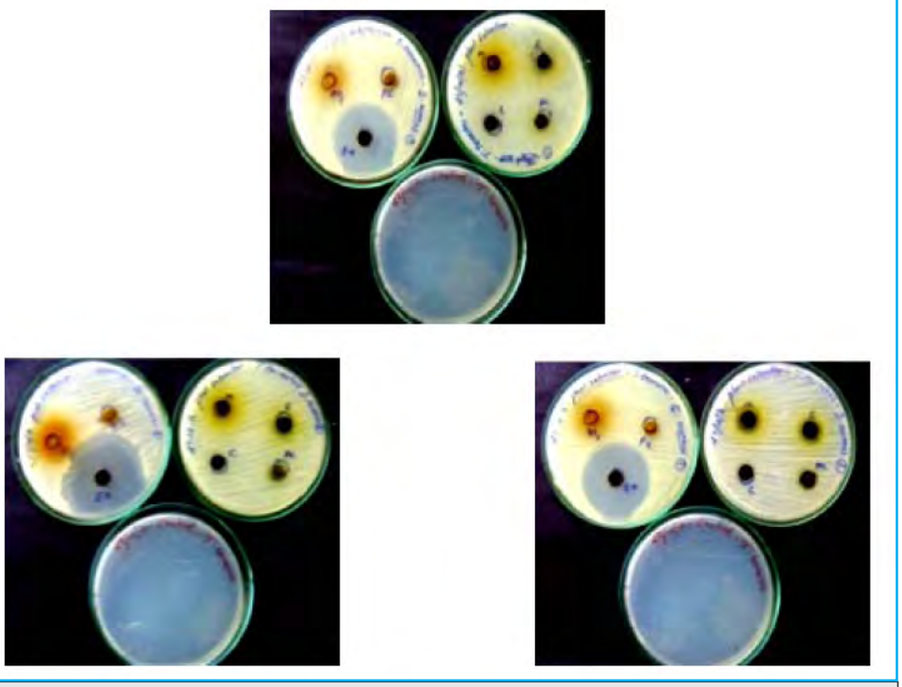
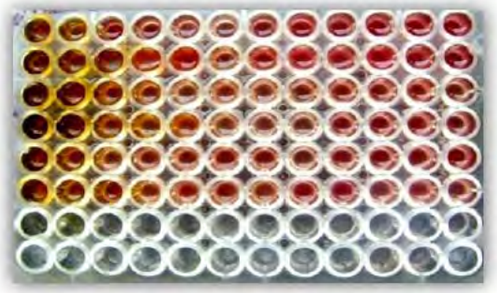


FIGURE 4. Antimicrobial activity of different *solvent* extract of *Tribulus terrestris* against *Staphylococcus aureus* shows that the maximum inhibition was seen in Ethyl acetate extract of *Tribulus terrestris*. The moderate inhibition was seen in Ethanol extract of *Tribulus terrestris*. The minimum inhibition was seen in the Acetone, Chloroform extract Tribulus terrestris. No inhibition of *Tribulus terrestris* was seen in the Aqueous and Petroleum ether extract of *Tribulus terrestris*. a) P.E-Petroleum ether, (b) AQ-Aqueous, (c) E.A-Ethyl acetate, (d) M-Methanol, (e) E-Ethanol , (f) CHL-Chloroform, (g) AC-Acetone.

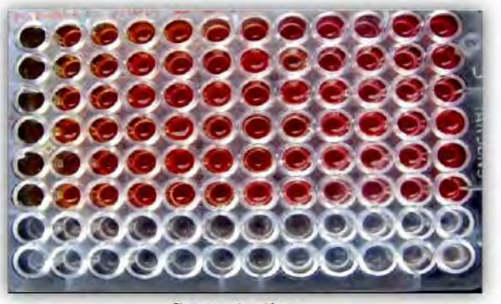


Concentration

100 (µl)	50 (µl)	25 (µl)	12.5 (µl)	6.25 (µl)	3.12 (µl)	1.56 (µl)	0.78 (µl)	0.39 (µl)	0.19 (µl)	0.09 (µl)	0.04 (µl)

The maximum inhibitory concentration was recorded in 25µl – 0.04µl con.of petroleum ether extract of *Tribulus terrestris* followed by minimum was recorded in 50 and 100µl con.of extract

FIGURE 5. MIC of different strains of Petroleum ether extracts of *Tribulus terrestris* by broth dilution method



Concentration

100 (µl)	50 (µl)	25 (µl)	12.5 (µl)	6.25 (µl)	3.12 (µl)	1.56 (µl)	0.78 (µl)	0.39 (µl)	0.19 (µl)	0.09 (µl)	0.04 (µl)

The maximum inhibitory concentration was recorded in 50µl – 0.04µl con.of aqueous extract of *Tribulus terrestris* followed by minimum was recorded in 100µl con.of extract

FIGURE 6. MIC of different strains of Aqueous extracts of *Tribulus terrestris* by broth dilution method

gram of STR-VAN-AMX-NEO-CHL-AMP-P-CIP; STR-VAN-AMX-NEO-AMP-P-GEN-CIP and STR-VAN-CHL-AMP-P-GEN-AK-CIP.

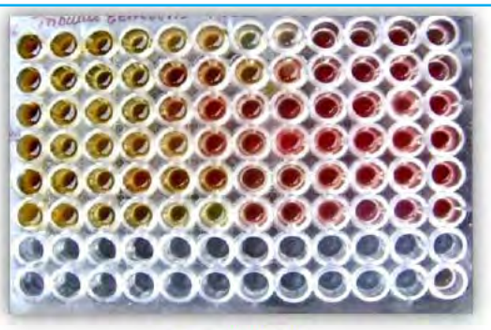
Totally 10 antibiotics were used to know the resistant pattern, among, 10 antibiogram were recorded. Ten antibiotics were showing more than 20% resistant to the isolates. All isolates (100%) were resistant of Ampicilin and Penicillin, while Vancomycin showed resistance (90%), Ciprofloxacin (60%), Streptomycin and Amoxyillin (50%), Neomycin (40%), Chlorompenical and Gentamycin (30%) followed by Amikacin (20%). All the *Staphylococcus aureus* isolates showed multiple antibiotic resistances. Such that one isolate resisted four, six and seven types of antibiotics, two isolates resisted 3 types of antibiotics, three isolates resisted eight antibiotics. The multiple antibiotic resistances (MAR) index was calculated according to the MAR index formula. The Maximum MAR index 0.8 was shown by MTUM08, MTUM06, MTUM07 and minimum MAR index 0.3 was shown by MBUM01 and MBUM08

Concentration

100 (µl)	50 (µl)	25 (µl)	12.5 (µl)	6.25 (µl)	3.12 (µl)	1.56 (µl)	0.78 (µl)	0.39 (µl)	0.19 (µl)	0.09 (µl)	0.04 (µl)

The maximum inhibitory concentration was recorded in 3.12µl – 0.04µl con.of Ethyl acetate extract of *Tribulus terrestris* followed by minimum was recorded in 100, 50,25,12.5 and 6.25 µl con.of extract

FIGURE 7. MIC of different strains of Ethyl acetate of *Tribulus terrestris* by broth dilution method



Concentration

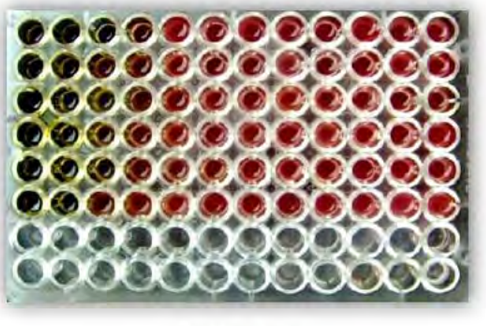
100 (µl)	50 (µl)	25 (µl)	12.5 (µl)	6.25 (µl)	3.12 (µl)	1.56 (µl)	0.78 (µl)	0.39 (µl)	0.19 (µl)	0.09 (µl)	0.04 (µl)

The maximum inhibitory concentration was recorded in 3.12µl – 0.04µl con.of Ethyl acetate extract of *Tribulus terrestris* followed by minimum was recorded in 100, 50,25,12.5 and 6.25 µl con.of extract

FIGURE 8. MIC of different strains of Methanol of *Tribulus terrestris* by broth dilution method

Based upon the MDR (Multiple Drug Resistance), seven strains of *Staphylococcus aureus* were selected for further studies. Finally, the strains were tested with purified leaf extract of various solvents using *Tribulus* for antimicrobial activity and showed maximum inhibition in Ethyl acetate, moderate inhibition in Ethanol, minimum inhibition was recorded in Acetone, Chloroform, Methanol and no inhibition observed in Aqueous and Petroleum ether solvents. Minimum inhibitory concentrations were performed to determine the minimum concentration of antibiotics, which is effective on the wound pathogens were also recorded and compared to currently used antibiotics, the plant extract of *Tribulus terrestris* shows effective results (Fig. 5 to fig. 11).

Post-operative wound infection still remains one of the most important causes of morbidity and is one of the most common nosocomial infection (Suljagic *et al*., 2010) in surgically treated patients. In the present study, an attempt has been made to know the predominant pathogen *Staphylococcus aureus* is the major respon-

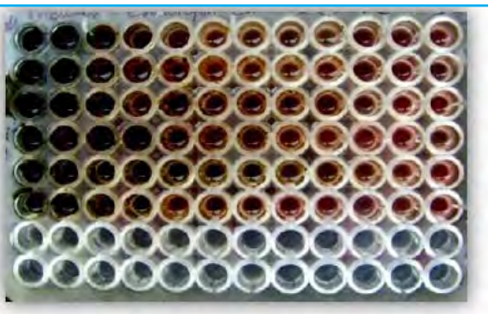


Concentration

100 (µl)	50 (µl)	25 (µl)	12.5 (µl)	6.25 (µl)	3.12 (µl)	1.56 (µl)	0.78 (µl)	0.39 (µl)	0.19 (µl)	0.09 (µl)	0.04 (µl)

The maximum inhibitory concentration was recorded in 12.5 µl – 0.04µl con.of Ethanol extract of *Tribulus terrestris* followed by minimum was recorded in 100, 50 and 25µl con.of extract

FIGURE 9. MIC of different strains of Ethanol of *Tribulus terrestris* by broth dilution method



Concentration											
100 (µl)	50 (µl)	25 (µl)	12.5 (µl)	6.25 (µl)	3.12 (µl)	1.56 (µl)	0.78 (µl)	0.39 (µl)	0.19 (µl)	0.09 (µl)	0.04 (µl)

The maximum inhibitory concentration was recorded in 12.5 µl – 0.04µl con.of Methanol extract of *Tribulus terrestris* followed by minimum was recorded in 100, 50 and 25µl con.of extract

FIGURE 10. MIC of different strains of Methonol of *Tribulus terrestris* by broth dilution method

sible for surgical site infections and their antibacterial susceptibility pattern. The rate of surgical site infection varies greatly worldwide and from hospital to hospital. The rate of SSI varies from 2.5% to 41.9% as per different studies. The incidence of SSI in the present study is 2.69% even though high, is in agreement with the various studies (Reichman and Greenberg, 2009).

Wound infections inflict clinical and societal consequences on the patients, but its bacteriological characteristic varies with different factors. Therefore, effective treatment and management of wound infections in hospital and community setting will require detailed epidemiological knowledge of the infecting bacterial pathogens and their antibiogram peculiar to the environment.

Concentration											
100 (µl)	50 (µl)	25 (µl)	12.5 (µl)	6.25 (µl)	3.12 (µl)	1.56 (µl)	0.78 (µl)	0.39 (µl)	0.19 (µl)	0.09 (µl)	0.04 (µl)

The maximum inhibitory concentration was recorded in 3.12µl – 0.04µl con.of Ethanol extract of *Tribulus terrestris* followed by minimum was recorded in 100, 50, 25, 12.5 and 6.25 µl con.of extract

FIGURE 11. MIC of different strains of Chloroform of *Tribulus terrestris* by broth dilution method

Table 2. MIC of different strains of Aqueous extract of *Tribulus terrestris by* broth dilution method

Test microorganism	Plant Extract (Aqueous) Concentration (500mg/ml)											
	100 (µl)	50 (µl)	25 (µl)	12.5 (µl)	6.25 (µl)	3.12 (µl)	1.56 (µl)	0.78 (µl)	0.39 (µl)	0.19 (µl)	0.09 (µl)	0.04 (µl)
MTUM02	-	+	+	+	+	+	+	+	+	+	+	+
MTUM03	-	+	+	+	+	+	+	+	+	+	+	+
MTUM05	-	+	+	+	+	+	+	+	+	+	+	+
MTUM06	-	+	+	+	+	+	+	+	+	+	+	+
MTUM07	-	+	+	+	+	+	+	+	+	+	+	+
MTUM09	-	+	+	+	+	+	+	+	+	+	+	+
(-) indicates absence of growth and (+) indicates presence of growth of microorganisms												

Table 3. MIC of different strains of Ethyl acetate extract of *Tribulus terrestris by* broth dilution method

Test microorganism	Plant Extract (Ethyl acetate) Concentration (500mg/ml)											
	100 (µl)	50 (µI)	25 (µI)	12.5 (µI)	6.25 (µI)	3.12 (µI)	1.56 (µI)	0.78 (µI)	0.39 (µI)	0.19 (µI)	0.09 (µI)	0.04 (µI)
MTUM02	-	-	-	-	-	-	-	+	+	+	+	+
MTUM03	-	-	-	-	-	-	-	+	+	+	+	+
MTUM05	-	-	-	-	-	-	+	+	+	+	+	+
MTUM06	-	-	-	-	-	-	-	-	-	+	+	+
MTUM07	-	-	-	-	-	-	-	-	-	+	+	+
MTUM09	-	-	-	-	-	-	-	-	+	+	+	+
(-) indicates absence of growth and (+) indicates presence of growth of microorganisms												

Based on this information, the author (Bularafa Mohammed Yasidi *et al.*, 2015) examined the prevalence and antibiogram of bacterial pathogens isolated from wound infection cases seen at the hospital over the study period. A total of 392 wound swabs/ and pus of different types of wound infections from different anatomical sites and associated clinical conditions were analyzed by standard bacteriological methods. Of the 392 clinical specimens analyzed, 301(76.8%) yielded the majority of pathogens were recovered from septic wound infections. Overall, 7 different bacterial pathogens were identified.

The authors state that among different kind of microorganisms the major pathogen *Staphylococcus aureus* was isolated and used for antimicrobial testing. The

Table 4. MIC of different strains of Methanol extract of *Tribulus terrestris by* broth dilution method

Test microorganism	Plant Extract (Methanol) Concentration (500mg/ml)											
	100 (µl)	50 (µI)	25 (µI)	12.5 (µI)	6.25 (µI)	3.12 (µI)	1.56 (µI)	0.78 (µI)	0.39 (µI)	0.19 (µI)	0.09 (µI)	0.04 (µI)
MTUM02	-	-	-	-	+	+	+	+	+	+	+	+
MTUM03	-	-	-	-	-	-	-	+	+	+	+	+
MTUM05	-	-	-	-	+	+	+	+	+	+	+	+
MTUM06	-	-	-	+	+	+	+	+	+	+	+	+
MTUM07	-	-	-	-	-	+	+	+	+	+	+	+
MTUM09	-	-	-	-	-	-	+	+	+	+	+	+
(-) indicates absence of growth and (+) indicates presence of growth Microorganisms												

Table 5. MIC of different strains of Ethanol extract of *Tribulus terrestris by* broth dilution method

Test microorganism	Plant Extract (Ethanol) Concentration (500mg/ml)											
	100 (µl)	50 (µl)	25 (µl)	12.5 (µl)	6.25 (µl)	3.12 (µl)	1.56 (µl)	0.78 (µl)	0.39 (µl)	0.19 (µl)	0.09 (µl)	0.04 (µl)
MTUM02	-	-	-	+	+	+	+	+	+	+	+	+
MTUM03	-	-	+	+	+	+	+	+	+	+	+	+
MTUM05	-	-	-	+	+	+	+	+	+	+	+	+
MTUM06	-	-	+	+	+	+	+	+	+	+	+	+
MTUM07	-	-	-	+	+	+	+	+	+	+	+	+
MTUM09	-	-	+	+	+	+	+	+	+	+	+	+
(-) indicates absence of growth and (+) indicates presence of growth of microorganisms												

Table 6. MIC of different strains of Chloroform extract of *Tribulus terrestris by* broth dilution method

Test microorganism	Plant Extract (Chloroform) Concentration (500mg/ml)											
	100 (µl)	50 (µl)	25 (µl)	12.5 (µl)	6.25 (µl)	3.12 (µl)	1.56 (µl)	0.78 (µl)	0.39 (µl)	0.19 (µl)	0.09 (µl)	0.04 (µl)
MTUM02	-	-	+	+	+	+	+	+	+	+	+	+
MTUM03	-	-	+	+	+	+	+	+	+	+	+	+
MTUM05	-	-	+	+	+	+	+	+	+	+	+	+
MTUM06	-	+	+	+	+	+	+	+	+	+	+	+
MTUM07	-	-	-	+	+	+	+	+	+	+	+	+
MTUM09	-	-	-	-	+	+	+	+	+	+	+	+
(-) indicates absence of growth and (+) indicates presence of growth of microorganisms												

bacterial pathogens demonstrated high resistance to Ampicillin (78%), Amoxicillin (66%), and Cotrimoxazole (78%), in contrast to the high sensitivity pattern observed with Ofloxacin 83%, Norfloxacin 71%, Ciprofloxacin 78%, Erythromycin 72%, Chloramphenicol 62%, Gentamicin 58% and Ceftazidime 60%. But in this present study demonstrates that all *Staphylococcus aureus* isolated from pus samples of infectious patients. The high resistance was observed in Ciprofloxacin (60%), Amoxicillin (50%) in contrast to high sensitivity pattern observed with Chloramphenicol (30%), Gentamicin (30%), Norfloxacin (40%).

Jain *et al.* (2014) states that in their study all *Staphylococci* were susceptible to Vancomycin and Teicoplanin.

Table 7. MIC of different strains of Acetone extract of *Tribulus terrestris by* broth dilution method

Test Microorganim	Plant Extract (Acetone) Concentration (500mg/ml)											
	100 (µl)	50 (µl)	25 (µl)	12.5 (µl)	6.25 (µl)	3.12 (µl)	1.56 (µl)	0.78 (µl)	0.39 (µl)	0.19 (µl)	0.09 (µl)	0.04 (µl)
MTUM02	-	-	-	-	-	+	+	+	+	+	+	+
MTUM03	-	-	-	-	+	+	+	+	+	+	+	+
MTUM05	-	-	-	-	-	-	-	-	+	+	+	+
MTUM06	-	-	-	-	-	+	+	+	+	+	+	+
MTUM07	-	-	-	-	+	+	+	+	+	+	+	+
MTUM09	-	-	-	-	-	+	+	+	+	+	+	+
(-) indicates absence of growth and (+) indicates presence of growth of microorganisms												

In contrast, the Vancomycin remains the first choice of treatment for MRSA and to preserve its value, its use should be limited to those cases where there are clear indications. But in this present study dissimilar results were obtained by predominant pathogen *Staphylococcus aureus* indicates resistant to Vancomycin, it proofs that the emerging of drug resistant ability of *Staph* to be currently initiated.

The result of this present study indicated that bacterial isolates demonstrated high sensitivity to Chloramphenicol being about 70% sensitive, whereas resistant to β-lactam antibiotic namely Penicillin (100%) was very high. These results are contrary to that obtained for anaerobes isolated from oro-facial infections in earlier study which reported good activities of the later agents against the anaerobes (Nicholes, 2004).

Recurrently, the medicinal plants are the oldest form of healthcare known to mankind. From the ancient time people are using different herbs or plants as the remedy for various diseases. But now a day's people have become dependent on synthetic medicines which have many side effects. So to reduce the side effects we can use medicinal plants for the treatment of common diseases rather than using drugs.

