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Efficacy of an Alcohol - Free, Citrate Based Agent 'Clinister' as a Surface Disinfectant Against Common Bacterial and Fungal Contaminants

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Abstract

Surface disinfection is essential for the prevention of infection in the households, hospitals, laboratories and other high-risk areas. The current surface disinfectants in use such as Phenolic compounds, Quaternary ammonium compounds, Aldehydes, Sodium hypochlorite etc., have associated health hazards. Clinister is an alcohol-free, citric acid based disinfectant routinely in use for disinfection in the Food Industry in Japan. In this study, the efficacy of Clinister for surface disinfection was assessed by contact plate method in a microbiological lab set-up in India. The Clinister powder was mixed with water at appropriate concentrations and applied over the surfaces of instruments, tables and the floor employing standard disinfection protocols. The microbiological sampling was done by applying the contact plate with the surfaces before and after application of Clinister, following which the plates were incubated. After incubation for six hours, there was a significant reduction in the number of colony forming units (CFUs) of the organisms isolated from these surfaces which were *Staphylococcus spp., Bacillus spp., Micrococcus spp., Penicillium spp and A. fumigates.* Thus, use of Clinister as a surface disinfectant is recommended in hospitals, laboratories, households, child care centres, public areas, poultry etc., wherever the risk of spread of infection is high.

Keywords: Clinister, surface disinfectant, antimicrobial agent

Introduction

Surfaces are considered "non-critical" as they are not directly in contact with intact skin and they are debated to be not commonly associated with transmission of infections in hospitals and other critical areas (1). However, they become important in situations of outbreak and in case of preventive measures against transmission of infection (2). Several studies have demonstrated the importance of surface disinfection (3-5). The common disinfectants employed for such surface disinfection are the following materials either individually or in combination: Phenol, Cresol, Benzalkonium chloride, Cetrimide, Isopropyl alcohol, Dichloroisocyarurates, Quaternary ammonium compounds like Didicyldimethylammonium chloride, Glutarldehyde, Sodium bicarbonate, Hydrogen peroxide, Sodium hypochlorite etc.

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(6-8). However most of these disinfectants have disadvantages like dermatitis, utricaria and de-pigmentation of the skin associated with use of Phenol (which is commonly used in developing nations), reduced efficacy in the presence of organic matter, unpleasant smell, irritation to skin, eyes and mucous membranes and corrosiveness to metals associated with Chlorine, irritation to skin, eyes and air ways, allergic asthma and contact eczema with the use of Aldehydes. Even the widely used Quaternary ammonium compounds (9) have disadvantages like reduced efficacy in the presence of organic matter (10) and their wide spread use has in fact increased the resistance of gram negative organisms (11). At this juncture, Clinister which is an alcohol- free citric acid based disinfectant developed by Kubota *et al* came into use for disinfection in food industry in Japan (12,13). Herein we report the efficacy of the Clinister as a surface disinfectant using appropriate microbiological testing methods in a microbiological lab set-up in India.

Materials and Methods

Clinister (supplied by GN Corporation Co. Ltd., Japan through Nichi Vision Life Sciences, Chennai, India) is a food additive based disinfection agent containing Anhydrous citric acid, Trisodium citrate; Dehydrate cetylpyridinium chloride and Lactose compounded in ratios described earlier by Kubota *et al* (12,13). The efficacy of Clinister in surface disinfection was analyzed by the contact plate method before and after application of the Clinister over the surfaces of instruments, tables and clean room floor in the Department of Microbiology, Aurolab, Madurai, India. Two grams of the Clinister powder was mixed with 3.5 liter of drinkable water and this solution was applied on the surface of instruments, tables and was used for mopping on epoxy floor surfaces in that lab. This application was performed under the supervision of a microbiologist following the standard surface disinfection protocols.

Sampling device: The surface sampling was accomplished by the use of Contact plate method. (Surface contact area 28 cm^2). Contact plate is a plastic Petri dish (65 mm in diameter and 12 mm in height) pre -filled with Agar to give a convex surface with an area of 25cm^2 approximately and the diameter of the convex surface agar is 60 mm. The contact plate was filled with Soyabean casein Digest Agar with Lecithin and Polysorbate 80 (SCDA).

Sample collection method: The SCDA contact plate was applied to the surfaces with a uniform and steady pressure to the area to be sampled, without allowing circular or linear movement (Fig. 1). The contact plate testing was done before and after the exposure of the surfaces to Clinister. The sampled contact plates were closed and incubated at 30 - 35 °C. The growth in the SCDA plates was monitored after 3 days and further incubated for another 3 days at 20 - 25°C. This was done to revitalize the stressed bacteria which have been exposed to air and surface in order to achieve conditions where they can again form colonies. This temperature also favors growth and isolation of fungi. Visible colonies were counted after the incubation period manually.