Phan *et al.* (2001) find out the secondary metabolites are synthesized by the plant as part of the defence system of the plant. The plant contains chebulinc acid, tannic acid, gallic acid, resin, anthroquinone and sennoside. It also contains glycosides, sugar, terpenoids, steroids, phosphoric acid and these compounds were proven to exhibit antibacterial, antifungal, antiviral and anticarcinogenic (Neamsuvan *et al.*, 2012). Hence, the present study to logically select a leaf part of bio-medically active *Tribulus* plant to exhibit a broad antimicrobial activity against wound pathogen *Staphylococcus aureus*.

Ali *et al.*, (2001) confirmed that their study the *Tribulus terrestris* had no detectable antibacterial activity against any of the infectious disease causing reference bacteria. For this reason, in this present study choose a plant material of *Tribulus* to check the vital activity against infectious disease that proved the Indian leaf of *T. terrestris* were active against clinically dominant pathogen *S.aureus* isolated from post-operative wound infection.

Recurrently, the plant extract is highly sensitive when compared to the standard antibiotic. These data indicate that Gram-positive bacteria are the most sensitive strains for the different extracts, which exhibited their main antibacterial activity on Gram-negative bacteria (Bakri and Douglas 2005). Our results are in good agreement with previous works (Al Maofari *et al.*, 2013) showing a weaker activity of essential oil of *Anis* and *Salvia tomentosa* and also Nair and Chanda, a (2008) also reported Gram positive were more sensitive. Ethanol extract showed more effect than aqueous extract in inhibiting the growth of the bacterial strains (Nair and Chanda, b 2007; Firas *et al.*, 2008). The growth of the Gram positive strains was found more inhibition than Gram negative. In this present study, similar results was observed the antimicrobial activity of 7 different solvent plant extract was tested against *S.aureus*. The maximum antibacterial activity was seen in Ethyl acetate extract (100µl) of *Tribulus terrestris*. Intermediate antibacterial activity was seen in Ethanol extract (100µl) of *Tribulus terrestris* (Fig. 3).

CONCLUSION

The present study revealed that the post operative wound infection is one of the most common chronic infectious diseases of human is also a serious problem among wound of post-operative patients caused by predominant pathogen *Staphylococcus aureus*. Therefore, effective treatment and management of wound infections in hospital and community setting will require detailed knowledge of the infecting bacterial pathogens and their antibiogram peculiar to the environment for drug selection. Resistant bacteria which are human pathogens may cause the disease, they may still be dangerous because they can transfer their antibiotic resistance genes to other organism. Antimicrobial resistances of bacterial pathogens are a major problem for the treatment of animal and human patients with bacterial diseases. There is an alarming increase of infections caused by antibiotic resistant bacteria to urgently need of antimicrobial compound to treat a pathogenic organism. Recurrently the plants have been one of the important sources of medicine since the beginning of human civilization. There is a growing demand for plant based medicines to activity against several infectious pathogens. Hence the current study concluded that the different solvent extract of *Tribulus* plant has different mode of action to eradicate the wound pathogen around the surgical site. The plant extracts can also be exploited in designing the wound care products.

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Role of certain tree species in absorption of air pollutants caused by heavy metals – Copper, Zinc and Uranium in Tehran

Mehdizadeh Mojdehi Aida[1] and Ronassi Ali Arash[2]

[1]*Ph.D. student Belarusian National Technical University, Minsk, Belarus*
[2]*Assistant Professor, Department of Physics, Payame Noor University, Tehran, Iran*

ABSTRACT

As environmental pollution to heavy metals is being increased every year, occurrence of serious risks to health of human, animals and plants can be expected. Heavy metals with all of their destructive effects are the main pollutants in the air of big cities. Hence, this study has been conducted with the objective of analysis of role of tree species in absorption of air pollution caused by copper (Cu), zinc (Zn) and (U) uranium in Tehran. According to the climate of studied stations, especially wind velocity and direction, the sampling operation was taken at the end of March and December. The number of samples was also determined based on number of tree species studied (*Pinus eldarica , Cupressus arizonica,Platanus orientails*), organs of tree species (leaves), number of stations (Mehrabad Airport, Damavand and Chitgar Park) and sampling time scopes (March and December). Sampling was done in frame of statistical plan of completely random block in 3 iterations and the data analysis was done using analysis of variance and using SAS software. The results obtained from this study show that the absorption of cooper, zinc and uranium metals in the leaves of studied trees in polluted stations is more than control station. According to obtained results, uranium concentration in the leaves of studied species increased respectively in *Pinus eldarica Medw, Cupressus arizonica Greene* and *Platanus orientails*. The leaves of Pinus eldarica Medwin Mehrabad Airport station have shown highest absorption of this metal. Moreover, obtained results show that the cooper absorbed by leaves of studied species is increased respectively in Pinus eldarica Medw, Cupressus arizonica Greene and Platanus orientails. In terms of the effect of station on absorption of cooper, the species in the Mehrabad station have shown high absorption of this metal. The concentration of absorbed zinc in leaves of studied species is also increased respectively in *Pinus eldarica Medw, Cupressus arizonica Greene* and *Platanus orientails* and the zinc absorbed by species of Mehrabad station has shown the highest level. It is concluded that the absorption level of Cu, Zn and U in leaves of studied trees in polluted stations has been higher than control stations and *Pinus eldarica Medw* can be used as an index of absorption of heavy metals such as Cu, Zn and U.

KEY WORDS: TREE SPECIES, HEAVY METALS, COPPER, ZINC, URANIUM

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INTRODUCTION

As environmental pollution to heavy metals is being increased every year and can ultimately lead to serious risks to health of human, animals and plants, heavy metals with all of their destructive effects can be the main pollutants in the air of big cities. Therefore, one of the most fundamental issues gaining attention of scientists to heavy metals is lack of metabolism of these metals in the body. According to increasing population and the industrialization phenomenon of societies, more and more use of fossil fuels, especially oil products to produce electricity, transport systems and industrial and house uses, has led to occurrence of environmental problems because of production of hydrocarbons, polluted gases and heavy metals (Naderi et al, 2012, Zhou et al., 2017, Suvarapu and Baek, 2017).

As a result of rapid industrial growth over the decades, soil pollution to heavy metals has been increased. Although heavy metals can be existed in soil naturally, high percentages of these metals could be the outcome of human activities such as use of chemicals,organic modifiers, animal fertilizers, mineral processing, sewage sludge and waste from the iron and steel industry, mines, road transport and so on .Vehicles can be one of the main sources of producing heavy metals at the cities, which can cause pollution of soil around the roads by production of pollutants and making them enter to the environment and into the air. According to various studies conducted in field of heavy elements, the metals such as cooper (Cu), lead (Pb) and zinc (Zn) have shown high importance because of long half lifetime in body of human and other animals and because of being toxic (Suvarapu and Baek, 2017, Qiutong & Mingkui, 2017 and Sistani et al, 2017, Xu et al., 2017).

In field of role of tree species in adsorption of air pollution caused by heavy metals, various studies have been conducted. Cheraghi et al (2012) have conducted a study under the title of "analysis of heavy metals in bed, leaves and stem of Avicennia marina in Khuzestan" and have determined concentration of heavy metals such as Cu, Pb, Ni and Cd in sediments of Imam Khomeini Port Zone and Avicennia Marina and analysis of mobility of these metals based on enrichment percentage. 9 stations were selected in theMangrove stations and some samples of leaves and stem of Mangrove were collected, along with sediments of the zone. The results showed that concentration of metals in step of plants was more than leaves. Moreover, there was significant correlation between concentration of metals in stem and sediments (Cheraghi et al, 2012).

Maddah et al (2013) measured the amount of sediment in extracts resulted from stem, shoot and leaves and soil of flowerpots at the end of Nov using ICP device. The results showed that *P.Sylvetris* specie is the suitable specie forphytoremediation of lead. In the concentration of 800ppm, the contamination of Pb in stem and shoots is more than leaves; although in concentration of 1600ppm, the highest accumulation of lead is in stem and leaves. Comparing different horizons of soil showed that Pb has been mainly contaminated in surface horizon (Maddah et al, 2013).

Kardar et al (2015) has studied cadmium adsorption in organs of *Fraxinus excelsior* and *Cupressus arizonica* in Isfahan. To this end, some samples of leaves and surface steps of trees were prepared linearly and randomly in 3 iterations in late spring and summer and the cadmium concentration in them was measured using atom adsorption device. The results show that cadmium absorption level in areal organs of *Cupressus arizonica* happens more than *Fraxinus excelsior* and cadmium absorption in leaves of studied species is more than stem. Moreover, cadmium absorption in different organs of species in polluted station was more than other stations and the highest level of cadmium absorption was observed in September and the lowest level was observed in June (Kardar et al, 2015).

Kord et al (2011) has conducted a study under the title of refinement of soil polluted to Zn by means of tree species of *Pinus Eldarica Medw*, *Cupressus arizonica Greene, Robinia peseudoacacia,Fraxinus rotundifolia Mill* and*Ulmus carpinifolia var umbraculifera Rehd* in Tehran. For this purpose, in summer in polluted stations (Azadi, Bahman and Bazar) and control station (Aghdasieh), a transect was selected due to wind direction and sampling was done in 3 iterations from leaves and surface stems of trees in frame of absolutely random statistical plan and the Pb concentration in each sample was measured using atom absorption device model Varian220. The results showed that *Pinus eldarica Medw*, *Cupressus arizonica Greene* and *Robinia peseudoacacia* species have shown respectively highest Pb concentration in aerial organs (14.39ppm, 11.91ppm and 9.72ppm) and highest coefficient of Pbtransfer from the underground to the limb (respectively 3.49, 2.99 and 2.82). Accordingly and due to adequate coping conditions, the 3 species have been used to refine Pb-contaminated soil in similar zones (Kord et al, 2011).

Afshari et al studied heavy metal pollution using pollution factor in soil of lands with various uses in central zone of Zanjan Province. To evaluate 241 samples of surface soil based on systematic approach, nesting was done in depth of 0-10c of agricultural, farming and urban uses. The results showed that 1time more of the pollutant is in field soil and 0.4, 5.0 and 2.0 and 1 of total Pb, Zn, Cd and Cu in samples soils and respectively the overall concentration of Fe, Mg, Co and Ni is lower than field concentration of these metals. High amounts

of pollution factor of Pb, Zn, Cd and Cu was observed in urban uses and the pollution factor of Fe, Mg, Cr, Co and Ni was observed in agricultural and farming uses. Finally, the results showed that the metals in first group are mostly under the effect of human activities and the metals in second group are mostly affected by mother materials in the zone.

Shabanian and Chanor (2013) studied thebiodegradability of wooden species used in urban foresting of Sanandaj. Contamination of some heavy metals such as Pb, Zn, Cd and Mg was measured in leaves ofOriental plain, elm, asparagus, Croissant and black pine in Sanandaj's City Center (as polluted zone) and the area of Kurdistan University (as control zone). The results showed that contamination of Pb, Zn and Cd in leaves of majority of species in polluted zone was significantly higher than control zone at the level of 95%. In the polluted zone, the highest contamination of Pb and Cd was observed inCroissant, most Zn contamination was observed in asparagus and the most Mg contamination was observed in elm, (Shabanian and Chanor 2013).

Sistani et al (2017) conducted a cross-sectional and descriptive-analytical research under the title of "heavy metals contamination in adjacent soil of Kerman Steel Industry: evaluation of metal enrichment and degree of pollution". In this study, they found that the most concentration has been belonged to steel and concentration of other metals such as Pb, Cd has been significantly under the effect of steel complexes and some plans should be made for the surrounding area to reduce emission of pollutants. Therefore, this study has been conducted to investigate the amount of heavy metal absorption in some tree species in Tehran.

MATERIAL AND METHODS:

In this study, to measure the amount of heavy metal absorption in some tree species in Tehran, urban areas such as parks, boulevards, streets and highways and industrial districts used various tree species are selected as stations and research station. Then, during certain time intervals, through sampling leaves, stems and shoots of species, the contamination of each organ was determined. Accordingly, species with highest level of heavy metal absorption and with higher resistance against these metals were detected and were used in the zones polluted to these metals.

As the status of pollution caused by studied heavy metals is not same in whole city, sampling operation should be taken in form of an index of pollution status, so that it can consider minimum and maximum and average levels of pollution. To this end and due to reports of air pollution by Environmental Protection Agency and Transport and Traffic Agency of Tehran, Mehrabad Airport station was selected as station with high air pollution; Damavand Station was selected as station with low pollution. Chitgar station was selected as control station due to low pollution to be the basis for comparison with other polluted stations.

After determining the polluted and control stations, the studied species were identified and selected in each station. The desired species were selected based on majority in Tehran and dominance in all studied area from Evergreen and Easter species andBroad leaf and needle leaf species. The tree species existed in all studied stations in common were detected and board leaf *Platanus orientails* and needle leaf *Pinus Eldarica Medw* and*Cupressus arizonica Greene* were selected to take required comparisons to determine heavy metal absorption level among different organs like leaves.

Sampling was done on leaves of trees in such way that leaf samples were carefully separated from petiole location. Moreover, according to position of the leaves on the crown of tree, an arrangement was taken to select leaves from whole crown of tree. Number of samples was also determined due to number of studied tree species (*Pinus Eldarica Medw*, *Cupressus arizonica Greene*, *Platanus orientails*), organs of tree species (leave), number of stations (Mehrabad Airport, Damavand and Chitgar Park) and sampling time scopes (March and December) and iteration of examinations (3 iterations). Hence, according to the climate of studied stations, especially wind speed and direction, sampling was done in late March and late December.

In this study, the effect of various factors such as tree species, research stations, and time intervals of sampling and concentration of heavy metals in leaves of trees was studied. In frame of absolutely random block plan and in 3 iterations, sampling was done and the data analysis was done using analysis of variance and using SAS software. Drawing diagrams and graphs was also done using Excel software.

RESULTS

The results obtained from analysis of variance of mean square in leaves of tree species for 3 elements of Cu, Zn and U in time scope of March and December in 3 studied zones are presented in table 1.

According to table 1, the results obtained from analysis of variance in March showed that the effect of specie is significant for all studied properties at the probability level of 1% and the results have been insignificant for Cu concentration in leaf. Moreover, the results obtained from analysis of variance in December showed that the effect of specie for all studied properties is significant at the probability level of 1% and the results obtained from

Table 1. analysis of variance of studied properties (March and December)

Sources of variations	Degrees of freedom	Mean Square					
		March	December	March	December	March	December
		Leaf Cu	Leaf Cu	Leaf Zn	Leaf Zn	Leaf U	Leaf U
Specie	2	**35.65	**1.45	**1.23	**1.47	**0.98	**0.85
Station	2	**43.55	**1.64	**1.32	**10.21	**0.21	**032/
Specie×Station	3	**2.36	**0.08	**0.12	**0.09	*0.12	**0.9
Error	30	0.35	0.09	0.05	0.01	0.18	0.15
Coefficient of variation (%)		10.38	15.35	8.11	15.11	0.17	0.75

uranium concentration in leaf and Cu contamination in leaf are not significant.

According to figure 1, the results of mean comparisons showed that the mutual effect of station on Zn concentration in leaves of these trees was significant at the level of 95% ($p<0.05$). On the other hand, the results of multiple comparisons using Duncan test showed that the highest Zn concentration in *Pinus eldarica Medw* leaf was at Mehrabad Airport station (1.07ppm) and the lowest concentration was at Chitgar station in *Platanus orientails* (1.00ppm).

According to figure 2, the results of mean comparisons showed that the mutual effect of station on Cu concentration in leaves of these trees was significant at the level of 95% ($p<0.05$). On the other hand, the results of multiple comparisons using Duncan test showed that the highest Cu concentration in *Pinus eldarica Medw* leaf belonged to Mehrabad Airport station being 2.04 ppm and the lowest concentration belonged to Chitgar station in*Platanus orientails* which was 1.63 ppm.

According to figure 3, the results of mean comparisons showed that the mutual effect of station on U concentration in leaves of these trees was significant at the level of 95% ($p<0.05$). On the other hand, the results of multiple comparisons using Duncan test showed that the highest U concentration in *Pinus Eldarica Medw* leaf was at Mehrabad Airport station being 0.99 ppm and the lowest concentration at Chitgar station in *Platanus orientails* was 1.00ppm.

According to figure 4, the results of mean comparisons showed that the mutual effect of station on Zn concentration in leaves of these trees was significant at the level of 95% ($p<0.05$). On the other hand, the results of multiple comparisons using Duncan test showed that the highest Zn concentration in *Pinus Eldarica Medw* leaf was at Mehrabad Airport station (3.36 ppm) and

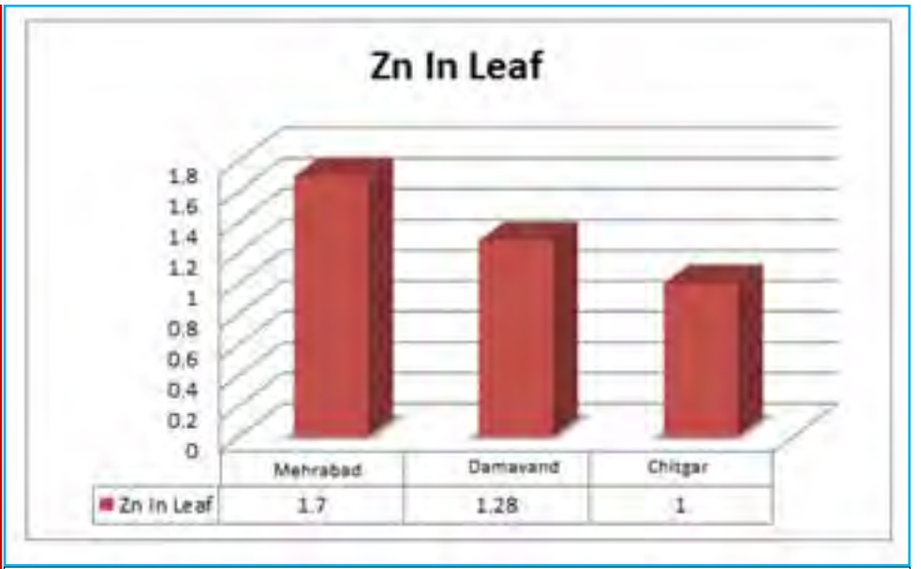

FIGURE 1. The effect of station on Zn concentration (ppm) in leaves of studied trees (March)

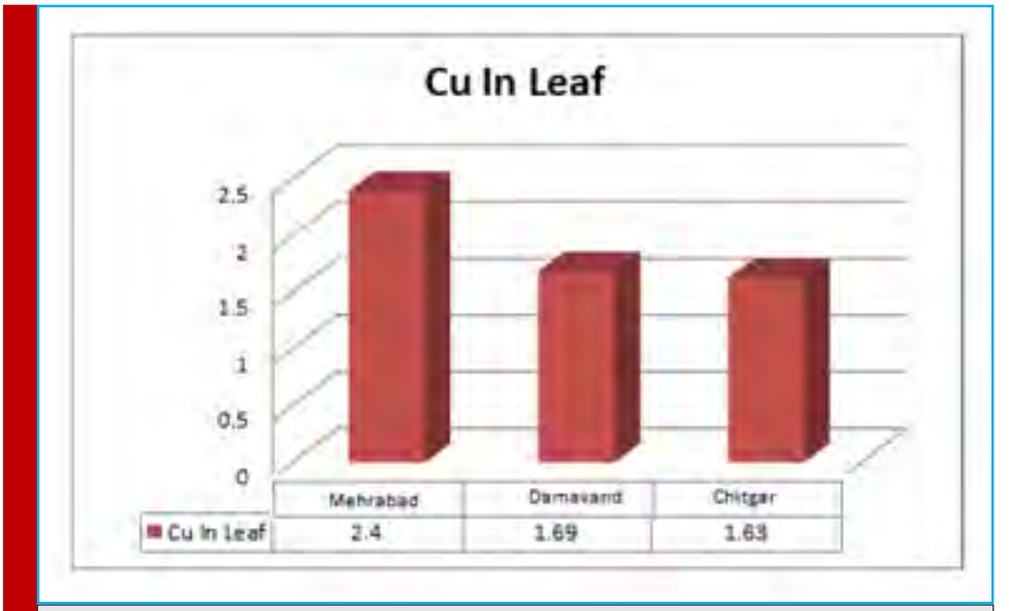

FIGURE 2. Cu concentrations (ppm) in leaves of studied trees (March) from various stations

the lowest concentration at Chitgar station in *Platanus orientails* was 1.18 ppm.