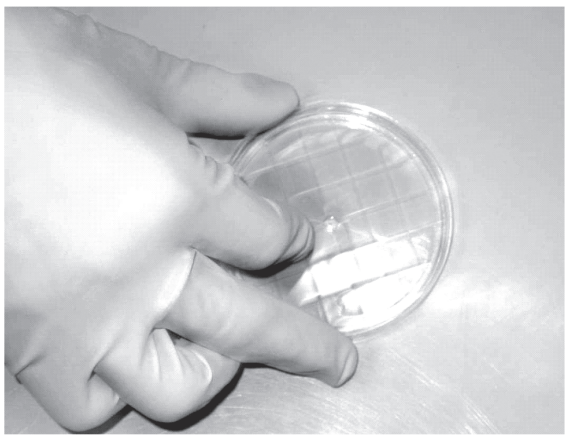

Fig. 1: Collection of floor samples by contact plate method

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Results

After six days of incubation, the colonies observed in the SCDA agar plates were counted and the respective genus was identified by staining methods like Gram stain and Lacto phenol cotton blue. The average number of microbial colonies reduced from 31 CFU/Plate to <1 CFU/plate after application of Clinister (Table 1). The most common surface contaminants isolated were *Staphylococcus spp., Bacillus spp., Micrococcus spp., Penicillium spp. A. fumigates* which were observed to be significantly reduced within five minutes after application of Clinister on the floor in the media preparation room (Fig. 2), over the surface of the stainless steel table (Fig. 3) and on the floor in the wash room (Fig. 4).

Discussion

Surface disinfection becomes important in several situations like households (14), health care facilities like hospitals and laboratories (3-5), child care centres (15), poultry (16) and other public areas where the risk of spread of infection is high. Though the transmission of infection through surface contact is still debated (1), particular importance to surface disinfection is attached during times of outbreaks of infections (2). Though several surface disinfectants are in use, they have associated side effects like skin infections associated with Phenol, Chlorine (9) and resistance of gram negative organisms imparted by widespread use of Quaternary ammonium compounds (11). In case of uses in situations like poultry, it has been reported that most of the commonly used Phenol, Quaternary ammonium compounds and Sodium hypochlorite have reduced efficacy in the presence of organic matter (10, 16). The Clinister used in this study as it is food additive based, does not have the health associated adverse effects as with other surface disinfectants described above. Clinister has been found to be non-toxic even when ingested (12, 13). It is already widely used in the food industry in Japan. The present study demonstrates the efficacy of Clinister in surface disinfection as observed by the significant reduction in the number of CFUs before and after application of Clinister.

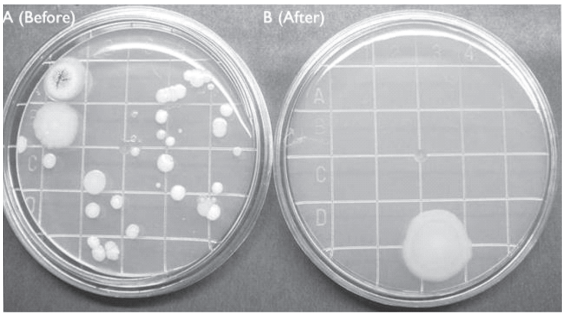

Fig. 2: Media preparation room A: Floor sample microbial colonies before application of Clinister; B. Microbial colonies after application of Clinister.

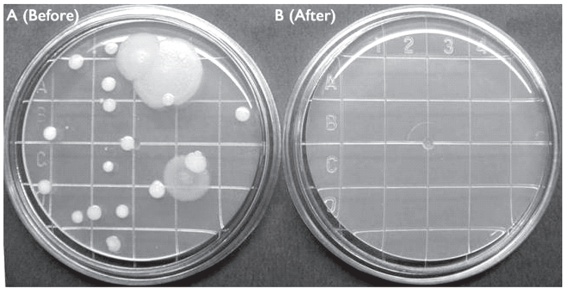

Fig. 3: Stainless steel table A: Surface sample microbial colonies before application of Clinister; B. Microbial colonies after application of Clinister.

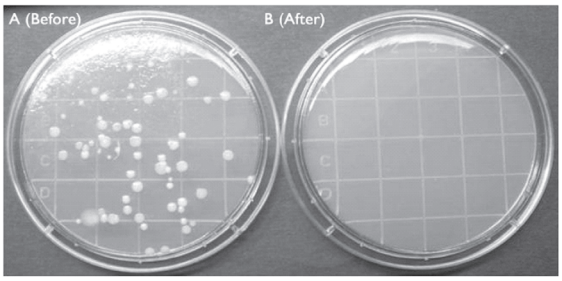

Fig. 4: Wash room A: Floor sample microbial colonies before application of Clinister; B. Microbial colonies after application of Clinister.

It was reported in a study that during the 2009 H1N1 outbreak in Thailand, the influenza virus RNA contamination could be documented on household surfaces and on the fingertips of ill children. Especially homes with younger children were more likely to have contaminated surfaces compared to older children (17). As Clinister has already been shown to be effective against the

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Table 1: Bacterial colonies in the SCDA plates before and after Disinfecting with Clinister.

S. No	Sample locations	Result obtained (CFU/plate)	
		Before exposure	After exposure*
1	Media preparation room	34	1
2	Media preparation room	21	<1
3	Microbial test room	21	<1
4	Microbial limit test room – change room	69	1
5	Instruments table	10	<1
6	Instruments table	21	<1
7	Laminar Air Flow	8	<1
8	Laminar Air Flow	11	<1
Average number of colonies		31	<1

**No growth denoted as <1 CFU per plate*

H5N3 Avian Influenza virus (18), the routine use of Clinister in households, child care centres, hospitals, public areas with high risk of infection and in poultry is likely to decrease the spread of such pathogenic organisms. The Clinister will also be highly useful in situations of outbreak. As Clinister comes both in powdered and tablet forms (18), its use in a variety of situations becomes easier. Wet tissue wipes or cotton soaked with Clinister solution would be an ideal solution for wiping the instruments, gadgets, devices, surfaces etc in hospitals, laboratories, kitchens, tables, operating rooms, clean rooms for cell culture, microbiological laboratories and other domestic, professional set-ups, where disinfecting the surfaces is essential. Further studies are advocated to validate the use of Clinister in different situations of infection control.

Conclusion

Use of Clinister as a surface disinfectant effectively reduces the bacterial and fungal microbiological burden as proven by the reduction in the number of CFUs of the microorganisms after its application in a microbiological lab set-up in India. Use of Clinister is advocated for disinfection of a variety of surfaces in health care set-ups, pathological and microbiological laboratories, clean rooms, child care centres, old age homes, households, public areas, poultry etc. where the risk of spread of infection is high on a routine basis to prevent spread of infections and a possible outbreak.

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Potential conflict of interests: Dr. Nobuyuki Yamaji and Dr. Sunao Kubota are applicants to the patents which have been filed regarding the development of the Clinister - technology

References

1. Rutala, W.A. and Weber, D.J. (2001). Surface disinfection: should we do it? J Hosp Infect, 48 Suppl A:S64-8.
2. Kossow, A., Schaber, S. and Kipp, F (2013). (Surface disinfection in the context of infection prevention in intensive care units). Med Klin Intensivmed Notfmed, 108 :113-8.
3. Donskey, C.J. (2013). Does improving surface cleaning and disinfection reduce health care-associated infections? Am J Infect Control. doi:pii: S0196-6553(13) 00055-2. 10.1016/j.ajic. 2012.12.010.
4. Petti, S., Polimeni, A. and Dancer, S.J. (2013). Effect of disposable barriers,

disinfection, and cleaning on controlling methicillin-resistant Staphylococcus aureus environmental contamination. Am J Infect Control. doi:pii: S0196-6553 (12) 01270-9. 10.1016/j.ajic.2012. 09.021

5. Exner, M., (2007) Divergent opinions on surface disinfection: myths or prevention? A review of the literature. GMS Krankenhhyg Interdiszip, 2 :Doc19.

6. Sui, Y.S., Wan, G.H., Chen, Y.W., Ku, H.L., Li, L.P., Liu, C.H. and Mau, H.S. (2012) Effectiveness of bacterial disinfectants on surfaces of mechanical ventilator systems. Respir Care, 57: 250-6.

7. Barbut, F., Yezli, S. and Otter, J.A., (2012) Activity in vitro of hydrogen peroxide vapouragainst Clostridium difficile spores. J Hosp Infect, 80 :85-7.

8. Singh, M., Sharma, R., Gupta, P.K., Rana, J.K., Sharma, M. and Taneja, N. (2012) Comparative efficacy evaluation of disinfectants routinely used in hospital practice: India. Indian J Crit Care Med,16:123-9.

9. Hegstad, K., Langsrud, S., Lunestad, B.T., Scheie, A.A., Sunde, M. and Yazdankhah, S.P. (2010) Does the wide use of quaternary ammonium compounds enhance the selection and spread of antimicrobial resistance and thus threaten our health? Microb Drug Resist, 16 :91-104.

10. Gélinas, P., Goulet, J. (1983). Neutralization of the activity of eight disinfectants by organic matter. J Appl Bacteriol, 54 : 243-7.

11. Holdsworth, S.R. and Law, C.J. (2013). The major facilitator superfamily transporter MdtM contributes to the intrinsic resistance of Escherichia coli to quaternary ammonium compounds. J Antimicrob Chemother, 68 :831-9.

12. Kubota, S., Matsuzawa, K., Wada, M. and Yamaji, N. (2008). Bactericidal Effect of a Disinfectant (Bio Io Nurse) Prepared by Adding Citric Acid and Low Concentration Alcohol to Strongly Acidic Electrolyzed Water. New Food Industry, 50:9. (Article in Japanese)

13. Kubota, S., Matsuzawa, K., Wada, M., Moei, I. and Yamaji, N. (2010). Usefulness of Bio Io Nurse as an Indoor Spray Disinfectant- Basic Examination of Kitchen Disinfection. New Food Industry, 52: 55-58. (Article in Japanese)

14. Exner, M., Vacata, V., Hornei, B., Dietlein E. and Gebel, J. (2004). Household cleaning and surface disinfection: new insights and strategies. J Hosp Infect, 56 Suppl 2:S70-5.

15. Jimenez, M., Martinez, C.I. and Chaidez, C. (2010). Disinfection alternatives for contact surfaces and toys at child care centers. Int J Environ Health Res, 20 :387-94.

16. Stringfellow, K., Anderson, P., Caldwell, D., Lee, J., Byrd, J., McReynolds, J., Carey, J., Nisbet, D. and Farnell, M. (2009). Evaluation of disinfectants commonly used by the commercial poultry industry under simulated field conditions. Poult Sci, 88:1151-5.

17. Simmerman, J.M., Suntarattiwong, P., Levy, J., Gibbons, R.V., Cruz, C., Shaman, J., Jarman, R.G. and Chotpitayasunondh, T. (2010). Influenza virus contamination of common household surfaces during the 2009 influenza A (H1N1) pandemic in Bangkok, Thailand: implications for contact transmission. Clin Infect Dis,51 :1053-61.