According to figure 5, the results of mean comparisons showed that the mutual effect of station on Cu concentration in leaves of these trees was significant at the level of 95% ($p<0.05$). On the other hand, the results of multiple comparisons using Duncan test showed that the highest Cu concentration in *Pinus Eldarica Medw* leaf belonged to Mehrabad Airport station being 2.74 ppm and the lowest concentration was at Chitgar station in leaves of *Cupressus arizonica Greene* being 1.85 ppm.

According to figure 6, the results of mean comparisons showed that the mutual effect of station on U concentration in leaves of these trees was significant at the level of 95% ($p<0.05$). On the other hand, the results of multiple comparisons using Duncan test showed that the highest

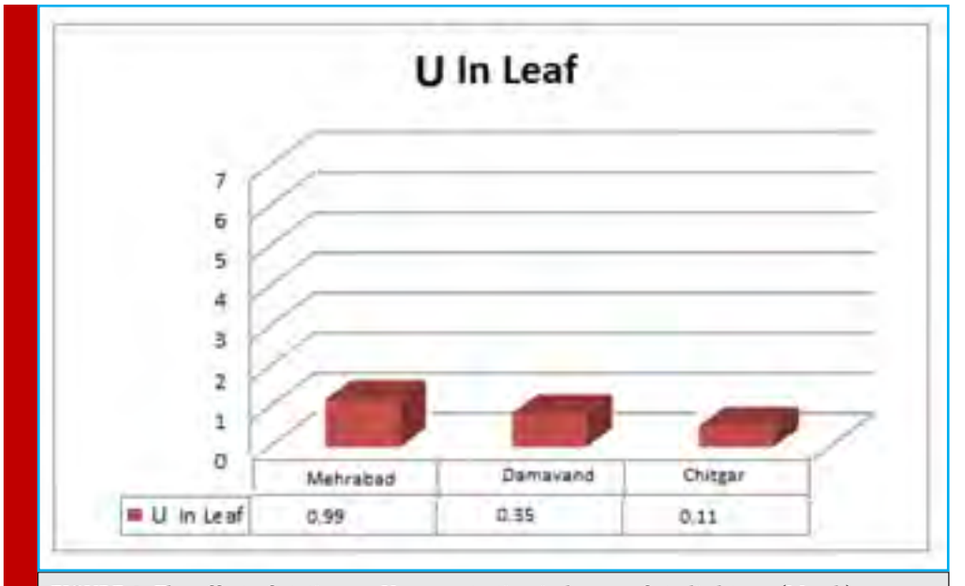

FIGURE 3. The effect of station on U concentration in leaves of studied trees (March)

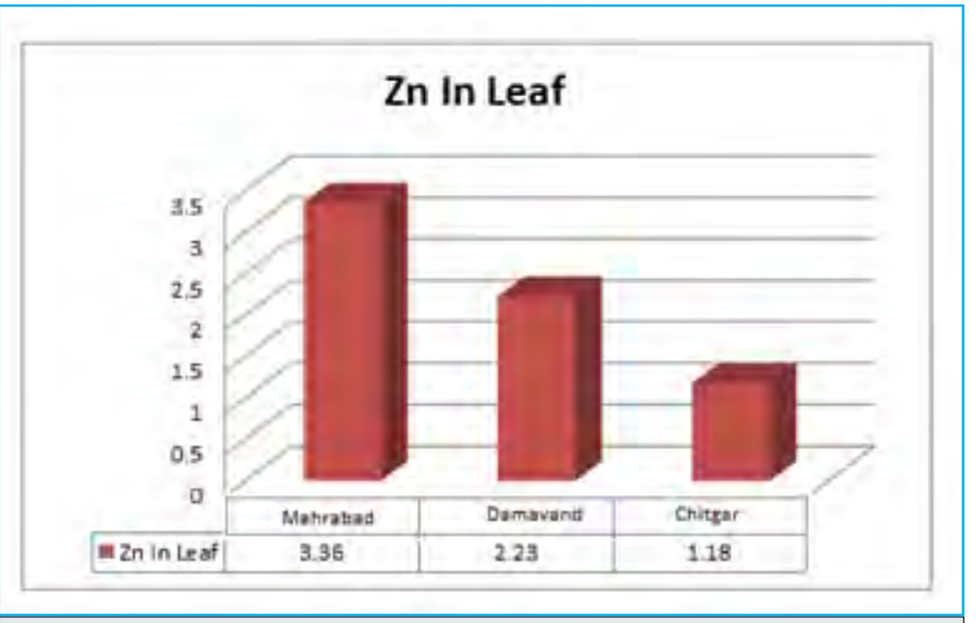

FIGURE 4. The effect of station on Zn concentration in leaves of studied trees (December)

U concentration in *Pinus Eldarica Medw* leaf belonged to Mehrabad Airport station being 0.99 ppm and the lowest concentration belonged to Chitgar station in leaves of *Cupressus arizonica Greene* which was 0.22 ppm.

According to figure 7, the results of mean comparisons showed that the mutual effect of specie on Zn concentration in leaves of these trees was significant at the level of 95% ($p<0.05$). On the other hand, the results of multiple comparisons using Duncan test showed that the highest Zn concentration was in *Pinus Eldarica Medw* leaf (1.61 ppm) and the lowest concentration was in leaves of n*latanus orientails* (1.29 ppm).

According to figure 8, the results of mean comparisons showed that the mutual effect of species on Cu

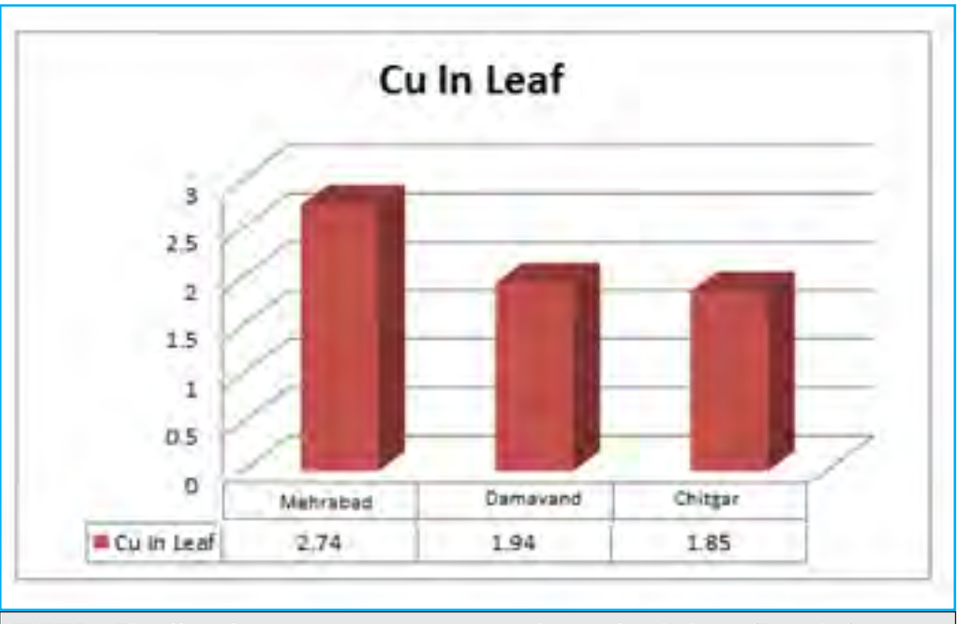

FIGURE 5. The effect of station on Cu concentration in leaves of studied trees (December)

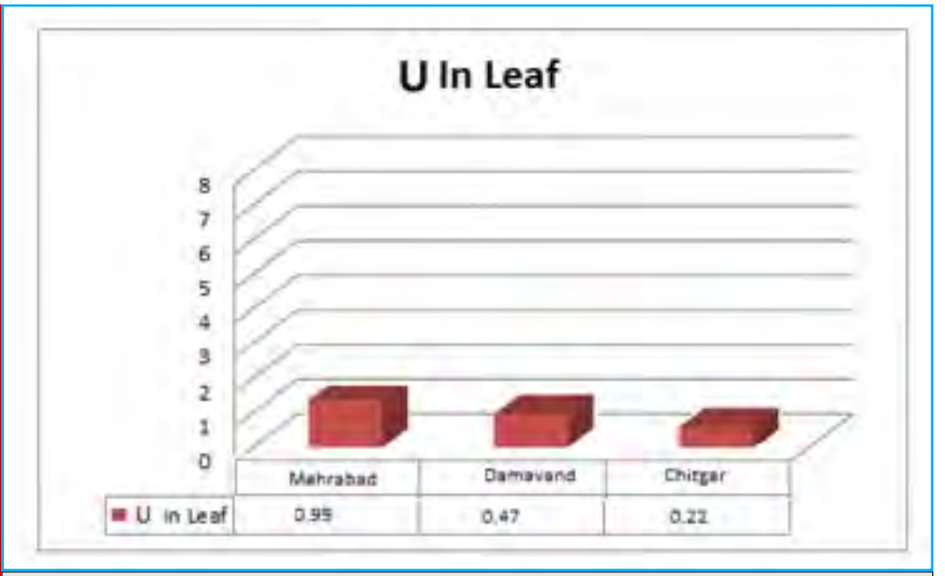

FIGURE 6. The effect of station on U concentration in leaves of studied trees (December)

concentration in leaves of these trees was significant at the level of 95% ($p<0.05$). On the other hand, the results of multiple comparisons using Duncan test showed that the highest Cu concentration was in the leaves of *Pinus eldarica Medw* (2.61 ppm) and the lowest concentration belonged to *Platanus orientails* which was 1.08 ppm.

According to figure 3, the results of mean comparisons showed that the mutual effect of specie on U concentration in leaves of these trees was significant at the level of 95% ($p<0.05$). On the other hand, the results of multiple comparisons using Duncan test showed that the highest U concentration belonged to *Pinus Eldarica Medw* leaf to 0.91 ppm and the lowest concentration belonged to *Platanus orientails* to 0.17 ppm.

According to figure 10, the results of mean comparisons showed that the mutual effect of specie on Zn con-

FIGURE 7. The Zn concentration ppm in leaves of studied trees (March)

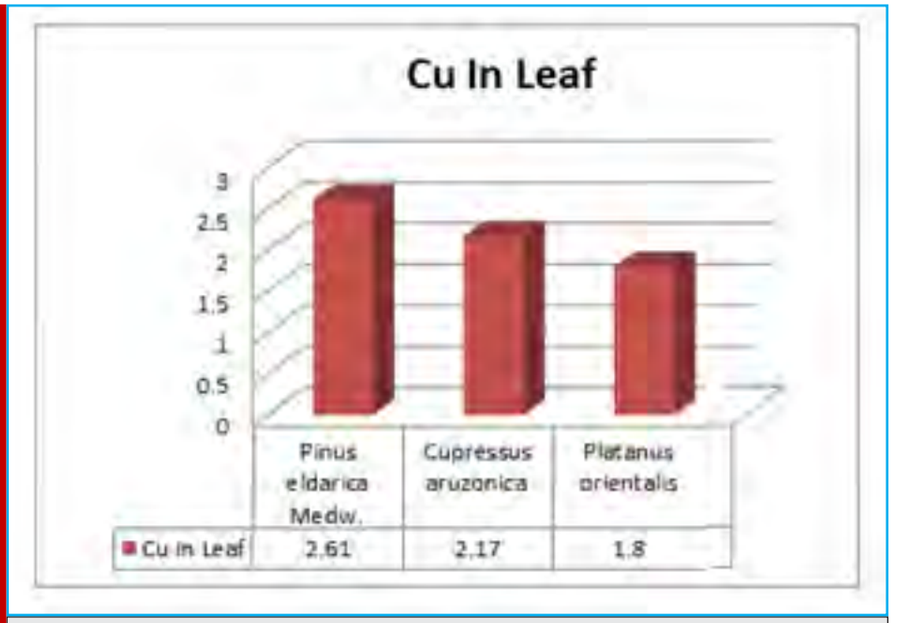

FIGURE 8. The Cu concentration ppm in leaves of studied trees (March)

centration in leaves of these trees was significant at the level of 95% ($p<0.05$). On the other hand, the results of multiple comparisons using Duncan test showed that the highest Zn concentration belonged to *Pinus eldarica Medw* leaf to 2.47 ppm and the lowest concentration was in *Cupressus arizonica Greene* (2.05 ppm).

According to figure 11, the results of mean comparisons showed that the mutual effect of specie on Cu concentration in leaves of these trees was significant at the level of 95% ($p<0.05$). On the other hand, the results of multiple comparisons using Duncan test showed that the highest Cu concentration was in *Pinus eldarica Medw*

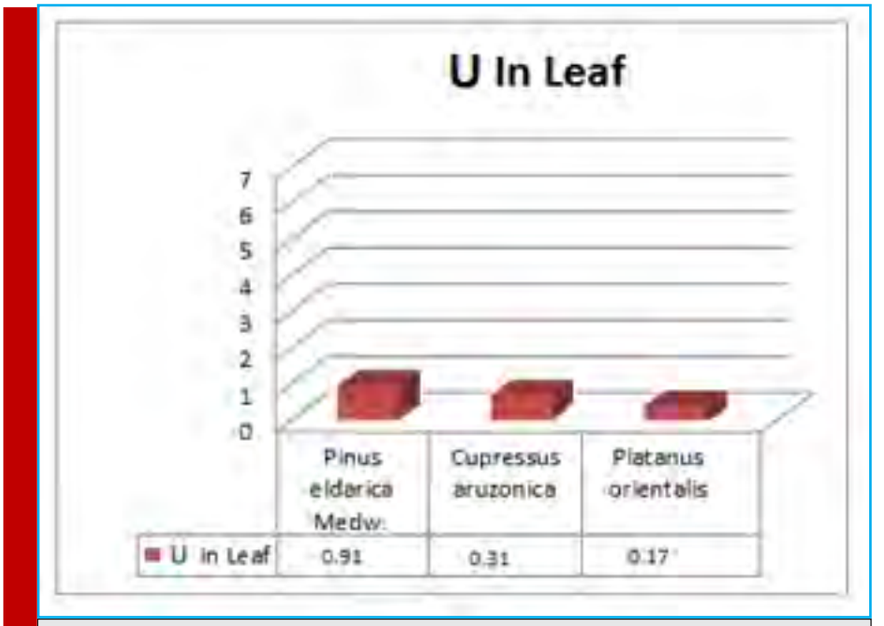

FIGURE 9. The U concentration in leaves of studied trees (March)

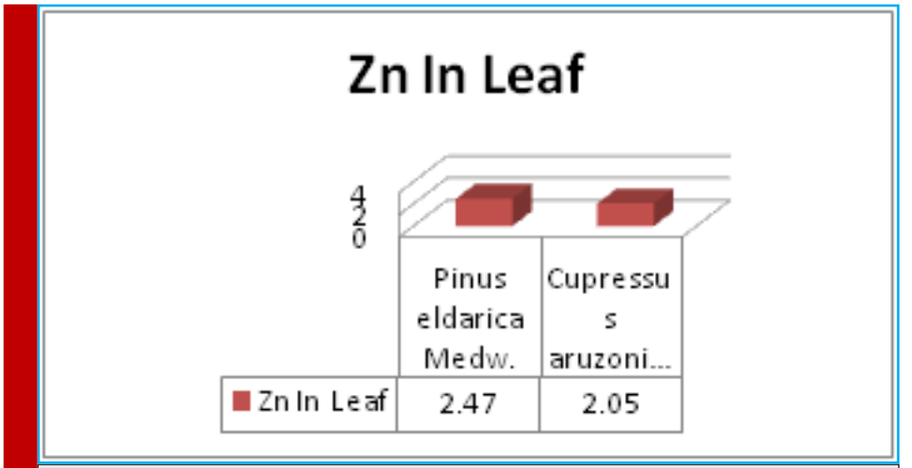

FIGURE 10. The Zn concentration in leaves of studied trees (December)

(2.38 ppm) and the lowest concentration was in *Cupressus arizonica Greene* being 1.97 ppm.

According to figure 12, the results of mean comparisons showed that the mutual effect of specie on U concentration in leaves of these trees was significant at the level of 95% ($p<0.05$). On the other hand, the results of multiple comparisons using Duncan test showed that the highest Uranium concentration was in *Pinus eldarica*leaves which was 0.27 ppm and the lowest concentration of 0.11 ppm was in the leaves of *Cupressus arizonica*.

DISCUSSION AND CONCLUSION

This study has been conducted with the aim of analysis of the role of three tree species in absorbing the air pollution caused by Cu, Zn and Uranium in Tehran. This study has been conducted in 3 stations including Mehrabad Airport, Damavand and Chitgar stations. In this study, it was found that absorption of heavy metals (Cu, Zn and Uranium) in leaves of studied trees in polluted stations was more than control station and it is increased respectively in Chitgar, Damavand and Mehrabad stations. As the environment plays key role in absorption of metals by plant species, it seems that Mehrabad Airport has shown higher level of absorption of the metal in leaves of tree species than other stations due to high concentration of metals in the air. Heydari et al (2005) have claimed that Pb absorption by plants can be increased due to concentration of this metal in the environment. Marry et al (1996) have also mentioned in their investigations that Pb absorption level by plants is in significant correlation with concentration of this metal in the environment.

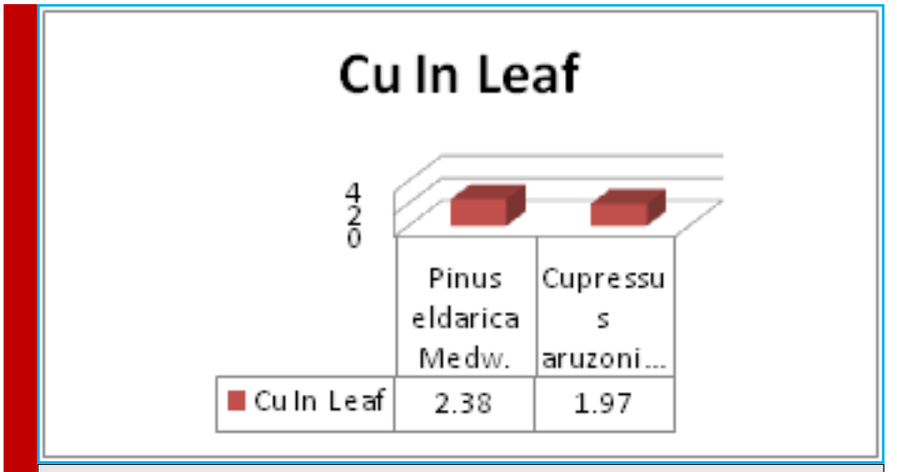

FIGURE 11. The Cu concentration in leaves of studied trees (December)

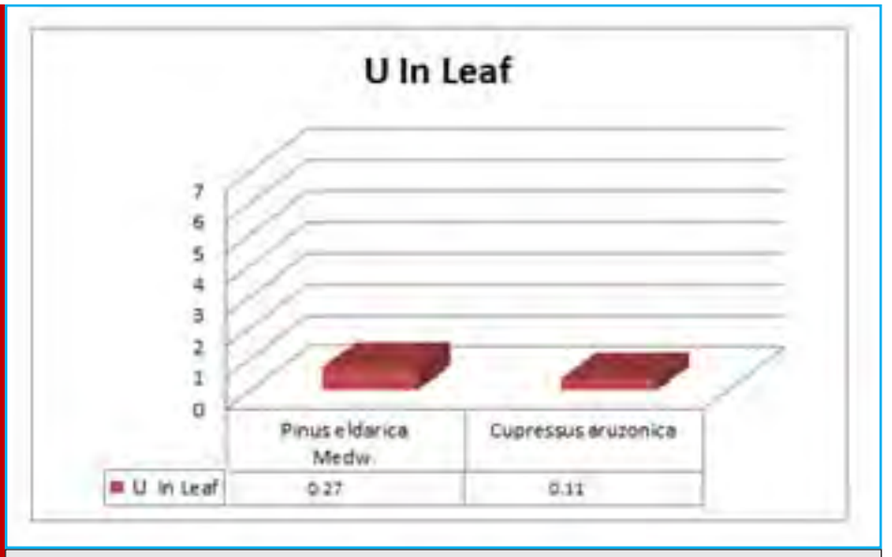

FIGURE 12. The U concentration in leaves of studied trees (December)

Cheraghi et al (2012) showed that concentration of metals in stem of plants was more than leaves. Moreover, significant correlation was observed between concentration of metals in stem and the sediments. Maddah et al (2013) found that forest pine is suitable specie for PbPhytoremediation. In concentration of 800ppm, the contamination of Pb is in highest level in stem and shoots; although in concentration of 1600ppm, the Pb contamination is mostly in stem and leaf.