18. Kubota, S. (2012). Effects of powdered Bio-Io Nurse on Highly Pathogenic Avian Influenza Viruses. Food Industry (Article in Japanese; in press).

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A single Amino Acid Deletion in Capsid Protein VP1 G-H Loop Region of a Serotype O Foot and Mouth Disease Virus Isolate from India

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Abstract

Foot and mouth disease is endemic in India with prevalence of O, A and Asia1 serotypes among which type O accounts for more than 80% of the outbreaks. All the Indian serotype O isolates were genetically belonging to a single group, Middle East-South Asia (ME-SA) topotype further divided into three distinct lineages and an unnamed minor group. Sequencing the VP1 region of a field isolate O/GOA/120/2002 revealed a deletion of one amino acid at 139th site. Further phenotypic characterization of the isolate by plaque assay showed that the plaques size was very small with a diameter of 1-2 mm. However, the isolate was antigenically homologous to the current Indian vaccine strain. The amino acid deletion did not cause any detectable antigen variation. However, the isolate showed smaller plaque size. Additionally this amino acid may not be essential for the virus multiplication and structural stability of VP1.The significance of the deletion is not clear.

Keywords: Foot and mouth disease virus, Serotype O, VP1 and Amino acid deletion.

Introduction

Foot and mouth disease (FMD) is an acute, systemic and highly contagious viral disease of the cloven foot animals covering a wide range of both domestic and wild animals. The disease is characterized by very high morbidity and low mortality with the development of vesicular eruptions on tongue epithelium and interdigital spaces of hoof (1). Highly contagious nature of the disease and its severe economic effects due to loss of trade in animal and animal byproducts (2) makes it one among the notifiable diseases in the list of OIE.

The disease is caused by foot and mouth disease virus, a member of family *Picornaviridae* and genus *Aphthovirus* and exists as seven distinct serotypes. Antigenic variation occurs in the virus due to alteration of the amino acids at critical residues involved in neutralizing antibody binding. The structure of foot-and-mouth disease virus (FMDV) is composed of 60 copies of four capsid proteins viral-protein1- 4 (VP1- 4). These capsid proteins share eight-stranded antiparallel β-barrel structure with formation of puffs, knobs and loops (3, 4). In FMDV, the surface loop joining β-strands in VP1 region forms the G-H loop region (formed by amino acid residues 134-160 of VP1). This major antigenic region contains the Arg-Gly-Asp (RGD) sequence that is involved in recognition of an integrin receptor (5). The G-H loop is highly disordered and one of the most mobile regions of the virus surface (6).

In India, at present three different serotypes namely O, A and Asia 1 are reported with type O accounting for more than 80 % of the outbreaks (7-9). All Indian type O FMD outbreaks are caused by Middle East and South Asia topotype (ME-SA) only. Within ME-SA topotype four lineages are in circulation, namely Ind 2001, PanAsia-1, PanAsia-2 and an unnamed minor

group. The Ind 2001 lineage dominated the recent outbreaks (8). The current Indian vaccine strain O/IND/R2/75 belongs to ME-SA group topotype and grouped under branch B. Since 2003, no outbreaks were reported due to an isolate from this branch of the phylogenetic tree (8). Phylogenetic analysis of type O isolates collected from Indian outbreaks from 2001 to 2012 was studied (10). While analyzing the VP1 region sequences of type O isolates a deletion of one amino acid was observed. The virus showing deletion was also phenotypically characterized by plaque assay (11).

Materials and Methods

Cell and virus : Baby hamster kideney-21 (BHK-21) cell line maintained in the cell culture laboratory, Indian Immunologicals limited (IIL), Hyderabad was used for passaging the viral cultures and plaque assay. O/GOA/120/2002 isolated during an FMD outbreak in Goa in 2002 and maintained in the virus repository, FMDV laboratory, IIL, Hyderabad was used in the study.

Genetic characterization : The viral RNA was extracted from monolayer adapted virus suspension by RNeasy mini kit (Qiagen, Germany) and VP1 gene was amplified using oligonucleotide primers ARS4 and NK61 as described earlier (12, 13). Positive PCR product of 1.3 kb was purified by Qiaquick® PCR Purification Kit (Qiagen, Germany) which was further used for direct cycle sequencing. Cycle sequencing was carried out in an automatic cycle sequencer (ABi Prism, Applied Biosystems, USA) using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA) as per the manufacturer's protocol. The forward and reverse primers (ARS4/NK61) were used individually for sequencing as two independent runs.

Sequence of O/GOA/120/2002 (GenBank Accession No. JX070590) was aligned with GenDoc and Clustal X 1.8 program (14) using O/IND/R2/75 sequence from GenBank as a reference. To confirm the deletion, sequencing of isolate was repeated with three passages of the virus on BHK-21 monolayer cells.

Phenotypic characterization: Phenotypic variation of O/GOA/120/2002 was studied by observing the plaques in BHK-21 monolayer plates. Briefly the plaque assay titration was done in BHK-21 cells grown in the sterile Petri dishes (Corning, USA). The fully grown BHK-21 monolayer was infected with log diluted virus suspension sample and incubated for 1 hr at 37°C followed by overlaying of the infected monolayer cells with plaque overlay medium. After 48 hrs of incubation at 37°C, the plates were stained with 0.08% w/v crystal violet mixed in saline and formaldehyde.

Antigenic characterization : Two-dimensional microneutralization test (2D-MNT) was carried out with the bovine vaccinate serum (BVS) of Indian serotype O vaccine strain O/IND/R2/75 and O/GOA/120/2002 (15). Briefly, in a 96 well cell culture plate (Nunclon™ Denmark) the serum samples was two fold diluted and added with different log dilutions of the virus suspension samples with appropriate controls. The BHK-21 monolayer cells were added to all wells and the plates were sealed and incubated for 48 hrs at 37°C. After 48 hrs the plates were stained with 0.4% naphthalene black stain containing formaldehyde. The serum neutralizing end point titer was calculated as reciprocal of the last dilution that neutralizes 100$TCID_{50}$ virus particles in 50% of the wells. The MNT was repeated three times and the mean antibody titers were statistically analyzed and average value was taken for calculation of *'r'* value The serum neutralizing antibody titers were calculated against its homologous virus and also against the field viruses which was used for the *'r'* value calculation (16). A field isolate with *'r'* value >0.30 was considered antigenically similar to the vaccine strain and those with *'r'* <0.30 as antigenically heterologous to the vaccine strain.

Results and Discussion

Phylogenetic analysis of the isolate with reference sequences revealed that O/GOA/120/

2002 belonged to ME-SA topotype falling under branch B along with O/IND/R2/75 (Fig.1). Deduced amino acid analysis of the sequence showed deletion of three nucleotides (a codon), between 415-417 in O/GOA/120/2002.

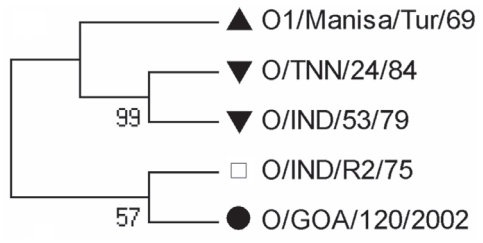

Fig. 1. Phylogenetic tree showing branch A and B of ME-SA topotype with the position of O/GOA/120/2002. ● - indicates O/GOA/120/2002, □ - indicates current Indian vaccine strain, ▼ - indicates earlier Indian vaccine strains, ▲ - indicates exotic vaccine strains.

Amino acid analysis of all the three viral passage sequences used for confirmation of deletion also showed the persistence of the deletion at 139th amino acid residue. None of isolates collected during that year showed any deletion (10). Recently a similar deletion at 139th amino acid residue had been reported by Das and colleagues (17) in three Ind 2001 lineage viruses isolated from Gujarat in 2009. Comparison of amino acid sequence of O/GOA/120/2002 VP1 with O/IND/R2/75, O/TNN/24/84 and O/IND/53/79 (earlier Indian vaccine strains) and O_1/Manisa/Tur/69 is showed in Fig. 2.

At nucleotide level O/GOA/120/2002 isolate showed a difference around 10 to 15 % with the reference sequences used in the study. This isolate was homologous to the vaccine strain O/IND/R2/75 by 2D-MNT with an *'r'* value of 0.48. The 139th amino acid site of VP1 region which is

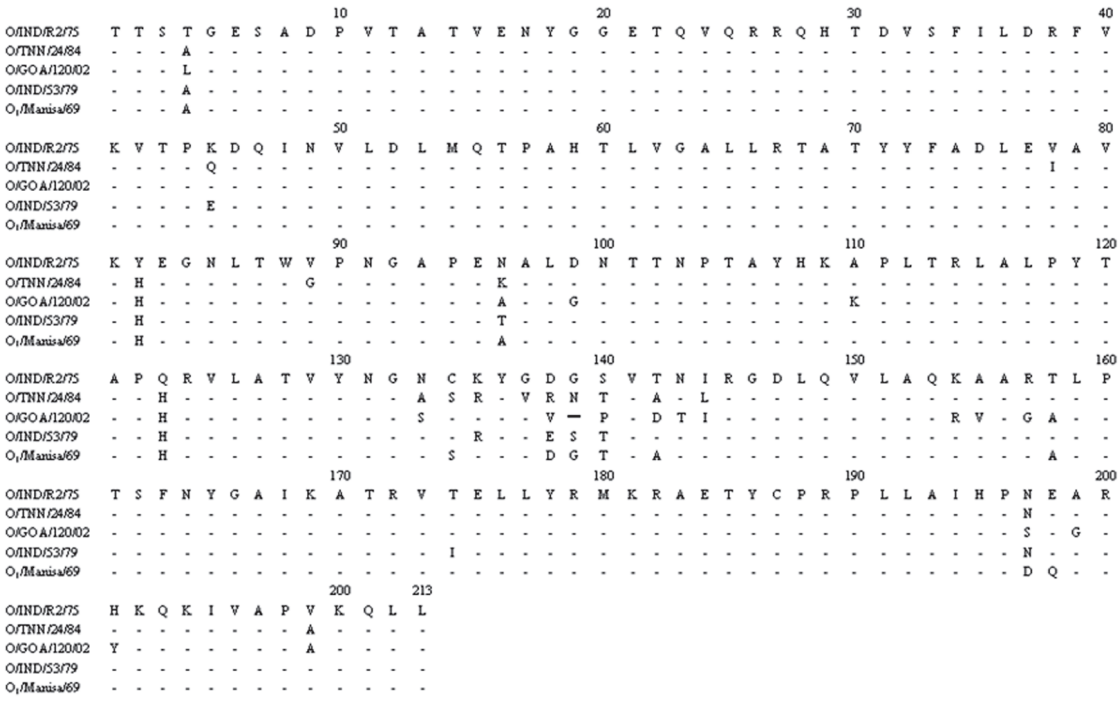

Fig. 2. Comparison of VP1 region amino acid sequences of O/GOA/120/2002 showing 139th amino acid deletion with the current and earlier Indian vaccine strains along with O_1/Manisa/Tur/69.

located within the G-H loop was demonstrated under positive selection (18) and any amino acid variation at this residue resulted in the reduction of neutralizing antibody binding (19).