The results obtained from the present study show that the absorption of these metals in tree species studied varies during different times and the process is increased in December and March. As absorption of these elements in plants is a physiologic phenomenon, absorption is decreased in cold months of the year with reduction of temperature and reduction of breathing. Poorfarhadi (1994) and Shahmansuri (1995) studied role of different seasons in absorption level of Pb in plants and found that the most absorption of Pb is in summer.Organization of Parks and Green Space of Tehran Municipality (1994) has also reported most Pb contamination level in plants in late September.

Kardar et al (2015) found that absorption of Cd in different organs of plant species in polluted station has been more than other stations and the highest cd absorption level has been attributed to September and the lowest level is observed in June. Shabanian and Cheraghi (2013) showed that contamination of Zn, Pb and Cd in leaves of majority of species in polluted zone has been significantly higher than control zone at the level of 95%. In polluted zone, the contamination of Pb and Cd has been observed in cedar and Zn in elm and Mg are also observed mostly in*Robinia peseudoacacia*.

In this study, in the studied treatments, absorption of metals existed in needle leaf trees has been more than broad leaf trees, so that its increasing process has been observed respectively in*Pinus Eldarica Medw*, *Cupressus arizonica Greene* and *Platanus orientails*. It seems that parameters such as being evergreen, more numbers of leaves, high growth speed, the Cu contamination in soil and ability of transfer from underground to aerial organs can affect increased absorption of metals in needle leaf plants. Lasat (2000) has found that growth speed of species and the coefficient of Cu transfer from underground to shoots could be the most important factor to increase the element in shoots (leaf and branches). Safdari (2005) has also mentioned that air pollutants can have more effect on needle leaf plants because of being evergreen and because of wider surface of leaf against the airflow.

Moreover, as one of the secondary results of this study, it could be mentioned that through conducting a field study in the zones with probability of emission of radioactive mainly caused by heavy metals such as surrounding areas of nuclear stations and the places for disposal of radioactive materials, through planting tree species with high absorption capability of heavy metals, especially radioactive metals such as U235, U238 and Pu239 found in nuclear wastes abundantly, a reliable method could be found in long-term to measure the amount of emission of radioactive materials from thenuclear disposal plant or reservoirs. Particularly, it could be applied in positions or places, at which Geiger Müller meters or other nuclear sensors can't be applied for any reason.

The results obtained from this study show that the Cu absorbed by leaves of studied species is increased

respectively in *Pinus eldarica Medw*, *Cupressus arizonica Greene* and *Platanus orientails*. Also, the highest absorption level of this metal is observed in leaf of *Pinus Eldarica Medw*. In terms of the effect of station in absorption of Cu, the species in Mehrabad Airport station have shown highest level of Cu contamination. The Zn contamination is also increased in leaves of studied species including*Pinus eldarica Medw*, *Cupressus arizonica Greene* and *Platanus orientails*and the *Pinus eldarica Medw*leaves have shown the highest contamination of this metal. The effect of stations on this metal has been also similar to two mentioned metals and has shown highest concentration in species in Mehrabad Station. In this study, it seems that due to the results obtained, uranium concentration absorbed by studied species is increased respectively in *Pinus eldarica Medw*, *Cupressus arizonica Greene* and *Platanus orientails*. The highest uranium contamination is also observed in *Pinus eldarica Medw*leaves. As it was mentioned, the concentration of uranium in studied species in polluted stations is more than control station and the leaves of *Pinus eldarica Medw*in Mehrabad Station have shown highest contamination of this metal. Therefore, it could be observed in this study that absorption of Cu, Zn and U in studied trees in polluted zones has been higher than control station and *Pinus eldarica Medw*can be applied as an indicator species for contamination of heavy metals such as Cu, Zn and Uranium.

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Comparing the analgesic effect of intranasal fentanyl and ketamine in children

Golikhatir Iraj[1], Montazer Seyed Hossein[1]*, Aghilli Elham[2], Aminiahidashti Hamed[1], Bozorgi Farzad[1], Jahanian Fatemeh[1] and Hosseininejad Seyed Mohammad[1]

[1]*Assistant Professor, Department of Emergency Medicine, Mazandaran University of Medical Science, Sari, Iran*
[2]*Emergency Medicine Resident, Mazandaran University of Medical Science, Sari, Iran*

ABSTRACT

Introduction: Pain control is one of the treatment priorities and the most important children's rights because children experience painful events since birth and during childhood due to common childhood illnesses or accidents. The aim of this study was to compare the analgesic effect of intranasal ketamine and fentanyl in children. Method: The present research is a double blind randomized clinical trial conducted on 80 children aged 3-13 years who were admitted to the Emergency Department of Sari Imam Hospital. The patients who met the inclusion criteria were randomly divided into two groups using random number generator and 40 patients were considered per group. Data were analyzed using SPSS, Mann-Whitney-U test and wilcoxon paired test. Findings: The findings showed that the analgesic effect of ketamine and fentanyl are similar among the studied children and there is no significant difference. In ketamine group there was significant difference between the mean of pain, systolic blood pressure, diastolic blood pressure, heart rate and respiratory rate variables before and after taking ketamine, according to Willcoxon statistics and sig. smaller than 0.05. In fentanyl Group, there was significant difference between the mean of pain, systolic blood pressure, heart rate and respiratory rate variables before and after taking fentanyl, according to Willcoxon statistics and sig. smaller than 0.05. There was no significant difference between ketamine and fentanyl analgesic effect in terms of parental satisfaction and there was also no significant difference between medical team's level of satisfaction with ketamine and fentanyl analgesic effect. Conclusion: Considering that the analgesic effect of intranasal ketamine and fentanyl on the pain control among the studied children is similar, their prescription is recommended. It is also recommended to simultaneously measure children's level of anxiety and effect of these drugs on their anxiety because children have different experiences of pain and anxiety affects measuring pain severity in future studies.

KEY WORDS: ANALGESIC, INTRANASAL, FENTANYL, KETAMINE, CHILDREN

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INTRODUCTION

Pain is one of the most common symptoms in patients admitted to the emergency room. Achieving desired analgesia and sedation before the procedures has become an elusive goal in many cases (Murphy et al., (2014).International Association of pain defined it as an unpleasant feeling and emotional experience associated with acute or potential tissue damage (Hazinski 2013). Children are usually brought to the emergency department due to painful diseases and injuries. Other diagnostic and painful or unpleasant therapeutic procedures may be required during the visits (Atkinson et al., (2009). Pain in children is more difficult than adults either in the evaluation or treatment process. This difficulty becomes more obvious when Intravenous analgesic is considered (Moore et al. (2010). Creating a practical approach to sedation and analgesia may vary in different parts of the world (Shahryariet al 2010 Majidi et al., 2017).

Many studies have been conducted on the analgesic effects of ketamine in the world. Many studies conducted on intranasal, oral and rectal administration of ketamine have shown that topical application of this drug is also possible. Many empirical evidence suggest that NMDA receptors exist in the central nervous system and peripheral nerves. Moreover, empirical studies have noted that the peripheral administration of NMDA receptor antagonists show analgesic effects of this drug (Hadayi M, Rezaeian M. (2011, Brian et al 2015).

Fentanyl is a potent opioid agonist that can be used in different ways. Fentanyl is the oldest synthetic opioid agonist and mainly exerts its effect on the hair receptor (Brian et al., (2015). Intravenous administration is the main method of medications to children but it is also stressful and painful and we sometimes need to spend a lot of time. Intramuscular injection is similar to the intravenous injection with the exception that longer period of time is required for the drug to exert its effect. According to the foregoing, intranasal and buccal methods seem to be more appropriate methods (Banks, et al. (2004). However, between these two methods, using buccal method requires need more interaction between the child and the therapist and even in voluntary cases of drug use, 56% of the drug remains in the mouth. Nasal cavity holds a rich vascular network and since this vascular bed is easily accessible, it is easy to be managed. Onset and peak effect usually occurs after 3-5 minutes and within 10-15 minutes (Veldhorst, 2013).

Studies have shown that a wide range of intranasal ketamine doses has been used in clinical practice and further research is proposed to determine the optimal dose of intranasal ketamine for analgesia (Marcia et al. (2013). So far, various medications are presented and provided for this purpose. But ketamine, which is a receptor agonist has gained much popularity in this field (Majidi et al., 2017).

Fentanyl is a short-acting strong industrial drug and is now widely used for pain relief. Few studies have compared the effects of intranasal fentanyl and ketamine. Graudins et al., (2015) conducted a study in American Academy of Emergency Medicine (AAEM) and compared the effect of intranasal fentanyl and ketamine in children 3 to 13 years who suffered from organ damage and had pain severity of at least 6 out of 10. The 40 children were assigned in each group in this comparison. As a result, a reduced pain which was reported in both groups was similar, but ketamine showed more side effects. Level of satisfaction with and side effects of ketamine and fentanyl was respectively 83%, 78% and 82%, 40%. Prior to this study, there was no study on intranasal fentanyl and ketamine for pain control in children.

In a study in 2013 in the Department of Emergency Medicine of British Columbia Hospital, Andolfatto investigated the intranasal ketamine effect in reducing pain in patients admitted to the emergency department. This study was performed in patients older than 6 years. A total of 40 patients were enrolled in the study and the mean change in pain was 34mm within 30 minutes and the average time for this reduction in pain was about 9.5 minutes. There were no reports of serious side effects during this time and all reported side effects were transient, requiring no intervention.

Murphy et al. (2014) conducted a study in the Department of Emergency Medicine at University College Dublin and studied intranasal fentanyl consumption for the treatment of acute pain in children. Patients were randomly selected and intranasal fentanyl was administrated for pain control and its efficacy was compared with intravenous medications. Side effects and level of satisfaction were also investigated. This study was carried out on children aged less than 21 years who weighed more than 10 kg with severe pain . Severity of pain was assessed every 5 minutes to 30 minutes and then at 30.60 and 120 minutes. A total of 30 patients were enrolled and the pain severity was dropped by about 13mm after 120 minutes. There were no severe side effects, for which intervention was needed. Also, small sample size of some studies caused the results not to be generalized to the entire population (Yeaman et al., (2013). Also, different doses of these two drugs were used in studies on the effects of intranasal ketamine or fentanyl in pain control among children (Saunders et al., 2010).

In our country, no comparison has been made in this way. Since there has been no similar study in this regard so far in the country, similar studies conducted abroad are few and the results of these few studies cannot generalized to all areas yet. On the other hand, taking into

account the speed and ease of use of the intranasal drug, we decided to compare the effect of intranasal fentanyl and ketamine as well as their effects and complications in pain management. The present study also aimed to achieve acceptable outcome with regard to recommending the use of these two drugs in the emergency department by measuring satisfaction of parents and medical team.

MATERIAL AND METHODS

This study is a double blind randomized clinical trial that was conducted on 80 children aged 3-13 years' old who were taken to Sari Imam Hospital. Individuals who were enrolled in the study were selected among all children aged 3-13 years who were admitted to emergency due to the trauma and needed remedial measures and painful procedures such as healing wounds with moderate to severe pain during procedure. The number of samples was considered 40 individuals for each group based on statistical formulas. Patients who met the inclusion criteria were randomly assigned in two groups using random number generator so that the number of children had relatively equal distribution at the end, allowing evaluation of the results for both age groups. A total of 40 patients were considered in each group. One group received Ketamine and the other one was given fentanyl. Among pain severity assessment scales, which are used at early age, FPS-R scale is a scale that is translated into 30 languages and can be used for free (Julie 2013).

VAS scale is also a measure commonly used in the age of 6 years, the patient shows the location of his/her pain on a horizontal line. Left and right sides of the line show point of no pain and unbearable pain, respectively. Pain score range is between the pain score in the left side and is expressed in millimeters. Based on the pain scales pain severity of 6 and higher shows moderate to severe pain. At the beginning of the project and during the procedure, FPS-R and VAS scales were respectively used to assess pain in children aged 3-6 and over 6 years. Eventually, after summarizing the pain assessment results and the effect of the drugs on it, qualitative evaluation of the results was performed.

According to ASA physical status classification standards, all ASA I and II class children were enrolled in the study. Also according to method of drug use, all children with severe colds, respiratory infections or major nasal damage, and all those with taking painkillers before referral, given the confounding effect on the evaluation of results, and contraindications for drugs, suspected cases of increased ICP, severe respiratory diseases or a history of seizure disorders were excluded. Also, after obtaining permission from the Medical Ethics Committee, registering the present trial in the Iranian database of clinical trials and providing adequate explanations on how to do pain management technique as well as obtaining consent letter from children's parents, patients were enrolled.

However, patients were not deprived of any treatment due to participation in this research and it does not cause any harm to them .Lidocaine was used for local anesthesia during the procedure. Both drugs produced by Rotemxmedica-Germany company, Ketamine and fentanyl were prepared by Saha Halal Pharmaceutical Company and Drug Administration of Mazandaran University of Medical Sciences, respectively. After the initial assessment and recording pain levels and vital signs at admission in the first questionnaire then in the second questionnaire during the procedure, the following standard recording and monitoring were performed: pulse rate (PR), respiratory rate (RR), blood pressure (BP), blood oxygen saturation (SpO2) and temperature body temperature (T).

Drugs were already drawn into 1cc syringes, to have equal volume of drugs, appropriate to 10 kg weight. The syringe were named A and B and were given to the head of the pharmaceutical ward who was unaware of their contents. The drugs were later administrated to patients in a double-blind, randomized manner during the project. One group received 1 mg / kg intranasal ketamine and 1mcg/kg intranasal fentanyl was administrated to another group. The procedure was started 5 minutes after administration is starting considering the time required for onset of action. The pain severity and vital signs were examined at 5, 15, 20, 30, 45 and 60 minutes in the studied patients, if necessary, an additional 1.4 dose was prescribed for pain control to at 15 minutes. In case of spo2 less than 92% for more than 10 seconds or apnea more than 20 seconds, jaw thrust maneuver and mask ventilation was started. Nausea and vomiting was measured by observing and questioning the patient and parents. Other side effects, including dizziness, and derealization were recorded by observing and questioning and each of them underwent treatment and monitoring based on the severity and type. Information was recorded by an individual (emergency resident or medicine specialists) who was unaware of the groups. Data analysis was carried out using SPSS v.16 and Mann-Whitney-U test and Willcoxon paired test.

RESULTS AND DISCUSSION

The mean ± standard deviation of pain before taking Ketamine and fentanyl were respectively, 8.9, 8.93 and 0.93, 0.92 and there was no significant difference between Ketamine and fentanyl groups in terms of pain before taking these drugs based on Mann-Whitney U z-statistic and sig. value of greater than 0.05. Thus, chil-

Table 1. Comparison of pre- to post-intervention change in pain intensity in both groups based on Mann-Whitney-U test

Variable	Group	Average	Number	Standard Deviation	Mann- Whitney Z statistics	Sig.
Pre medication pain	Ketamine	40	8.90	0.93	0.14-	0.900
	Fentanyl	40	8.93	0.92		
Post medication pain	Ketamine	40	2.68	0.86	0.00	0.001
	Fentanyl	40	2.68	0.86		

dren in both groups were not statistically different in terms of the premedication pain and are homogeneous.

The mean ± standard deviation of pain after taking Ketamine and fentanyl were respectively, 2.68, 2.68 and 0.86, 0.86 and there was no significant difference between Ketamine and fentanyl groups in terms of pain after taking these drugs based on Mann-Whitney U z-statistic and sig. value of greater than 0.05. Thus, children in both groups were not statistically different in terms of the post-medication pain and are homogeneous. In other words, it can be said that the analgesic effect of these drugs is similar in children is not significantly different (Table 1).

According to U Mann Whitney Test, mean ± SD of systolic blood pressure before taking ketamine and fentanyl were 107.63, 107.38 and 5.43, 4.86, respectively. Based on U Mann Whitney Test z-statistic and sig. value of greater than 0.05, there is no difference between the two groups of children in terms of blood pressure before using the drug. According to U Mann Whitney Test, mean ± SD of diastolic blood pressure before taking ketamine and fentanyl were 66.38, 65.63 and 5.06, 4.83, respectively. Based on U Mann Whitney Test z-statistic and sig. value of greater than 0.05, there is no difference between the two groups of children in terms of diastolic blood pressure before using the drug. Mean ± SD of heart rate before taking ketamine and fentanyl were 108.30, 108.35 and 7.05, 7.49, respectively. Based on U Mann Whitney Test z-statistic and sig. value of greater than 0.05, there is no difference between the two groups of children in terms of heart rate before using the drug.

Mean ± SD of respiratory rate before taking ketamine and fentanyl were 18.4,18.8 and 1.82,2.02, respectively and based on U Mann Whitney Test z-statistic and sig. value of greater than 0.05, there is no difference between the two groups of children in terms of respiratory rate before using the drug.

Mean ± SD of oxygen saturation before taking ketamine and fentanyl were 99.5, 99.55 and 0.96,0.85, respectively and based on U Mann Whitney Test z-statistic and sig. value of greater than 0.05, there is no difference between the two groups of children in terms of oxygen saturation before using the drug. Thus, we conclude that children in two groups are homogeneous in terms of the above variables and there is no significant difference between them before treatment (Table 2).

Based on U Mann Whitney Test, mean ± SD of systolic blood pressure after taking ketamine and fentanyl were 103.75,103.25 and 4.63,5.13, respectively and based on U Mann Whitney Test z-statistic and sig. value of greater than 0.05, there is no difference between the two groups of children in terms of systolic blood pressure after using the drug.

Mean ± SD of diastolic blood pressure after taking ketamine and fentanyl were 64,65 and 4.56,4.24, respec-

Table 2. Comparison of variable blood pressure, heart rate and oxygen saturation before taking the drug among children in groups based on Mann-Whitney U test

Variable	Group	Number	Average	Standard deviation	Mann-Whitney Z statistics	Sig.
Pre- systolic blood pressure	Ketamine	40	107.63	5.43	1.86-	0.062
	Fentanyl	40	107.38	4.86		
Pre diastolic blood pressure	Ketamine	40	66.38	5.06	0.64-	0.52
	Fentanyl	40	65.63	4.83		
Pre-heart rate	Ketamine	40	108.30	7.05	0.07	0.95
	Fentanyl	40	108.35	7.49		
Pre-RR	Ketamine	40	18.40	1.82	0.76-	0.45
	Fentanyl	40	18.80	2.02		
Pre- oxygen saturation	Ketamine	40	99.50	0.96	0.05-	0.96
	Fentanyl	40	99.55	0.85		

Table 3. Comparison of blood pressure, heart rate and oxygen saturation variables after drug consumption among children in both groups based on Mann-Whitney-U test

Variable	Group	Number	Average	Standard deviation	Mann-Whitney Z statistics	Sig.
Post- systolic blood pressure	Ketamine	40	103.75	4.63	0.69-	0.4896
	Fentanyl	40	103.25	5.13		
Post diastolic blood pressure	Ketamine	40	0.64	4.56	1.21-	0.2268
	Fentanyl	40	0.65	4.24		
Post –heart rate	Ketamine	40	102.15	5.57	1.54-	0.1242
	Fentanyl	40	0.100	5.83		
Post-RR	Ketamine	40	17.45	1.50	0.52-	0.6063
	Fentanyl	40	17.60	1.45		
Post –oxygen saturation	Ketamine	40	99.70	0.72	1.11-	0.2666
	Fentanyl	40	99.50	0.88		

tively and based on U Mann Whitney Test z-statistic and sig. value of greater than 0.05, there is no difference between the two groups of children in terms of diastolic blood pressure after using the drug.