Isolate O/GOA/120/2002 showed a pronounced amino acid sequence variation within the G-H loop region, in comparison with the other regions of VP1. G-H loop residues 133, 138, 142, 143, 144, 154, 155, 157 and 158 showed variation from most of the field isolates (data not shown). The deletion of 139th residue within the G-H loop along with changes at various other amino acid residues was tolerated by the virus.

In serotype O isolates, the G-H loop region contains critical amino acid residues at 144, 148, 154 and 208th positions that are involved in the formation of antigenic site 1 (20). Usually any alteration in amino acid at these residues results in the reduced binding of neutralizing antibodies. Sometimes, though the critical amino acid residues remain unchanged, substitution of amino acid at some other sites indirectly alters the structural conformation of G-H loop, bringing about a dramatic reduction in the proportion of antibody attachments (21).

The phenotypic characterization of the isolate was done by plaque assay with BHK-21 cells. The size of the plaques was observed to be much smaller in diameter of 1-2 mm when compared with the Indian vaccine strain O/IND/R2/75 plaques (Fig. 3).

Amino acid analysis of the VP1 region of O/GOA/120/2002 showed a variation at 110th site from the reference sequences. A substitution of Lysine (K) instead of alanine (A) was observed in O/GOA/120/2002 isolate at the 110th site which is placed within the βF-βG loop at the five fold axis of the virion. The presence of lysine at 110th site had been reported earlier (22) to increase the positive charge in the local surface of the virus capsid. In Southern African Territory 1 (SAT1) serotype, the presence of Lysine (K) as 110th amino acid in VP1 region had also reported to be responsible for a smaller plaque size in BHK-21 cells due to charge-based interaction, perhaps

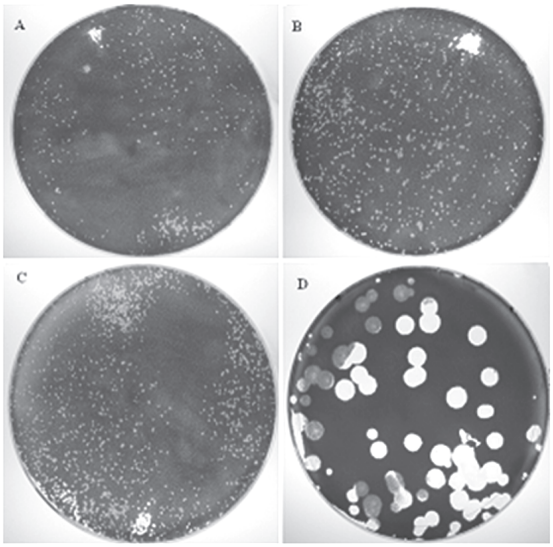

Fig. 3. Plates showing the plaque assay results of O/GOA/120/2002 (A, B & C) and O/IND/R2/75 (D). The size of O/GOA/120/2002 isolate plaques were observed to be smaller in diameter (1-2 mm) in A, B & C showing passage number 1, 2 and 3 in BHK-21 monolayer.

involving binding to cell surface sulphated polysaccharides (22). Further they (22) had found that those SAT1 isolates with Lysine at 110th site invariably had Glycine (G) (a non-polar amino acid) at their 112th site. In O/GOA/120/2002 isolate amino acid Lysine was present at 110th site with a different non-polar amino acid Leucine at the 112th site.

For FMD vaccine strains the amino acid at 140th site is either Serine or Threonine. However our observation for the field isolates is mostly Proline. Vaccine strains did not show any deletion at 139th amino acid. In the present case deletion at 139th site has no bearing on the amino acid residue at 140th site being Proline or Serine / Threonine.

The appearance of the newer antigenic variants in the field complicates the control strategies and necessitates continuous monitoring of the isolates. Since the isolates with 139th amino acid residue deletion was not

reported except for three Ind 2001 lineage isolates (17) for more than 10 years in any part of the country there may not be of much significance for such a deletion in the immune evasion strategy of the virus.

Conclusion

Phylogenetic analysis of the field isolates is necessary to monitor the variations occurring under field conditions. Genetically all the Indian type O isolates belonged to ME-SA topotype. An isolate from a FMD outbreak in Goa in 2002 belonged to branch B and showed a deletion of amino acid at 139th VP1 site. The phenotypic characterization of the isolate showed smaller plaques on BHK-21 monolayer with a diameter of 1-2 mm.

References

1. OIE Terrestrial Manual. (2012). Foot And Mouth Disease World Assembly of Delegates of the OIE in May 2012. Chapter 2.1.5.1.
2. Forman, S., Le Gall, F., Belton, D., Evans, B., François, J.L., Murray, G., Sheesley, D., Vandersmissen, A. and Yoshimura, S. (2009). Moving towards the global control of foot and mouth disease: an opportunity for donors. Revue cientifique et technique de l office international des epizooties, 28: 883-896.
3. Mateu, M. (1995). Antibody recognition of picornaviruses and escape from neutralization: a structural view. Virus Research, 38: 1-24.
4. Minor, P.D. (1990). Antigenic structure of Picornaviruses. Current Topics in Microbiology and Immunology, 161: 121-154.
5. Mason, P.W., Reider, E. and Baxt, B. (1994). RGD sequence of foot-and mouth disease virus is essential for infecting cells via the natural receptor but can be bypassed by an antibody-dependant enhancement pathway. Proceedings of the National Academy of Sciences of the United States of America, 91: 1932–1936.
6. Logan, D., Abu-Ghazaleh, R., Blakemore, W., Curry, S., Jackson, T., King, A., Lea, S., Lewis, R., Newman, J., Parry, N., Rowlands, D., Stuart, D. and Fry, E. (1993). Structure of a major immunogenic site on foot-and-mouth disease virus. Nature, 362: 566-568.
7. PD-FMD Annual Report, 2010-11. (2011). Project Directorate on Foot and mouth disease, Mukteswar.
8. Subramaniam, S., Pattnaik, B., Sanyal, A., Mohapatra, J.K., Pawar, S.S., Sharma, G.K., Das, B. and Dash, B.B. (2012). Status of Foot-and-mouth Disease in India. Transboundary and Emerging Diseases, DOI: 10.1111/j.1865-1682.2012.01332.x
9. Pattnaik, B., Subramaniam, S., Sanyal, A., Mohapatra, J.K., Dash, B.B., Ranjan, R. and Rout, M. (2012). Foot-and-mouth Disease: Global Status and Future Road Map for Control and Prevention in India. Agricultural Research, 1: 132–147.
10. Yuvaraj, S., Madhanmohan, M., Nagendrakumar, S.B., Kumar, R., Mohana Subramanian, B., Mohapatra, J.K., Sanyal, A., Pattnaik, B. and Srinivasan, V.A. (2013). Genetic and antigenic characterization of Indian foot-and-mouth disease virus serotype O isolates collected during the period 2001 to 2012. Infection, Genetics and Evolution, 13: 109–115.
11. Bachrach, H.L., Callis, J.J., Hess, W.R. and Patty. R.E. (1957). A plaque assay for foot-and-mouth disease virus and kinetics of virus reproduction. Virology, 4: 224-236.
12. Knowles, N.J. and Samuel, A.R. (1995). Polymerase chain reaction amplification and cycle sequencing of the 1D (VP1) gene of foot-and-mouth disease viruses. Report of the Session of the Research Group of the Standing Technical Committee of the

European Commission for the Control of Foot-and-Mouth Disease held jointly with the FMD Sub-group of the Scientific Veterinary Committee of the Commission of the European Community, Mödling, Vienna, Austria. Rome: FAO. pp 8, 45-53.

13. Knowles, N.J. and Samuel, A.R. (1998). Polymerase Chain Reaction amplification and cycle sequencing of the 1D (VP1) gene of foot-and-mouth disease viruses. RT-PCR and sequencing protocols for the molecular epidemiology of exotic virus diseases of animals. Institute for Animal Health, Pirbright, UK, pp. 26-35. http://www.wrlfmd.org/fmd_genotyping/genotyping_protocol.pdf

14. Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994). CLUSTAL W 18x: improving the sensitivity of progressive multiple sequence alignment through sequence weighting positions-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22: 4673-4680.

15. Rweyemamu, M.M., Booth, J.C., Head, M. and Pay, T.W.F. (1978) Microneutralization tests for serological typing and subtyping of foot-and-mouth disease virus strains. Journal of Hygiene (Cambridge), 81: 107–123.

16. Rweyemamu, M.M. (1984). Antigenic variation in foot-and mouth disease: studies based on the virus neutralization reaction. Journal of Biological Standards, 12: 323–337.

17. Das, B., Sanyal, A., Subramaniam, S., Mohapatra, J.K. and Pattnaik, B. (2012). Field outbreak strains of serotype O foot-and-mouth disease virus from India with a deletion in the immunodominant âG-âH loop of the VP1 protein. Archives of Virology, 157: 1967-1970.

18. Mart´ýn, M.J., Nu´n˜ez, J.I., Sobrino, F. and Dopazo, J. (1998). A procedure for detecting selection in highly variable viral genomes: evidence of positive selection in antigenic regions of capsid protein VP1 of foot-and-mouth disease virus. Journal of Virological Methods, 74: 215–221.

19. Rojas, R.E., Carrillo, E.C., Schiappacassi, M. and Campos, R. (1992). Modification of foot-and-mouth disease virus O1 Caseros after serial passages in the presence of antiviral polyclonal sera. Journal of Virology, 66: 3368–3372.

20. Xie, Q.C., McCahon, D., Crowther, J.R., Belsham, G.J. and McCullough, K.C. (1987). Neutralization of Foot-and-Mouth-Disease Virus can be mediated through any of at least 3 separate antigenic sites. Journal of General Virology, 68: 1637-1647.