Mean ± SD of heart rate after taking ketamine and fentanyl were 102.15,100 and 5.57,5.83, respectively and based on U Mann Whitney Test z-statistic and sig. value of greater than 0.05, there is no difference between the two groups of children in terms of heart rate after using the drug.

Mean ± SD of respiratory rate after taking ketamine and fentanyl were 17.45,17.60 and 1.5,1.45, respectively and based on U Mann Whitney Test z-statistic and sig. value of greater than 0.05, there is no difference between the two groups of children in terms of respiratory rate after using the drug.

Mean ± SD of oxygen saturation after taking ketamine and fentanyl were 99.70,99.50 and 0.72,0.88, respectively and based on U Mann Whitney Test z-statistic and sig. value of greater than 0.05, there is no difference between the two groups of children in terms of oxygen saturation after using the drug. Thus, we conclude that there is no statistically significant difference between children in the two groups after taking the drugs and the effect of these drugs on children is similar in this study (Table 3).

Now, after examining the mean differences between studied variables in two groups of children, we investigate the differences between these variables in each group before and after drug use in this section. According to Willcoxon statistics and sig. value of smaller than 0.05, there was statistically significant difference in the ketamine group among mean values of pain, systolic blood pressure, diastolic blood pressure, heart rate and respiratory rate before and after taking ketamine. In other words, ketamine is effective in reducing blood pressure (systolic and diastolic), heart rate and respiratory rate except for oxygen saturation variable (Table 4).

Table 4. Comparison of variables between children groups treated with ketamine based on Willcoxon paired test

	Drug type	Average	Number	Standard deviation	Willcoxon statistics	Sig.
Ketamine	Pre medication pain	8.90	40	0.93	5.630-	0.00009
	Post-medicationpain	2.68	40	0.86		
	Pre medication systolicblood pressure	107.63	40	5.43	3.489-	0.0005
	Post medication systolicbloodpressure	103.75	40	4.63		
	Pre-medicationdiastolicbloodpressure	66.38	40	5.06	2.747-	0.0060
	Post-medicationdiastolicbloodpressure	0.64	40	4.56		
	Pre-medicationHeart ratePre	108.30	40	7.05	5.191-	0.00009
	Post-medicationheart rate	102.15	40	5.57		
	Pre-medicationRR	18.40	40	1.82	4.359-	0.00009
	Post-medicationRR	17.45	40	1.50		
	Pre- medication oxygen saturation	99.50	40	0.96	1.232-	0.2180
	Post- medication oxygen saturation	99.70	40	0.72		

Table 5. Comparison of variables between children groups treated with fentanyl based on Willcoxon paired test

	Drug type	Average	Number	Standard deviation	Willcoxon statistics	Sig.
Fentanyl	Pre-medication pain	8.93	40	0.92	5.630-	0.00009
	Post-medication pain	2.68	40	0.86		
	Pre-medication systolic blood pressure	109.88	40	4.87	3.489-	0.0005
	Post-medication systolic blood pressure	103.25	40	5.13		
	Pre-medication diastolic blood pressure	65.63	40	4.83	2.747-	0.0060
	Post-medication diastolic blood pressure	0.65	40	4.24		
	Pre-medication Heart Pre rate	108.35	40	7.49	5.191-	0.00009
	Post- medication heart rate	0.100	40	5.83		
	Pre- medication RR	18.80	40	2.02	4.359 -	0.00009
	Post-medication RR	17.60	40	1.45		
	Pre-medication oxygen saturation	99.55	40	0.85	1.232-	0.2180
	Post-medication oxygen saturation	99.50	40	0.88		

According to Willcoxon statistics and sig. value of smaller than 0.05, there was statistically significant difference in the fentanyl group among mean values of pain, systolic blood pressure, heart rate and respiratory rate before and after taking fentanyl. In other words, fentanyl is effective in reducing blood pressure (systolic and diastolic), heart rate and respiratory rate except for diastolic blood pressure and oxygen saturation variable (Table 5)

Based on the Mann-Whitney test, mean ± SD of parents' satisfaction with analgesic effect of ketamine and fentanyl were respectively 73.75, 75.25 and 7.40 6.79; so based on the Mann-Whitney z-statistic and sig. value of more than 0.05, there is no significant difference between parental satisfaction with the ketamine and fentanyl analgesic effect.

Mean ± SD of medical team satisfaction with analgesic effect of ketamine and fentanyl were respectively 74.75, 75.75 and 5.06, 5.01; so based on the Mann-Whitney z-statistic and sig. value of more than 0.05, there is no significant difference between medical team satisfaction with the ketamine and fentanyl analgesic effect (Table 6).

The results showed that there is no significant difference between analgesic effects of these two drugs in studied children. These results are consistent with results obtained by Gradyns (2015) in America where the level of pain reduction was similar in both groups. The result of the current study are also consistent with the results of the study conducted by Yeamen et al., (2013) who examined the effects of fentanyl and ketamine on pain and pain caused by intraoperative ulcer and concluded that both fentanyl and ketamine are effective in pain relief and analgesia compared with placebo, but are not significantly different from each other: but the results of this study are inconsistent with results of the study conducted by (Julie 2013) in Iran, in which the difference between ketamine and fentanyl is relatively low and negligible and this effect has been reported to be due to the analgesic properties of ketamine.

The results of studies on the effects of fentanyl and ketamine are different, which could be due to different injury, choice of different patients, lack of blinding in the studies and type of the study design. The results showed that except for the oxygen saturation variable, ketamine

Table 6. Comparison of parents and the medical team satisfaction with analgesic effect of Ketamine and fentanyl based on the Mann-Whitney U test

Variable	Group	Number	Average	Standard deviation	Mann Whitney Z statistics	Sig.
Parental satisfaction	Ketamine	40	73.75	7.40	0.991	0.322
	Fatanyl	40	75.25	6.79		
Medical Team Satisfaction	Ketamine	40	74.75	5.06	0.890-	0.374
	Fantanyl	40	75.75	5.01		

was effective in reducing blood pressure (systolic and diastolic), heart rate and respiratory rate and fentanyl was also effective in reducing systolic blood pressure, heart rate and respiratory rate except for diastolic blood pressure and oxygen saturation variables. Very limited side effects were observed that were not tested due to being limited. Five derealization cases in the ketamine Group and three vomiting and three nausea cases in the fentanyl Group were reported. The results of the present study are consistent with the results of the study conducted by Gradyns (2015) who showed that ketamine has more side effects.

In a study on changes in respiratory rate, Javaherforooshet al., (2006)showed that ketamine and fentanyl lead to the highest and lowest respiratory rates, which may be due to the respiratory stimulant and respiratory depression effects of ketamine and fentanyl, respectively. In a study, (Tsze et al., (2012) showed that ketamine was well tolerated in pediatric pain management and one case led to vomiting. The results showed that the difference between the satisfaction of parents and the medical team of ketamine and fentanyl analgesic effect is not significant. Parents' average satisfaction with the analgesic effect of ketamine in children is 73.75% and medical team's average satisfaction with the analgesic effect of fentanyl was equal to 74.75%. The same amount for ketamine and fentanyl was 83% and 82%, respectively in a study conducted by Gradyns et al. (2015).

In a study on the effects of intranasal fentanyl for pain relief in children with musculoskeletal trauma, Saunders et al. (2010) reported satisfaction percentage of 79% and 74% respectively for parents and the medical team. One of the limitations of this study include that patients might have usednarcotic or any other analgesic drug for pain relief prior to admission to the emergency center. So, this issue could affect the outcome of the research and that's why this issue should be considered in future studies and prevents the entry of similar cases to the study. Considering the small number of similar studies, further studies are needed to prove the effectiveness and compare the intranasal effect of these drugs. Another limitation was that pain was different for children because they had different experiences of pain and assess pain in children were not easy. Therefore, it is recommended to consider larger sample size in next studies and level of anxiety in children and the effect of these drugs on anxiety be measured simultaneously because children have different experiences of pain and anxiety is effective in measuring the severity of pain. Also, ketamine and fentanyl analgesic effects and side effects and different methods of administration should be investigated in future studies.

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Antagonistic activity of ZrO_2 against typhoid fever causing *Salmonella typhi*, isolated from retail poultry shops in and around Tirupur District

A. Chithira, M.I. Farheena and A. Mohankumar

Division of Microbial Technology, PG and Research Department of Zoology, Chikkanna Government Arts College, Tirupur, Tamilnadu, India

ABSTRACT

Recurrently typhoid fever, caused by *Salmonella typhi*, remains a significant cause of mortality and morbidity in many regions of the world. So predominant pathogen *S.typhi* is one of the major causes of food and water borne gastroenteritis in human and remains an important health problem. So fecal samples were collected from the poultry retail shop in tirupur city. Totally 50 multidrug resistant *Salmonella* spp were isolated from 75 fecal samples and confirmed by using routine laboratory techniques. Later, the antimicrobial pattern of this isolates were studied by using 11 antibiotic discs which include Amikacin (10mcg), Co-trimoxazole (25mcg), Ciprofloxacin (30mcg), Tetracycline (30mcg), Cephalothin (30mcg), Ceftriaxone (30mcg), Entrofloxacin (10mcg), Gentamicin (10mcg), Ampicillin (10mcg), Trimethoprim (10mcg), Cefoxitin (30mcg). Among these strains (12%), (62%), (28%), (80%), (12%), (4%), (6%), (26%), (36%), (100%), and (8%) were found to be exhibit a significance degree of resistance to different groups of antibiotics. Further, plasmid profile were performed for the five multidrug resistance isolates and observed the molecular weight was 1500bp and 700bp respectively. Recurrently, the metal oxide nanoparticles are currently the most promising tools applied as antimicrobial agents for diagnosis of diseases. Nanoparticle Zirconium oxide was used to against *Salmonella* spp. Different concentration of Zirconium oxide 50µl, 100µl and 150µl were used against *Salmonella* spp. Among the three concentration of nanoparticle, maximum zone of inhibition 16mm was observed against the isolate CH36 at 150µl concentration of nanoparticle. Minimum zone of inhibition 13mm was observed against the isolate CH37. So hence the present study Zirconium oxide was used and it shows prominent antibacterial activity against typhoid causing organism.

KEY WORDS: TYPHOID FEVER, *SALMONELLA TYPHI*, ANTIBIOTIC RESISTANCE, PLASMID PROFILE AND ZRO_2

ARTICLE INFORMATION:

**Corresponding Author: nanochithira@gmail.com*

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INTRODUCTION

Typhoid fever is a major health problem in developing countries, for thousands of years, thriving in conditions of poor sanitation, crowding and social chaos, contaminated water, milk, food or fruits vegetables or via convalescent or chronic carrier (Harish Menezes, 2011). Typhoid fever is the most serious form of enteric fever and in 2000 it was estimated that the global number of typhoid causes exceeded 21,00,000 with more than 2,00,000 death. Globally, up to 27 million infections occur per year, with over $2x10^5$ attributable deaths annually, predominantly among children under the age of five years (Clark et al., 2010). The predominant *Salmonella* species are Gram-negative rod shaped bacteria that are members of the family Entero bacteriaceae and are considered threatened food borne pathogens facing food safety and public health. Salmonella is a serious threat facing poultry industries as it has the ability to infect chickens causing diarrhoea. In the last few years, consumption of contaminated poultry, eggs, and their products become the most common sources of foodborne human salmonellosis (Mahmoud et al., 2015; Park et al., 2015; Hsu et al., 2016). Arena et al., 2017; Pashazadeh et al., 2017 and Kalupahana et al., 2017).

A variety of food products, especially poultry and other types of meat products, are the most important sources of human *Salmonella* spp. infection, but water borne outbreaks have also occurred birds are mainly infected through feed, drinking water or environmental sources. The organisms route of infection is the faecal – oral route via food or water contaminated with faeces or urine of previously infected persons or animals. Most environmental concerns over land application of animal manure have focussed on either the effect of applied nutrients, especially N and b.

Although poultry production is considered as secondary agricultural production systems and it has an important role in high quality protein. Poultry provide globally important sources of animal protein and are amongst the most intensively reared of all livestock species several microbial diseases have been affecting the poultry and it is a major concern, both locally and international levels. The low productivity is mainly due to high mortality, which is caused particularly by bacterial disease and the mortality has been estimated in the range of 80-90% (Debnam and Jackson, 2005).

Food borne diseases are main problems, particularly in developing countries and cause the majority of illnesses and death around the world. Food is the most important vehicle that transmits the microorganisms to human (Varnam, 1991) among microorganisms *Salmonella* still a major cause of food – borne human disease in most parts of the world (Soultose et al., 2003 and Carraminana et al., 2004). Poultry and poultry products are frequently contaminated with *Salmonella* that can be transmitted to humans through the handling of raw poultry carcasses and products, or through consumption of undercooked poultry meat (Kimura et al., 2004). Young chick, mortality up to 100%, week chicks, loss of appetite, diarrhoea, and adult birds: no sings depression, diarrhoea, and drop in egg production, low mortality. The poultry farms bird flu has become a lethal condition that is occurring around the world more frequently (Julie et al., 2004).

The sub therapeutic use of antibiotics in poultry has become a popular practice and these is a growing body of scientific evidence to the effect that the increasing incidence of antibiotic resistant bacteria is closely associated with the heavy use of these antibiotics in poultry and other related agricultural practices. Despite the great progress in antimicrobial development, many infectious diseases, especially intracellular infections, remain difficult to treat. One major reason is that many antimicrobials are difficult to transport to cell membranes and have low activity inside the cell, there by imposing negligible inhibitory or bactericidal effects on the intracellular bacteria (Zhang et al., 2010).

In 2013 many techniques were used by several researchers to *Salmonella* spp. but these techniques could not completely cure *Salmonella* spp. Pathogenic bacteria still remain a major health concern, which are responsible for causing a large number of deaths and hospitalizations each year. Although we have current treatments such as antibiotics, bacteria are gaining resistance to these therapeutics at an alarming rate. That is why new therapeutic and diagnostic treatments are necessary if we want to be prepared against known and unknown pathogenic bacterial infections. A large group of these studies includes the implementation of nanotechnologies and nanomaterials to create new antibacterial nano-medicines that increased effectiveness and efficiency.

Moreover, nanotechnology is through to be technology of the future with several opportunities for applications one of the most important nanotechnology applications areas that hold the expectations of providing create benefits for humanity in the future is medicine (Neuberger et al., 2005). Therefore, it is important to find another efficient treatment for *Salmonella* infection instead of antibiotic. In the last few years, there has been a growing interest in nanotechnology. Indeed, nanoparticles have been gaining importance in recent years and became an effective revolution therapy against pathogenic bacteria due to their bactericidal properties. The nanoparticles size and surface area are significant agents to which their bactericidal mechanism of action attributed to several pathogens (Devi et al., 2017).

ZrO_2 nanoparticles with antimicrobial activity when embedded and coated and the surface can find immense applications in water treatment, synthetic textiles, biomedical and surgical device, food processing and packaging. Moreover, the composites prepare using ZrO_2 and polymers can find better utilization due to the enhanced antimicrobial activity.The multi drug resistant pathogens due to antigenic shift are ineffectively managed with current medications. This resistance to medication by pathogens has become a serious problem in public health and therefore mandating the need to develop new bactericides and virucides. Zirconium oxide nanoparticle (ZrO_2), having a long history of general use as an antiseptic and disinfectant, are able to interact with disulphide bonds of the glycoprotein / protein contents of microorganisms, viruses, bacteria, fungi. The ZrO_2 nanoparticle change the three dimensional structure of proteins by interfering with S- bonds and block the functional operation of the microorganisms. ZrO_2 nanoparticles with antimicrobial activity when embedded and coated and the surface can find immense applications in water treatment, synthetic textiles, biomedical and surgical device, food processing and packaging. Moreover, the composites prepare using ZrO_2 and polymers can find better utilization due to the enhanced antimicrobial activity.

Recently the antibiotics such as tetracycline, amikacin, co-trimoxazole ciprofloxacin, cephalothin, ceftriaxone, entrofloxacin, gentamicin, ampicillin, trimethoprim, cefoxitin are used for the poultry bacterial disease. One of the earliest nanomedicine applications particularly, an antimicrobial agent from ZrO_2 nanoparticle for the treatment of various microbial diseases is being emerged. However, studies related the ZrO_2 nanoparticle against *S.typhi* is too limited. Hence, the present study has been made an attempt to point out the bioactive medical properties of metal oxide nanoparticle (ZrO_2) against *Salmonella typhi* isolated from retail poultry shop in and around Tirupur District.

MATERIALS AND METHODS

Sterile spatulas were used to collect samples of freshly passed poultry droppings in sterile universal sampling bottles. 75 samples were collected from different poultry retail shop in Tirupur city. The droppings were collected from litter at random points and transported to the laboratory where they were analyzed within one hour from the time of collection. Pre-isolation enrichment of the faecal samples were carried out by inoculating each sample directly in to tryptone soy broth (TSB) and incubated at 35°C for 18-24 hrs. Immediately after enrichment, the organisms were serially diluted from $10^{-1}$to 10^{-9} and the dilutions 10^{-4} to 10^{-6} were plated on to Xylose lysine decorboxylase media (XLD) agar it was inoculated at 35°C for 24 hours onto XLD agar plate for the isolation of strains of *Salmonella* spp. respectively. The individual colonies with different morphology were picked using sterile tooth pick and grown in Tryptone soy broth and it was incubated at 37°C for 24 hours. Further it was plated to check for purity.

The isolated bacteria were primarily identified on the basis of the Gram staining, IMViC, Citrate Utilization, Triple sugar Iron agar, Nitrate reduction, Motility, Catalase, Oxidative. All *Salmonella typhi*. Strains used in this study were grown in TSB broth at 37°C for 24 hours. The following antibiotic discs: Amikacin, Co-trimoxazole, Ciprofloxacin, Tetracycline, Cephalothin, Ceftriaxone, Entrofloxacin, Gentamicin, Ampicillin, Trimethoprim, Cefoxitin were used for antibiotic sensitivity assay.

Selected colonies were inoculated into nutrient broth then incubated at 37 °C for 12 hrs. These cultures were used for further experiment.In this present study antibiotic susceptibility of *Salmonella typhi* was performed using Kirby Bauer disc diffusion method (1979). Plasmid were isolated from *Salmonella typhi* using the method of alkaline lysis (Niels, 1994) and the presence of plasmid was checked by 0.7% agarose gel was with visualized under UV light on transilluminator and photographed. Size of the plasmids was determined with the help of the standard molecular marker.

The antibacterial activity of the ZrO_2 nanoparticles was performed by using well diffusion method. About 20 ml of sterile molten Mueller Hinton agar was poured into the sterile petriplates. Triplicate plates were swabbed with the overnight culture (10^8cells /ml) of pathogenic bacteria S*almonella* spp. Different concentration of nanoparticles (50µl, 100µl, 150µl) was prepared with DMSO. The different concentrations of nanoparticles were screened against fifty isolates of *Salmonella* spp. The isolates were selected on the basis of *Salmonella* spp. should above 50% antibacterial activity against the antibiotics tested: Amikacin, Co-trimoxazole, Ciprofloxacin, Tetracycline, Cephalothin, Ceftriaxone, Entrofloxacin, Gentamicin, Ampicillin, Trimethoprim, Cefoxitin. The solid medium was gently punctured with the help of cork borer to make a well. Finally the nanoparticle samples with the concentration: 50µl, 100µl, 150µl were added from the stock into each well and incubated for 24h at 37±2°C. After 24 hrs of incubation, the zone of inhibition was measured and expressed as millimetre in diameter.

RESULTS AND DISCUSSION

Totally 75 samples were collected from different poultry retail shops in Tirupur city. 50 isolates of *Salmonella typhi* were isolated from the samples. The *Salmonella typhi* strains were confirmed by comparing the results

PLATE 1. Isolated colonies of *Salmonella typhi*

with standard biochemical test of *Salmonella typhi* such as gram negative in rod shape as result of gram staining. Indole negative, MR-VP- positive, Voges Proskauer – negative, Citrate positive, positive results were observed in case of Catalase, Nitrate reduction, Motility, Triple sugar iron agar, Oxidase. Selective media like XLD (Plate: 1) and Mac-Conkey agar media were used to isolate the *Salmonella typhi*. It showed black centre colony and white colony respectively. These colonies were isolated and stored for further experiment.

Antimicrobial susceptibility patterns were determined by using commercial antimicrobial disc (HIMEDIA, Mumbai): Amikacin (10mcg), Cotrimoxazole (25mcg), Ciprofloxacin (10mcg), Tetracycline (30mcg), Cephalothin (30mcg), Ceftriaxone (30mcg), Entrofloxacin (10mcg), Gentamicin (10mcg), Ampicillin (10mcg), Trimethoprim (10mcg), and Cefoxitin (30mcg). Antimicrobial susceptibility testing was performed in accordance with the standard guidelines of Kirby – Bauer (1979) disc diffusion method.

Totally 11 antibiotic discs were used for this assay, among that strain CH12 showed maximum resistance of 72.72% and the antibiogram AK- TET- COT- CTR- CEP- GEN- AMP-TR was recorded. Strain CH32 showed minimum resistance of 9.09% and the antibiogram TR was recorded (Plate: 2). The isolates were analyzed for antibiogram as described to determine the antibiotic susceptibility pattern along with the tendency of current resistance against widely used drugs. Among 50 isolates, 30 different antibiogram were found in this study and the resistance was found against Amikacin (12%), Cotrimoxazole (62%), Ciprofloxacin (28%), Tetracycline (80%), Cephalothin (12%), Ceftriaxone (4%), Entrofloxacin (6%), Gentamicin (26%), Ampicillin (36%), Trimethoprim (100%), and Cefoxitin (8%) (Table: 1).

Five strains CH12, CH22, CH23, CH36 and CH37 showed more than 50% percentage frequency among the fifty isolates of *Salmonella typhi*.

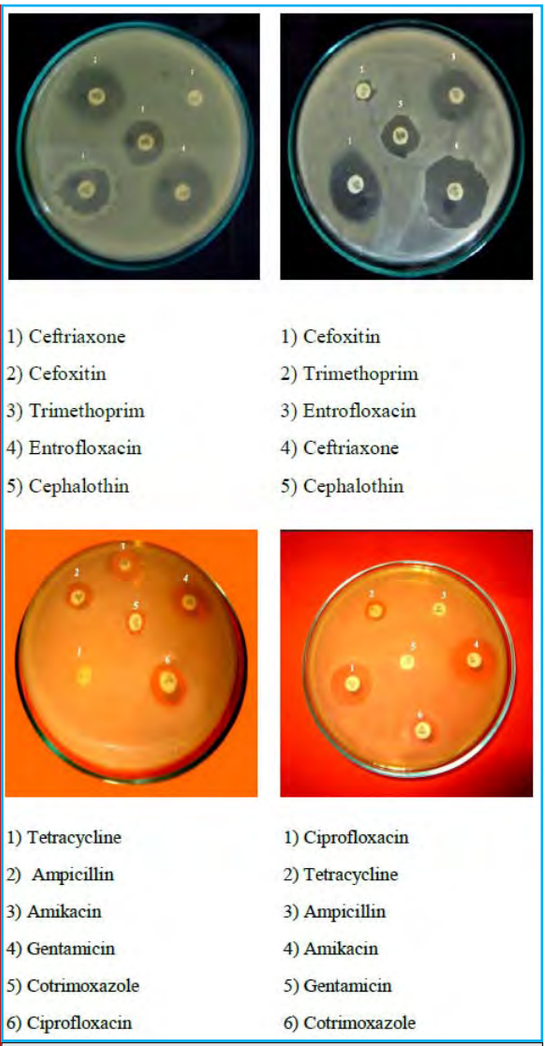

PLATE 2. Antibiotic susceptibility test of *Salmonella typhi*

$$\text{MAR index for isolates} = \frac{\text{No. of antibiotics to which isolate is resistant}}{\text{No. of antibiotics} \times \text{No. of isolates}}$$

$$\text{MAR index for isolates} = \frac{\text{No. of antibiotics resistant to the isolates}}{\text{No. of antibiotics} \times \text{No. of isolates}}$$

Multiple Antibiotic Resistance (MAR) index was calculated according to the formula

Maximum MAR index 0.7272 was showed by CH12 and minimum MAR index 0.0909 was showed by CH32. Strains which showed more than 50% resistance was taken for isolation of plasmid, by the following method of Niels (1994), two fragments were obtained from the strains: CH12, CH22, CH23, CH36, and CH37, but all the strains were plasmid born *Salmonella* spp. 100bp DNA ladder (MEDOX, Chennai), was used to know the

Table 1. Antibiotic Resistant Percentage of *Salmonella typhi*

S: No	Antibiotics	Percentage of resistant
1	Amikacin	12%
2	Ceftriaxone	4%
3	Ciprofloxacin	28%
4	Cephalothin	12%
5	Entrofloxacin	6%
6	Gentamicin	26%
7	Ampicillin	36%
8	Cefoxitin	8%
9	Tetracycline	80%
10	Trimethoprim	100%
11	Co trimoxazole	62%

molecular weight of the strain, it showed 1500 bp and 700 bp (Plate:3). The medical application of nanoparticles is gaining popularity with an increasing number of nanoparticle based therapeutics currently in clinical development. We expect that with the introduction of safer nanomaterials together with novel engineering approaches that result in optimally designed nanoparticles, enter the clinic in future.

Different concentration of nanoparticle Zirconium oxide 50µl, 100µl, and 150µl were prepared with Dimethyl sulphoxide (DMSO), well diffusion method was used; Different concentration of nanoparticle were impregnated into well on the seeded Mueller Hinton Agar (MHA) media. The plates were incubated at 37°C for 24hrs. Zone

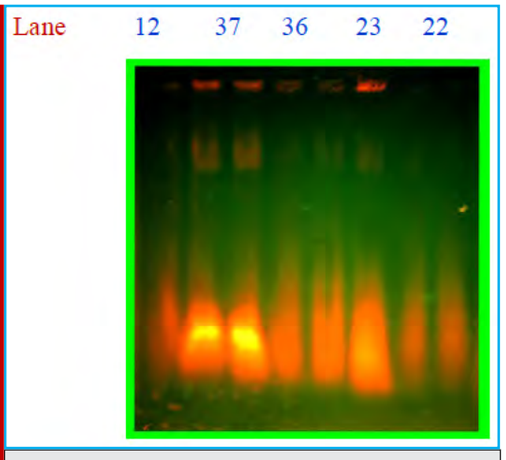

PLATE 3. Isolation of plasmid from *Salmonella typhi*

PLATE 4. Activity of Zro_2 Nanoparticle against of *Salmonella typhi*

of inhibition was recorded (Plate 4). The strains CH12, CH22, CH23, CH36 and CH37 which showed more than 50% resistant against 11 antibiotics were used to test against Zirconium oxide Nanoparticle. *Salmonella* spp.

Among the three concentrations of nanoparticles tested against five strains, maximum zone of inhibition (16mm) was observed against CH36 at 150µl, followed by 15mm against CH23 at 150µl. The minimum zone (13mm) was recorded in the strain CH37. Although in many areas of endemic city in Asia and the Indian subcontinent typhoid outbreaks in Sub-Sharan Africa are rarely documented, and data on incidence and antimicrobial susceptibility patterns are scarce. The observed rise in MDR *S.typhi* in Kenya is particularly alarming. For example during the period of their study, most of the *S.typhi* isolates from blood culture of patients prior to 1993 were fully sensitive to all antimicrobials (Bhay et al. 2005). But in the present study the strain CH12 showed maximum resistance 72.72% against all the antibiotics tested. *Salmonella typhi* recorded 100% resistance to tetracycline, 66.7% resistance to gentamicin and ampicillin respectively. These antibiotics are very common and are readily available as over the counter drugs to consumers in Nigeria (Funso Omojaasola and Folakemi Omojaasola, 2001). 80% of resistance showed by tetracycline, 26% resistance to gentamicin, and ampicillin showed 36% of resistance in the present study.

The resistance pattern of 101 strains of *S.typhi* to 11 drugs was determined by using the plate dilution method. All 101 strains tested were inhibited by Cephalothin; Gentamicin, (Olarte and Galindo, 1973), similar method was followed in the present investigation, 50 strains of *S.typhi* isolated were inhibited by Cephalothin (12%) and Gentamicin (26%).A total of 323 *S.typhi* isolates from three hospitals covering the Nairobi region of Kenya, 54 (16.7%) isolates were fully susceptible to all eight antibiotics tested, (Kariuki et al. 2010), 50 strains of *S.typhi* isolated from retail poultry shop in around Tirupur, in all isolates (2%) was fully susceptible to all eleven antibiotics tested. Further the authors were reported that

a total of 74 (22.9%) isolates were resistant to ampicillin or tetracycline, similar results was recorded in the study but total of fifty isolates showed 36% and 80% resistant to Ampicillin and tetracycline respectively.

One hundred and thirty two (132) bacteria were isolated from 1000 cow dung samples, among that 18 isolates of *Salmonella typhi* were cultured. 100% resistance to tetracycline by all the isolates, ampicillin showed 85.6% resistance (Omojowo and omojasola, 2013), in the present research work 75 faecal samples of retail poultry shop, were collected among them, 50 isolates of *Salmonella typhi* were isolates, ampicillin showed 36% resistance against all tested antibiotics and tetracycline showed 80% resistance.Six strains of *Salmonella typhi* were resistant to ampicillin, trimethoprim, tetracycline and gentamicin, these strains were isolated from the blood with typhoid patients. These strains showed multiple antibiotics resistance (MDR) and all the six strains were harbored plasmid about 50 kb (Pai et al. 2003). Similar results was obtained, fifty strains of *Salmonella typhi* were showed resistant to all tested antibiotics expect trimethoprim and all strains showed multiple antibiotic resistant. These strains were harboured plasmid about 1500 bp and 700 bp.

Hirose et al. (2001) focused the antibiotics susceptibilities of 62 strains of *S.enterica serovar typhi* of *S.enterica serovar paratyphi* were investigated with 18 antibiotics. Eighteen *S.enterica serovar typhi* isolates and five *S.enterica serovar paratyphi* A isolates were resistant to one or more antimicrobial agents among which 10 *S.enterica serovar typhi* isolates were susceptibility against ciprofloxacin. But our findings showed the isolates *Salmonella typhi* were showed antibiotics susceptibility against all tested antibiotics except trimethoprim, and these strains were resistant to one or more antibiotics.

Sisak et al. (2006) isolated 126 *Salmonella* spp from pigs were tested against 14 antibiotics. They found that the isolates showed resistance 1-8 antibiotics, *S.typhimurium* strains were found to the most resistant to streptomycin (91.5%), sulphonamides (88.1%), ampicillin (86.4%), and chloramphenicol (83.0%), in the present study, eleven antibiotics were tested against the isolates, among that ampicillin showed lesser percentage of inhibition while compare with work done by Sisak et al. (2006), but it showed 36% resistance only. Ashok et al. (2010) reported that they collected data for 2007 and 2008, ampicillin showed 18.2% and 52.8% of resistance against *Salmonella typhi,* there as tetracycline showed resistance of 9% and 33.4%, none of the antibiotics resistance against ciprofloxacin and ceftriaxone antibiotics, there uses controversy while compare the results with present study, tetracycline showed second highest resistance (80%), ciprofloxacin 28%, ceftriaxone 4% showed resistance.

The antibacterial potential of metal oxide nanoparticles viz, Al_2O, Fe_3O4, CeO_2, ZrO_2 and MgO against poultry pathogens: *Klebsiella* spp., *E.coli, Staphylococcus* spp. *and Salmonella* spp. The ZrO_2 showed maximum antibacterial activity against *Salmonella* spp. followed by *E.coli* respectively. The author reported in their study that, the ZrO_2 nanoparticles could be used as effective antibacterial agent against poultry pathogens. In the present investigation, ZrO_2 was used as a nanoparticles against the *Salmonella* spp isolates which is isolated from retail poultry shop. Totally five isolates CH12, CH22, CH23, CH36, and CH37 of *Salmonella typhi* were tested against different ZrO_2 concentration (50µl, 100µl, and 150µl), as the zone of inhibition were rapidly increased from 50µl - 150µl concentration of nanoparticles. Among five isolates tested, CH36 showed maximum zone of inhibition (16mm).

Mrithunjai singh et al. (2008) found that the nanoparticles increase chemical activity due to crystallographic surface structure with their large surface to volume ratio. They used silver ions and silver based compounds including silver nanoparticles, there has promoted research and this effect was size and dose dependent and was more pronounced against Gram – negative bacteria than Gram – positive organisms, in the present investigation, ZrO_2 was used as a nanoparticles, this showed maximum activity, the activity was dependent on dose.

CONCLUSION

Development of resistance to antibiotics by bacteria is inevitable, not only because of their rates in mutation and transferability of drug resistant genes. This constitutes a significant public health risk due to possible cross-contamination with antibiotic resistant bacteria of food and drinking water meant for public consumption, which always culminates in human illnesses, mostly typhoid fever. The growing incidence of multi-drug resistant *Salmonella typhi* has become a global phenomenon and antibiotic resistant bacteria are increasingly isolated from a wide array of sources, in the clinical environments, poultry. There is some scientific evidence of the growing rate of recovery of antibiotic resistant *S. typhi* from poultry products. So It is concluded from the present study that species of *Salmonella typhi* isolated from retail poultry shop in around Tirupur Dt. The rapid emergence of drug resistant strains of microbial *Salmonella typhi* pathogen especially those with multi drug resistance characteristics and the organism link with a plasmid. Due to the development of drug resistant urgently need new therapeutic among drug to combat the infectious disease. So in this study the biomedical properties of metal oxide nanoparticles was used in different concentration to treat against multidrug resistant *Salmonella typhi*.

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Dimentional accuracy of intraoral and laboratory scanners: A literature review

Iman Shafiei[1], Mehran Bahrami[2] and Saied Nokar[3]*

[1]*Prosthetic Department, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran*
[2]*Assistant Professor, Dental Research Center, Department of Prosthodontics, School of Dentistry, Tehran University of Medical Sciences, North Amirabad Ave, Tehran, Iran*
[3]*Associate Professor, Department of Prosthodontics, Dental Research Center, Tehran University of Medical Sciences, Tehran, Iran*

ABSTRACT

Restoration of dental implants remains one of the most challenging aspects of implant dentistry. Although it is not clear whether prosthetic misfit could affect osseointegration, mechanical complications of implant-supported prostheses can be avoided by achieving a good passive fit between the framework and the implants. Passive fit is a difficult concept to define. Obtaining absolute passive fit of the prosthetic framework on implants has been reported to be nearly impossible. Dimentional accuracy of intraoral and laboratory scanners play deniable role on producing desirable restorations. So, the aim of the current research was to determine dimentional accuracy of intraoral and laboratory scanners using the PubMed and Medline database English literature by the terms “Dimentional accuracy”, “Intraoral”, Laboratory scanners”.

KEY WORDS: DIMENTIONAL ACCURACY, INTRAORAL SCANNERS, LABORATORY SCANNERS, DIGITAL IMPRESSION, TRUNESS, PRECISION, CAD CAM PROSTHODONTICS

INTRODUCTION

Technique of computer aided design and computer aided manufacturing (CAD/CAM) is used to produce ceramic restorations such as all-ceramic crowns and fixed dental prostheses since decades ago (Su et al. 2015). Numerous CAD/CAM systems are capable of designing and fabricating prostheses on plaster cast made from conventional silicone impressions (Mo¨rmann, 2006). Non-standard operation during impression taking and deformation of clinical material will affect the accuracy of plaster model, consequently affecting the accuracy of three-dimensionalmodels (3D) model data and prostheses quality (Stimmelmayr et al. 2012). So, it is desirable

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to develop a facility which can take digital impressions directly from oral cavity to eliminate error and also economize on impression materials used in conventional impression procedures. The primery digital intraoral impression system commercially available was CEREC 1 system (Rekow, 2006). Laboratory digitizing starts with a conventional impression that is poured, and the leads model is digitized, using one of numerous optical or mechanical systems (Beuer et al. 2004). Also, some systems offer the possibility to scan the impression directly without cast fabrication (Güth et al. 2013).

In addition, discomfort for the patient like sweating, gagging, pain and partially inconvenient taste is a known issue associated with conventional impression taking. In these situations, this instability and discomfort factor might be avoided by direct data capturing, which represents a logical direct access to dental CAD/CAM (Steinhäuser-Andresen et al. 2011). To date few published literature exists on the performance of digital intraoral impression system, especially concerning the accuracy and precision of intraoral scanners. So, the aim of the current research was to determine dimentional accuracy of intraoral and laboratory scanners.

MATERIAL AND METHODS

The keywords used for the literature search for this review was peer-reviewed articles following key-words: Dimentional accuracy × Intraoral scanners and Laboratory scanners. Among them, the papers were fit the criteria selected and available full-text articles read. Related articles were also scrutinized. Hand search was also driven. The search was carried out using Biological Abstracts, Chemical Abstracts, and the data bank of the PubMed and Medline database updated to 2017. The references found in the search were then studied in detail.

HISTORY OF DIGITAL SYSTEMS IN DENTISTRY

To achieve a correct adaptation between the prosthesis and the implants, the first step is to obtain a highly accurate impression. Many clinical factors affect the accuracy of the impressions, such as tray type, impression technique, impression material used and its particular hydrophobic or hydrophilic characteristics, mixing methods and impression disinfection. Impressions can be made at either the implant level or the abutment level (Giménez et al. 2015). Computer-aided design/computer aided manufacturing (CAD/CAM) systems have evolved over the last two decades and have been used by dental health professionals for over twenty years (Duret et al. 1998). Francois Duret introduced CAD/CAM in restorative dentistry (Priest, 2005). One of the main lines of implementation was the intraoperative use for dental restoration using prefabricated ceramic monoblocks (Mörmann, 2004). Dental CAD/CAM's evolution over the past30 years has centered on the chairside market, beginning with CEREC® (Sirona). This is in part because the appeal of the CAD/CAM concept is that it offers dental professionals and their patients the convenience of same-day dentistry (Davidowitz and Kotick, 2011). A further development in CAD/CAM technology is the transition from closed file format to open access file systems, which opens up access to a much wider range of manufacturing technology such that the most appropriate manufacturing processes and associated materials can be selected (van Noort, 2012).

The CAD/CAM systems have been used mostly for the manufacturing of prosthetic fixed restorations, such as inlays, onlays, veneers and crowns. During the last decade technological developments in these systems have provided alternative restorations using different materials such as porcelain, composite resin and metallic blocks, which could not be prosecuted previously because of technical limitations. To date several optical impression systems have been developed with which direct impressions could be made in the oral cavity including Cerec AC (Sirona, Behnheim), Lava Chairside Oral Scanner (Lava COS, 3 M ESPE), E4D Dentist (D4D Technologies, LLC) and iTero (Cadent, Carlstadt) (Giménez et al. 2015). Despite numerous advantages introduced for dental implants, several challenges, since excellent accuracy is a prerequisite to achieve proper fit of the subsequent prosthesis (Giménez et al. 2014). There is scarce information on the accuracy of intraoral digital impression systems for dental implants including the implant-related factors and other clinical aspects such as the experience of the operator.

APPLICATION OF SCANNERS IN DENTISTRY

For the acquisition of digital images of teeth, different procedures have been described: digitization of plaster casts, digitization of impressions, and intraoral digital impressions (Morris et al. 2010). Digital work flow has been proposed to improve treatment planning, give higher efficiency, and allow new methods of production and new treatment concepts (Galovska et al. 2012). Data storage and reproducibility are facilitated, and treatment documentation and communication between professionals as well as between dentists and patients have become more convenient (Al Mortadi et al. 2012). Currently, there are a major digital impression devices: iTero (Align Technologies, San Jose, Calif), Lava COS (3M ESPE, Seefeld, Germany), and Trios (3Shape, Copenhagen, Denmark) for image acquisition; and CEREC AC (Sirona, Bensheim, Germany) and E4D (D4D Technologies, Richardson, Tex) for digital imaging and in-office manufacturing (Flugge et al. 2013). All scanning devices

need drying and powdering of intraoral surfaces (CEREC, E4D, Lava COS). Also, digital impressions are acquired without contact to the gingival tissues (Ender and Mehl, 2011). Direct acquisitions systems have been constantly improved because this are less invasive, quicker and more precise than the conventional methods. Besides the digital image can be easily store for a long time (Ramsey and Ritter, 2012).

TYPES OF INDIRECT IMPRESSIONS

Lost-wax

Lost-wax is the traditional technique for fabricating the metal substructure is the lost-wax technique and using various metal alloys for casting (Ucar et al. 2009). Conventionally, wax patterns were fabricated with wax and waxing instruments for example the popular PKT instruments. Wax is used to make the patterns because it can be conveniently manipulated, precisely shaped and can also be completely eliminated from the mold by heating. The fabrication of the wax pattern is the most critical and labor-intensive step in making the porcelain fused-metal crown (Vojdani et al. 2013). To fabricate a restoration prepared using the lost-wax technique, the dentist must first make an impression and the impression appointment may be uncomfortable for the patient because of the retraction procedure and need for anesthesia. Subsequently, time is required by the dental laboratory technician for careful pouring of the stone die or cast from the impression, preparation of the cast, then fabrication of the wax pattern, investing, and casting. Considering the lower unit cost of base metal alloys, a more economical dental laboratory technique would be helpful to replace the previously described technique for preparing cast restorations (Ucar et al. 2009).

Selective laser sintering (SLS) is a manufacturing technology recently introduced in dentistry. SLS, is one of the fast prototyping production techniques, uses a high-temperature laser to beam selectively substructure metal powder based on the CAD data with the fixed dental prostheses design. A thin layer of the beamed area becomes burnt and the fixed dental prostheses is completed by laminating these thin layers. The metal-ceramic crown is formerly one of the most commonly used fixed dental prostheses and the lower core is mostly produced by the lost wax technique and casting method. However, SLS system has several benefits such as material, time and expenses saving was well as the production is simpler compared to the existing methods (Akova et al. 2008).

CEREC

With CEREC 1 and CEREC 2, an opticalscan of the prepared tooth is made with a couple charged device (CCD) camera, and a 3-dimensional digital image is generated on the monitor. The restoration is then designed and milled. With the newer CEREC 3D, the operator records multiple images within seconds, enabling clinicians to prepare multiple teeth in the same quadrant and create a virtual cast for the entire quadrant. The restoration is then designed and transmitted to a remote milling unit for fabrication. While the system is milling the first restoration, the software can virtually seat the restoration back into the virtual cast to provide the adjacent contact while designing the next restoration (Estafan et al. 2003).

DCS Precident

The DCS Precident system is comprised of a Preciscan laser scanner and Precimill CAM multitool milling center. The DCS Dentform software automatically suggests connector sizes and pontic forms for bridges. It can scan 14 dies simultaneously and mill up to 30 framework units in 1 fully automated operation. Materials used with DCS include porcelain, glass ceramic, In- Ceram, dense zirconia, metals, and fiber- reinforced composites. This system is one of the few CAD/CAM systems that can mill titanium and fully dense sintered zirconia (Sjogren et al. 2004).

Procera

Procera/AllCeram was introduced in 1994 and according to company data, has produced 3 million units as of May 2004. Procera uses an innovative concept for generating its alumina and zirconia copings. First, a scanning stylus acquires 3D images of the master dies that are sent to the processing center via modem. The processing center then generates enlarged dies designed to compensate for the shrinkage of the ceramic material. Copings are manufactured by dry pressing high-purity alumina powder (>99.9%) against the enlarged dies. These densely packed copings are then milled to the desired thickness. Subsequent sintering at 2,000°C imparts maximum density and strength to the milled copings. The complete procedure for Procera coping fabrication is very technique-sensitive because the degree of die enlargement must precisely match the shrinkage produced by sintering the alumina or zirconia (Posselt et al. 2003).

Lava

Lava introduced in 2002, Lava uses a laser optical system to digitize information from multiple abutment margins and the edentulous ridge. The Lava CAD software automatically finds the margin and suggests a pontic. The framework is designed to be 20% larger to compensate for sintering shrinkage. After the design is complete, a properly sized semisintered zirconia block is selected for milling. The block is bar coded to register the special design of the block. The computer- controlled

precision milling unit can mill out 21 copings or bridge frameworks without supervision or manual intervention. Milled frameworks then undergo sintering to attain their final dimensions, density, and strength. The system also has 8 different shades to color the framework for maximum esthetics (Bindl et al. 2004).

Everest

Marketed in 2002, the Everest system consists of scan, engine, and therm components. In the scanning unit, a reflection-free gypsum cast is fixed to the turntable and scanned by a CCD camera in a 1:1 ratio with an accuracy of measurement of 20 µm. A digital 3D model is generated by computing 15 point photographs. The restoration is then designed on the virtual 3D model with Windows-based software. Its machining unit has 5-axis movement that is capable of producing detailed morphology and precise margins from a variety of materials including leucite-reinforced glass ceramics, partially and fully sintered zirconia, and titanium. Partially sintered zirconia frameworks require additional heat processing in its furnace (Blatz et al. 2003).

Cercon

The Cercon System is commonly referred to as a CAM system because it does not have a CAD component. In this system, a wax pattern (coping and pontic) with a minimum thickness of 0.4 mm is made. The system scans the wax pattern and mills a zirconia bridge coping from presintered zirconia blanks. The coping is then sintered in the Cercon heat furnace (1,350°C) for 6 to 8 hours. A low-fusing, leucite-free Cercon Ceram S veneering porcelain is used to provide the esthetic contour. In an in vitro study the marginal adaptation for Cercon all-ceramic crowns and fixed partial dentures was reported as 31.3 µm and 29.3 µm, respectively (Ariko et al. 2003).

INTRAORAL AND LABORATORY SCANNERS

Several intraoral scanners have been introduced in the recent decades, and an increasing number of dental clinics have decided to adopt these powerful devices for capturing digital impressions (Mangano et al. 2016). Capturing of digital impressions of the dental arches using this system can done by only a light beam, without the need of individual trays and materials (alginate, silicone, polyether) that are traditionally used to take impressions (Logozzo et al. 2014). Because of the unpleasant procedure, especially for those with a pronounced gag reflex conventional impressions are generally not appreciated by patients (Zimmermann et al. 2015). The possibility to effectively replace conventional impressions is the main advantage of intraoral digital impressions, which leads to decrease materials costs (Yuzbasioglu et al. 2014). Immediate control of the quality of the impression, and the possibility of obtaining 3D which can be electronically sent to the laboratory is known as advantages for this system (Schepke et al. 2015).

Digital impression making has improved this process and the ability to evaluate the preparation in real-rime. Having the capability of acquiring a scan of a prepared tooth and visualizing it on a computer monitor eliminates the issues associated with conventional impressions. The dentist is now able to see a magnified high-resolution image of exactly what is present in the oral cavity and not just a negative representation. This improved visualization enables the dentist to see and evaluate, in exquisite detail, the quality of the preparations, while the patient is still in the chair. Factors such as preparation taper, quality of margins, undercuts, inter-occlusal clearance, and path of draw can be color-coded displayed and directly corrected if necessary, and a new digital impression can be made within seconds. All of the currently available conventional impression materials exhibit some degree of dimensional change that builds distortion and inaccuracy into the final restoration. Digital impressions can reduce the possibility of dimensional change (shrinkage) that is evident with all conventional impression materials. Voids, tears, and pulls that are routinely experienced with conventional materials are no longer an issue with digital scans (Gebhards et al. 2010).

Laboratory digitizing starts with a conventional impression that is poured, and the resulting model is digitized, by using one of several optical or mechanical systems (Beuer et al. 2008). As well, some systems offer the possibility to scan the impression directly without cast fabrication (Quaas et al. 2007). However, in either instance, the initial step of the highly precise digital workflow is an analogue impression. Conventional high precision impression materials, like hydrocolloid, polyether, polyvinyl or polysulfide in combination with stone casts, offer a well-known procedure to transfer the clinical situation into the laboratory. However, some drawbacks are related to the sensitive process steps of this technique (Haim et al. 2009).

The CAD-CAM system includes three parts, which correspond to the three basic steps of the process: (I) First, a device is used to input the existing dental shapes into the system. This device includes a laser source (diode) which, through the first endoscope, projects light on the desired picture area. A second endoscope, adjacent to the first, allows a camera to take pictures in the mouth. This camera is connected to a system that digitizes the information and correlates the different views (Duret et al. 1985). (II) The CAD system, including all necessary hardware and software, allows the operator to create an electronic model of the impression, display

it on the screen, an d use it to design the prosthesis. The CAD system is linked to a proprietary articulator, called the Access Articulator, which provides the data relating to the dynamic movements of the jaw. (III) The CAM system, which includes a numerically controlled machine tool with four-axis capability. This machine will automatically mill the prosthesis from conventional or special materials (Belser et al. 1985).

Comparison of the accuracy of direct and indirect

To date numerous researches have been done on comparison of the accuracy of direct and indirect dental scanners. However, proces and cones reported for each system, here we highlighted the most significsnt findings of the previous reports. For the precision of direct digital impression, Guth et al. (2013) compared the 3D average and standard deviations of intraoral digital impression (Lava Chairside Oral Scanner) with those of extraoral desktop scanner (Lava Scan ST laboratory scanner) in an in-vitro study where a molar and premolar were scanned. It revealed the 3D standard deviations of 19 µm for the intraoral digital scanner and 31 µm for the extraoral scanner. Ender and Mehl (2011) compared the trueness and precision of digital impressions of the full arch with those of conventional impressions using a reference scanner on a in-vitro model, and the results showed that precision was 61.3 ± 17.9 µm for conventional impression, 30.9 ± 7.1 µm for digital impression with the Cerec Bluecam and 60.1±31.3 µm for digital impression with Lava C.O.S.

Guth et al. (2013) found that digital impressions made using Lava C.O.S. were more accurate than a laboratory scan of a conventional impression and conversion to a digital file. Mangano et al. (2016) in research on trueness and precision of four intraoral scanners in oral implantology revealed in the partially edentulous maxilla, CS 3500 had the best general trueness (47.8 µm) and precision (40.8 µm), followed by Trios (trueness 71.2 µm, precision 51.0 µm), Zfx Intrascan (trueness 117.0 µm, precision 126.2 µm), and Planscan (trueness 233.4 µm, precision219.8 µm). With regard to general trueness, Trios was significantly better than Planscan, CS 3500 was significantly better than Zfx Intrascan and Planscan and Zfx Intrascan was significantly better than Planscan; with regard to general precision, Trios was significantly better than Zfx Intrascan and Planscan, CS 3500 was significantly better than Zfx Intrascan and Planscan and Zfx Intrascan was significantly better than Planscan. In the totally edentulous maxilla, CS 3500 had the best performance in terms of general trueness (63.2 µm) and precision (55.2 µm), followed by Trios (trueness71.6 µm, precision 67.0 µm), Zfx Intrascan (trueness 103.0 µm, precision112.4 µm), and Planscan (trueness 253.4 µm, precision 204.2 µm). With regard to general trueness, Trios was significantly better than Planscan, CS 3500 was significantly better than Zfx Intrascan and Planscan and Zfx Intrascan was significantly better than Planscan with regard to general precision, Trios was significantly better than Zfx Intrascan and Planscan, CS 3500 was significantly better than Zfx Intrascan and Planscan and Zfx Intrascan was significantly better than Planscan. Local trueness values confirmed these results (Mangano et al. 2016).

On Precision of intraoral digital dental impressions with iTero and extraoral digitization with the iTero and a model scanner Flugge et al. (2013) reported scanning with the iTero is less accurate than scanning with the D250. Intraoral scanning with the iTero is less accurate than model scanning with the iTero, suggesting that the intraoral conditions (saliva, limited spacing) contribute to the inaccuracy of a scan. For treatment planning and manufacturing of tooth-supported appliances, virtual models created with the iTero can be used. An extended scanning protocol could improve the scanning results in some regions. In a study on accuracy 3M Lava C.O.S., 3Shape D900, Cadent iTero, CEREC Bluecam, and E4D Dentistdigital impression systems revealed digital impressions from the Cadent iTero system were the most accurate (Ali, 2015).

Recently, on comparison of repeatability between intraoral digital scanner and extraoral digital scanner, Su et al. (2015) revealed precision decreases with the increased scanning scope. Precision was clinically acceptable when scanning scope was less than half arch. Precision of extraoral scanning was acceptable in scanning any scope of arch region. Güth et al. (2013) revealed the direct digitalisation with Lava C.O.S. showed statistically significantly higher accuracy compared to the conventional procedure of impression taking and indirect digitalisation.

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The production and evaluation of biologically synthesized anticancer Chlorambucil – DTPA – Methionine

Elham Poonaki[1], Leila Pishkar[2], Mahdyeh Zarandooz[3], Hadi Hejazinia[3], Marzyeh Hosseini[4], Artin Assadi[3], Mohammad Zahedi[3] and Shahin Hadadian*[5]

[1]*Department of Biotechnology, I.A.U of Damghan, Damghan, Iran*
[2]*Young Researchers and Elite Club, Islamshahr Branch, Islamic Azad University, Islamshahr, Iran*
[3]*Department of Radiopharmacy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*
[4]*Department of Cellular and Molecular Biology, Islamic Azad University, Tehran North Branch,Tehran, Iran*
[5]*Nanobiotechnology Department, Pasteur Institute of Iran, Tehran, Iran*

ABSTRACT

Chromabucil is a direct acting direct acicular anticancer drug which is still widely used in the treatment of some cancers as a primary treatment, but its use is often limited due to the unwanted side effects of this drug due to the lack of specificity in targeting cancer cells. In this project, our effort to increase the specificity of Chlorambucil using methionine amino acid has led to the production and evaluation of biological antimicrobial Nano conjugate Chlorambucil-DTPA-Methionine. Research has shown that the consumption and harvesting of cancer cells increases significantly over nitrogen and polyamide compounds, while some amino acids, including methionine amino acids, are more expressed on the surface of the cancer cells, resulting in tissue Cancer cells increase to methionine amino acid, so in our study, we have been using methionine amino acid as a carrier and enhancer of the uptake of Chlorambucil antimicrobial to produce this conjugate from the connector We used DTPA. After studying this Nano conjugate and examining its structure, we investigated the therapeutic and biological effects of this nanoagonergic drug as compared to Chlorambucil on the MCF-7 and HT-29 cell line (breast cancer and colon cancer), including The MTT assay tests the determination of cell death and cell necrosis and a test for the determination of conjugate toxicity on mice, which ultimately led to the understanding that the new nano conjugate Chlorambucil-DTPA-Methionine not only retained its anti-cancer properties against the Chlorambucil drug But has shown less abnormal toxicity.

KEY WORDS: BREAST CANCER / CHLORAMBUCIL / METHIONINE / DTPA / CONJUGATE

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INTRODUCTION

Since cancer is a fatal illness worldwide, it is the second leading cause of death in the world after cardiovascular disease and, according to annual reports, causes many deaths from cancer. Today's treatments are very costly and have unpleasant side effects in the patient's body. Today, efforts are being made to find newer and more effective treatments, including these treatments, for chemical treatments. Nowadays, there is a lot of research to discover new drugs, appropriate drug delivery routes, and optimal drug targeting with less side effects. Recently, therapeutic studies have been conducted on the transmission of antibody-dependent drugs, but since the therapeutic method of drug delivery using antibodies also has inefficiencies and transitional problems, costs and side effects, in particular, itself, a newer research for the transfer of anticancer drugs is taking place. Since then, research has shown that the consumption and removal of cancer cells increases significantly over nitrogen and polyamide compounds, and the expression of some amino acids such as methionine amino acids on the surface of the cancer cells is more pronounced and therefore cancer tissue cells to increase the amino acid methionine, (Levine et al., 2000, Palmer et al. 2009, Roché et al. 2011).

In this study we have used methionine amino acid as carrier and antimicrobial agent for the treatment of Chlorambucil. Cancer treatment as an anticancer drug, like other anticancer drugs, has side effects on the cancerous patient's body, the reason is that by designing an anticancer drug conjugate using methionine amino acid (as an enhancement of cellular uptake and DTPA interface and conducting biologic studies of the effect of this conjugate on cancer cells through drug delivery to reduce side effects and Increase the efficacy of Chlorambucil to as an appropriate drug delivery method, we tried to do that. So far, hybrids have been made of non-interfacing Chlorambucil, such as Chlorambucil glucose and Chlorambucil –tyrosine (Gupta A et al. 2010).

But the proper interface for efficient drug delivery is very important. We have been working to produce this conjugate for the amino acid linkage of methionine We used the DTPA connector for Chlorambucil amine, which looks very good interface due to amine groups, because amine receptors from cancer cells increase and drug delivery will be more successful. We then performed the relevant biological tests on the MCF-7 and HT-29 cell line breast cancer cells.

MATERIAL AND METHODS

Following chemicals were used in the study.Chlorambucil (Sigma-Aldrich, USA) USA • Cell line MCF-7 and HT-29 (Pasteue Institute of Tehran) • Sulfo-NHS (Sigma-Aldrich, USA) USA) • EDC (Sigma-Aldrich, USA) • PBS (Merck, Germany) • DMSO (Merck, Germany) • Sephadex G-10 Fine (Sigma-Aldrich) • Chloroform (Merck, Germany) • FCS (Seromed Biochrom, Germany)) • FBS (Merck, Germany) • RPMI medium (Sigma-Aldrich, USA) • Penicillin powder (Sigma-Aldrich, USA) • Streptomycin powder (Sigma-Aldrich, USA) • The TNF-alpha kit (The RayBio® Human TNF -alpha ELISA) Kitin AnnexinV-PI (BD Pharmingen, UK).

Combination or hybridization of an antimicrobial agent of Chlorambucil and methionine amino acid, produced by the interface of DTPA herein. 2.320 g of methionine (if used 3 mg of Chlorambucil) is added in 5-5 milliliters of water Soluble. 2. Add 1 mg DTPA to the solution and dissolve it by sterilizer. 3. After 1 to 2 minutes, add twice as much amino acid as EDC to the previous solution. To the product of the first stage after 1 to 5 minutes of production, 758 mg / kg of Chlorambucil is added. 2. To complete the Chlorambucil dissolution, dissolve it with sterilization. 3. The product is ready for the next reaction. Use of DSMO is due to fat-loving Chlorambucil (CBL) and DMSO helps to dissolve it and accelerate the reaction. At this stage, purification of the conjugated product is studied. The purity of the compound produced was investigated by TLC chromatography.

A 10 milliliter TLC solvent was prepared containing 3 milliliters of chloroform (nonpolar solvent to dissolve lipophilic compounds) and 7 milliliters of methanol (polar solvent to dissolve hydrophilic compounds) (30% chloroform and 70% methanol) TLC papers were cut in rectangles with dimensions, length 8-7 cm and width of 5-4 cm. Then 1 millimeter above the solvent line (the highest part of the paper that is placed inside the solvent), with the capillary tube, there were delicate spots spaced apart, which included, respectively, methionine, DTPA, Sulfur-NHS, conjugate synthesis They were Chlorambucil and EDC. Each paper was placed in a solvent containing a 45° angle inside the container and due to the evaporation of chloroform and methanol, as well as the toxicity of chloroform, it should be closed in a container. The required amount of 25-26 C° and the required time in this method is about 20 minutes. Be Due to the fact that all materials and stains are colorless, UV light was used to observe the movement rate of the material, and stains were observed in the range of 280-220. The patches created with DTPA, sulfur-NHS, EDC, and CBL stain were a blurry and sharp spot. By this method, the result was that the synthesis product, conjugate Chlorambucil-methionine-DTPA, was present in the fourth to seventh test tubes.

Mass Spectroscopy analysis was carried out to confirm methionine - DTPA- Chlorambucil

Conjugation. MTT is a colorimetric assay which is used to assess cell viability based on metabolic activity. This assay is based on reduction of yellow tetrazolium salt (MTT) to form dark colored formazan dye by dehydrogenase enzymes in metabolically active cells the cells (MCF7) and (HT-29) were brooded with different concentrations of methionine - DTPA- Chlorambucil (0.1, 0.2, 0.5 mg/ml and Chlorambucil 0.5 mg/ml and untreated cell (as a negative control) for 48h.. After incubation (48 h), 50 µl XTT detection solution was added to each well of 96-well plate and the plate is kept in the incubator for 2 h. The formazan dye is soluble in aqueous solution and can be measured by evaluating the absorbance at 450 nm using a spectrophotometer. The results were compared to the untreated control cells. Statistical data analysis was done using Prism and excels software (Microsoft Office 2013). For quantitative data analysis paired One Way ANOVA in case of cluster comparison, were applied. $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

In gel filtration chromatography, the solution was removed from the chromatography column with 1 ml (20 drops) in 20 separate test tubes. Then, each tube was subjected to thin layer chromatography (TLC) to verify the accuracy of the final product. On the TLC paper, one millimeter above the solvent line with a capillary tube, delicate spots were separated from each other, which consisted of methionine, DTPA, Sulfo-NHS, synthetic conjugate, Chlorambucil-methionine- DTPA, Chlorambucil (CBL), and EDC. The stain was then investigated by the UV lamp and the presence of the product in the fourth to seventh tubes was determined, and then the Rf of each compound was calculated according to the formula given in Chapter 2, as follows (Table 1)

LC / MC mass spectrometry (LC / MC) spectroscopy was used to study the molecular structure and identify the conjugated Chlorambucil -methionine DTPA. The results are presented Figure 1.The 1983 molecular weight spectrum shows well that the molecular weight of 1983 is exactly equal to the conjugate molecular weight, which is proof of conjugated synthesis. The syntactic conjugate molecule is well visible

In Figure 2 and 3 the results of the compound toxicity on the MCF-7 and HT-29 cell line respectively indicate that our combination at 5 mg / ml dose has a significant toxicity ($p < 0.05$), and this toxicity is completely equal to Chlorambucil. Making targeted drugs is a very important part of cancer treatment. The use of non-cancerous anticancer drugs causes various side effects in the patient. One of the oldest anti-cancer drugs is Chlorambucil, which belongs to the group of alkalizing drugs, its effects on the cells are non-specific and mechanically unknown and have many side effects for patients. The production of a molecular conjugate of Chlorambucil -methionine was performed with the goal of having anticancer properties and also acting purposefully. The ability to kill Conjugate Chlorambucil-Methionine-

Table 1. The values of Rf are calculated from the TLC paper from the distance that the material goes through to the distance that the solvent travels.

Material	Methionine	DTPA	Sulfo-NHS	CBL-Met DTPA	CBL	EDC
Rf	72.0	82.0	49.0	40.0	32.0	88.0

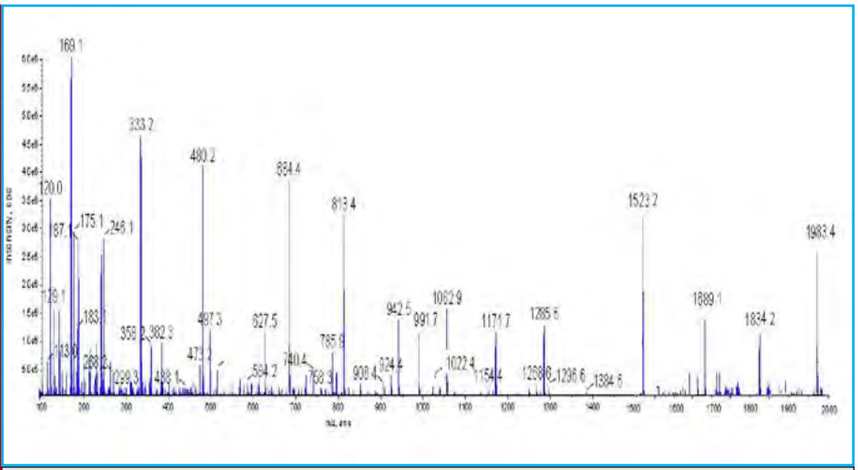

FIGURE 1. Mass Spectrum Display

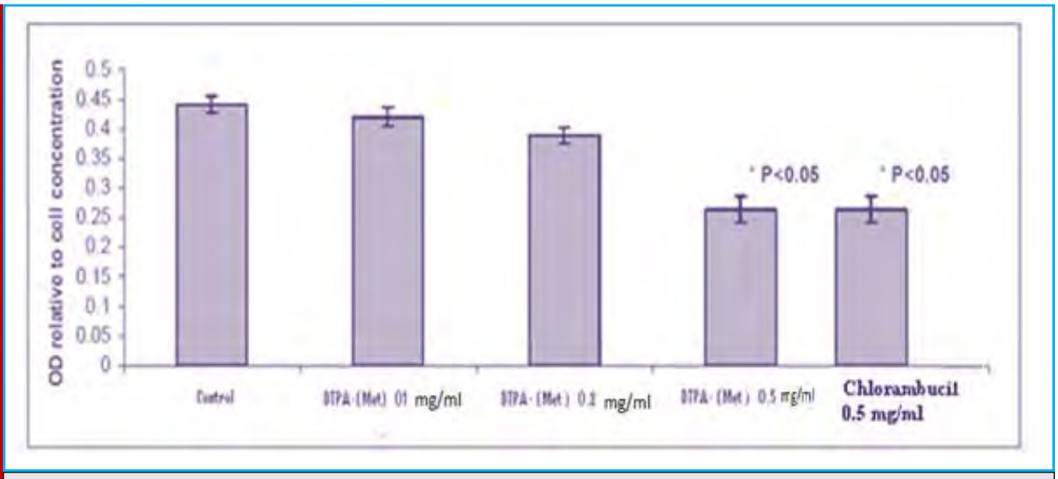

FIGURE 2. Showing the results of the MTT assay test on the MCF-7 cell line

DTPA in similar doses compared to Chlorambucil on the HT-29 and MCF-7 cell line was higher.

According to the results of experiments on conjugate and its comparison with Chlorambucil, the results were that: the binding of methionine amino acid to Chlorambucil not only does not eliminate the anticancer effect of this drug, but also increases its anticancer effect and increases the conjugated effect Because cancer cells tend to receive high amounts of polyamine compounds, the binding of the methionine amino acid to Chlorambucil will result in a targeted formulation that can absorb cancer cells of this conjugate. In 2004, according to Bothenichen's research on polyamine and cancer, it was discovered that the accumulation of polyamide compounds in cancerous tissues increased, as well as the concentration of these compounds in the body fluids of patients with cancer, (Buchrach et al. 2004) and in 2005 researchers it was found that the transfer of some essential amino acids in cancer cells increases, because in cells with high metabolism, the absorption of amino acids also increases. Therefore, using drugs reduces the synthesis of polyamine or inhibits the carriers of these amino acids, it can inhibit cancer, and these drugs can be a good option for cancer treatment (Yoon et al. 2005). In 2009, Palmer and colleagues in their research stated that increasing the concentration of polyamines plays an important role in the development of cancer, from the onset to the maintenance of the phenotype of the transformation, (Palmer et al. 2009).

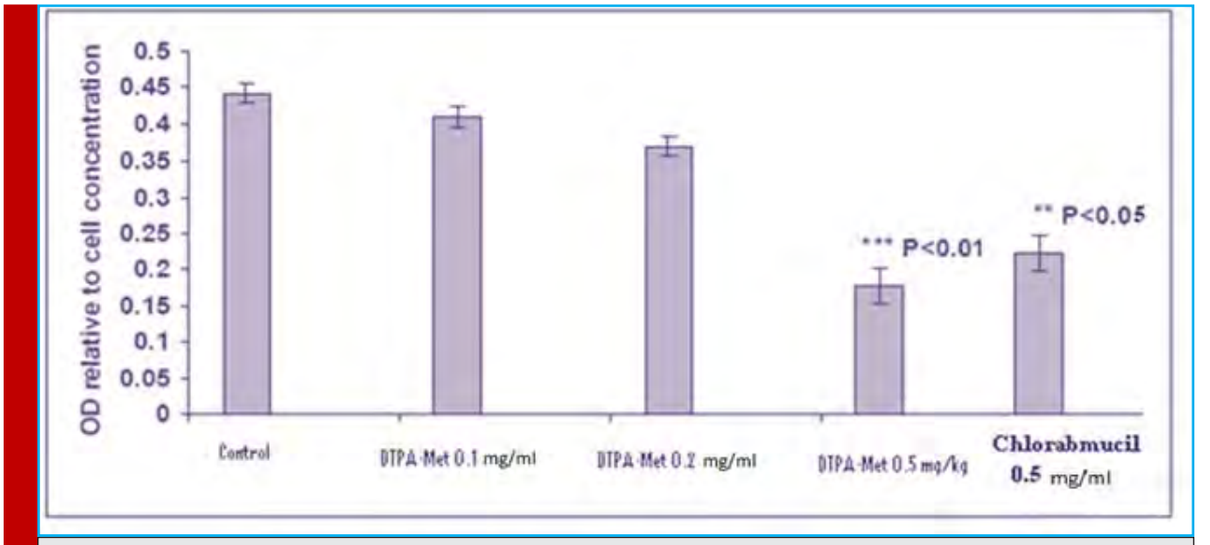

FIGURE 3. Showing the results of the MTT assay test on the HT-29 cell line

One of the ways of cancer cells to increase the concentration of polyamine compounds is the absorption of polyamines by the polyamine (PTS) transfer system in these cells. PTS is a rugged system that transmits a large amount of polyamines. Therefore, it may be possible to transfer cytotoxic drugs by binding to polyamine vesicles and using this system to target cancer cells selectively (Yoon et al. 2005). In 1997, the dependence of the growth of a number of solid tumors on high levels of methionine and the increased need for tumor cells to methionine were observed in relation to normal cells, which suggests that methionine can be considered as an appropriate choice for tumor treatment. (Buchrach et al. 2004). Conjugate Chlorambucil -methionine DTPA-D, due to its high chemical content, is considered as a polyamide compound and also has a methionine-like acid component in its structure, which is due to two reasons for its absorption in cancer cells. So far, much research has been done to transfer targeted drugs through specific molecules to tumors. The use of transfusion carriers and the manufacture of drug conjugates can have a more effective and specific effect on drugs. Due to the high side effects of Chlorambucil anticancer drugs and its nonspecific effects on the cells of the body, the purpose of this drug and its side effects have been many studies on Chlorambucil conjugation.

In 2010, they used Chlorambucil-estradiol for chemotherapy for breast cancer and found that they had better and better efficacy compared to Chlorambucil (Gupta et al. 2010). In 2011, researchers, given the frequent expression of receptors Folate (FRS) at the level of the malignant tumor cells synthesized two new conjugated folate carrying Chlorambucil, and they were biologically evaluated on the leukemia cell line. The results of the antitumor activity showed more conjugates compared to non-conjugated Chlorambucil (Guaragna et al. 2011).

By reviewing the articles, it was concluded that the synthesized conjugates of Chlorambucil had a positive effect and their effectiveness was much better than that of Chlorambucil, and these considerations made the conjugated Chlorambucil -DTPA-methionine II as a novel drug in The treatment of breast cancer has also been strengthened. Also, according to the aforementioned articles, the synthesis of conjugate Chlorambucil and the study of binding methods led to the conclusion that the binding of Chlorambucil to amino acids was carried out from its carboxyl side, not from the chlorine region. Because chlorine is a very important factor for killing cells by alkylating them. The carboxyl side of the Chlorambucil has a pharmacokinetic aspect (no dynamic aspect). Through this position, the drug enters the body, it traverses and excretes a pathway. Therefore, the manipulation and binding of the amino acid in this region, with the exception of the effect on absorption and its shelf-life in the body will not have an effect on inhibiting the effectiveness of the medicine.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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A novel method of cultivation of different varieties of tomato without using soil

B. Kiran Bala and R. Sasi Kumar

Department of Computer Science and Engineering K. Ramakrishnan College of Engineering, Trichy, Tamil Nadu, India

ABSTRACT

In our proposed system we have taken various varieties of tomatoes like Brandywine, Mortgage Lifter, Super Sweet 100 and Tiny Tim for cultivation by using the hydroponics technology. Closed hydroponic systems provide the same nutrient solution which is re-circulated and the nutrient concentrations are monitored and adjusted accordingly. In open hydroponic systems a fresh nutrient solution was introduced for each irrigation cycle. The big challenge behind the hydroponics technique was to maintain the nutrient levels in water periodically, for which sensors were used to monitor and ensure that water level and minerals level were in correct propositions as the sensor is very important criteria in hydroponics technique to grow the plants.

KEY WORDS: MINERALS, SENSORS, GERMINATION, COCO PEAT, SEEDS, NUTRIENT

INTRODUCTION

Day by day our environment is getting polluted not only air, noise, water even soil also is getting polluted due to real estate issues so many agriculture land has been converted into plots (Nguyen et al. 2016). Due to nuclear waste has been mixed in soil example in Japan still cultivating the plants in soil that particular vegetables, fruits those who are eating they still get affected by diseases (Alaeldin et al. 2017). There are several plants which can be grown in the hydroponics technique even single plant has several types of seeds and variety will be there. In our proposed system we have taken tomato plant to cultivate by using the hydroponics technology (El-Kazzaz et al. 2017).

To cultivate the tomato in different types of varieties but without soil to grow the plant due to the pollution in soil to overcome the problem and also to analyze different type of proposition of minerals added to plant and to see the variation in plant grows and taste as well as

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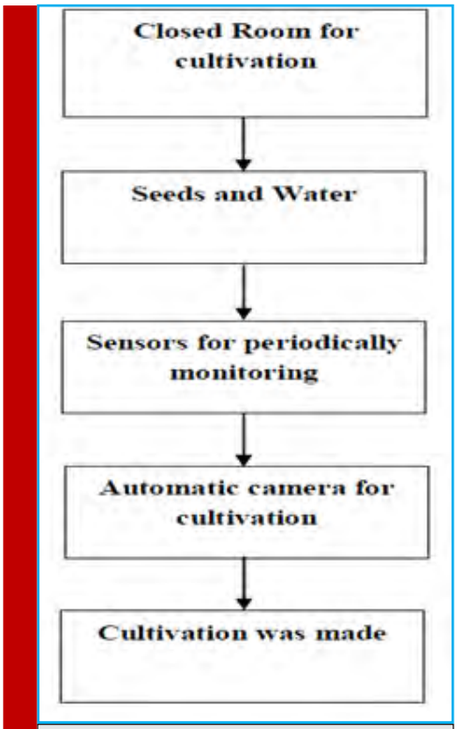

FIGURE 1. Diagram for the system

to analyze any side effect will come in this type of plant or not, (Charumathi et al. 2017).

Day by day our environment is getting polluted both in soil as well as water. In soil there are two types of problems firstly, most of the rural cultivation lands are converting into real estate plots and the second is pollution of left over soil with waste dumping of agrochemicals, pesticides, and other pollutants which are mixing in the soil destroying the agriculture, as plants do not grow properly and will produce the less fruits as well as vegetables, along with several diseases and toxicological manifestations. To overcome these problems recent focus is on cultivation of plants without soil which gives better solution to the problems, (Bala et al. 2017a). In this proposal there is no simulation required to grow the plants, within a room making green house effect using different seeds in different proposition and there by growing plants (Bala et al. 2017b).

The basic need for the seeds are nutrients as we have directly added the nutrients to water by recycling the process and less water with more yield is the concept in the entire process. To identify the nutrient is very difficult task in the entire process. There are 7 sensors, which have been used to identify the level of the nutrients in the water level as per the ratio indication is available in the sensor itself.

Selecting a room for growing plants. Selecting best seeds for cultivation and to design a sensor for implementation of this project. Using sensor concentration of the nutrient is identified; monitored and required feed is given for improving yield. By changing the variety of seeds as well as minerals/nutrient supplement for achieving result. There is a need of sensor to monitor the nutrient level in water so setup the minerals sensor is a major issue and to make automation of capturing

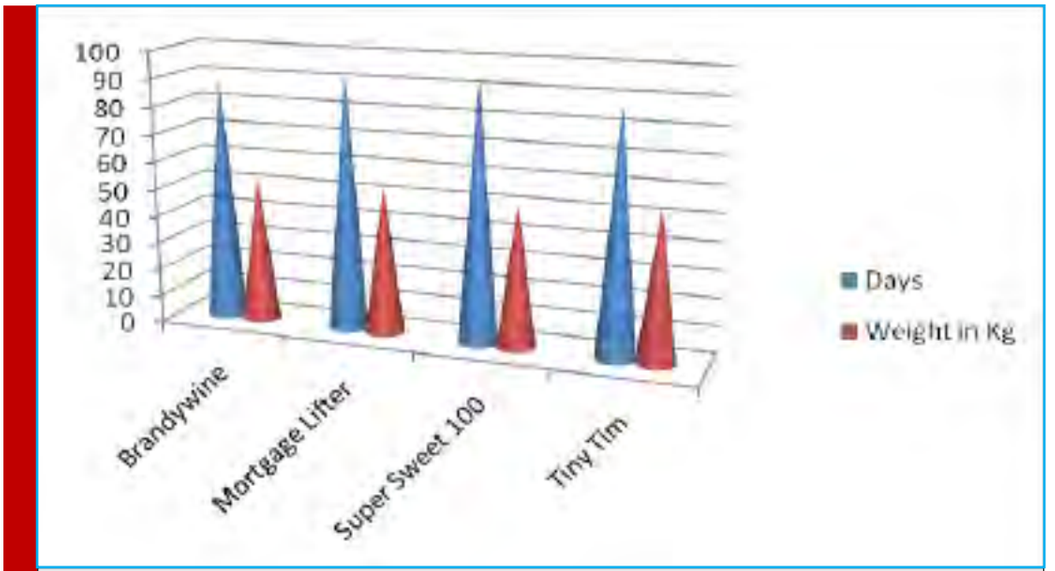

FIGURE 2. Showing yields of various tomato varieties in different days with their weight

the image is also a necessary think available and want to buy different types of seeds and in different environment along with necessary things. For establishing the experimental setup and to make the system automation there is a need of camera. To measure the minerals level there is a need for sensor which checks the nutrient level in the water to be pumped for growing the plants, also green house effect and electricity back up is mandatory for implementing this project. In the implementation part the entire process taken 95 days to cultivate the vegetables as per the proposed system. We have tried different types of seeds and total time taken for the yield was 95 days. In the closed system 10X10 SQ.M 53.33 Kg of tomato has been cultivated.

Growing the plants without soil as well as with low amount of water by recycling the same water and adding the nutrients directly to water and monitoring the nutrients level in water through sensor has been quite successful in the present study. In the proposed system same thing has been implemented but 7 sensors as well s different variety of tomato has been used for the cultivation.

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(2) Book: Falconer DC (1960) Introduction to Quantitative Genetics. Oliver & Boyd, Edinburgh 165-185.
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Name of Fellow/Member	Life Member, Fellow Society Science & Nature and Member BBRC	Designation and Address
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Dr. R. Khalique	BBRC	Department of Zoology, University of Kashmir, Srinagar
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