21. Parry, N., Fox, G., Rowlands, D., Brown, F., Fry, E., Acharya, R., Logan, D. and Stuart, D. (1990). Structural and serological evidence for a novel mechanism of antigenic variation in foot-and-mouth disease virus. Nature, 347: 569–572.

22. Maree, F.F., Blignaut, B., Aschenbrenner, L., Burrage, T. and Rieder, E. (2011). Analysis of SAT1 type foot-and-mouth disease virus capsid proteins: Influence of receptor usage on the properties of virus particles. Virus Research, 155: 462–472.

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Effect of Physical Pre-treatment on Cellulases Production by *Cladosporium* sp. NCIM 901 in Submerged Fermentation using Bagasse as a Substrate

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Abstract

The production of cellulases (filter paper activity, endoglucanase and β-glucosidase) by *Cladosporium* sp. NCIM 901 on different media in shake flask culture was compared. The culture filtrate of the organism exhibited highest total cellulolytic activity and extracellular protein content on 7 days interval during the course of its growth on Mary Mandel's mineral medium supplemented with 1% cellulose compared to other media. Various soluble carbon sources were tested in this study, cellulose supported the maximum production of cellulolytic enzymes (1.64, 0.75 and 1.1 U/ml of FPase, CMCase and β-glucosidase). Enhanced production of cellulolytic enzymes was observed when KNO_3 was used as nitrogen source (1.71, 0.93 and 1.23 U/ml of FPase, CMCase and β-glucosidase). Among the lignocellulosic materials, sugarcane bagasse increased cellulolytic enzymes production (1.94, 0.95 and 0.98 U/ml of FPase, CMCase and β-glucosidase) and the next best was saw dust. Maximum yield of cellulolytic enzymes were observed when bagasse was physically pretreated (1.972, 1.103 and 1.112 U/ml of FPase, CMCase and β-glucosidase) and the reducing sugar concentration of 0.52 mg/ml was obtained.

Keywords: *Cladosporium* sp. NCIM 901, Celllulolytic enzymes, FPase, CMCase, β-glucosidase, Pretreatment.

Introduction

Lignocellulose is the major component of biomass, comprising around half of the plant matter produced by photosynthesis and representing the most abundant renewable organic resource on earth. It consists of three types of polymers, cellulose, hemicellulose and lignin that are strongly inter-meshed and chemically bonded by non-covalent forces as well as by covalent cross linkages (1). Small amounts of the cellulose, hemicellulose and lignin produced as by-products in agriculture or forestry is used, the rest being considered as waste. Many microorganisms are capable of degrading and utilizing cellulose and hemicellulose as carbon and energy sources during their growth process.

Proper biotechnological utilization of the cellulosic wastes in the environment will eliminate pollution and may be converted them into useful by-products. The potential of cellulose as an alternative energy source has stimulated research into bioconversion processes which hydrolyse cellulose to soluble sugars which further used as feedstocks in alchoholic fermentations and other industrial processes (2). Cellulose, an insoluble polymer consisting of β-(1-4)-linked glucose residues, has been the subject of intense research for more than a century, and new insights into a better understanding of its molecular architecture continue to emerge. It is well known that native cellulose molecules (cellulose I) are found in fibril form, and that its molecular architecture has a high degree of individuality, depending on its source (cell wall layer or plant type) (3).

Currently, there are two major ways of converting cellulose to glucose: chemical versus

enzymatic. Enzymatic hydrolysis of cellulose is an important reaction in nature as it marks the first step in the decay of cellulose. Even other processes of pretreatment of cellulose are necessary in the production of ethanol from cellulosic material since it makes the recalcitrant cellulosic biomass has limited accessibility to enzymatic hydrolysis (4). Physical, chemical and hydrothermal processes involved in the pretreatment of lignocellulosic substrate. Enzymatic hydrolysis has been recognized as an attractive and cost effective method for hydrolysis of the pretreated biomass (5). Cellulases are inducible enzymes, which are synthesized by many microorganisms during their growth on cellulosic materials. Microorganisms known to produce cellulases include bacterial species of *Clostridium* and *Bacillus* and species of filamentous fungi from *Penicillium, Asperigillus* and *Trichoderma* (6). Cellulase enzymes provide a key opportunity for achieving tremendous benefits of biomass utilization through the bioconversion of the most abundant cellulosic wastes into glucose (7).

The primary functions of cellulolytic and hemicellulolytic enzymes are to hydrolyse the β-1,4-glycosidic linkages present in the majority of plant structural polysaccharides, cellulose and hemicellulose respectively (8). Cellulase hydrolyzes cellulose by synergistic action of its three constituent enzymes viz., β-1,4-endoglucanase, cellobiohydrolase and β-glucosidase which cleaves respectively internal β-1,4-glycosidic bonds, cellobiose from cellulose and hydrolyses cellobiose to glucose (9). Even though there are many reports on cellulase producing fungi (10), only few have high activities for commercial success (11).

The primary goal of pretreatment is to enhance the formation of sugars or the ability to form sugars by subsequent hydrolysis of the carbohydrate fraction of the (pretreated) biomass. It is imperative to achieve high yield and minimize the breakdown of sugars into decomposition products. The rate of formation of sugars is relative to their destruction which must be maximized to achieve a high yield pretreatment (12). Milling is a mechanical pretreatment of the lignocellulosic biomass that reduces the particle size and crystallinity (13). The reduction in particle size leads to an increase of available specific surface and a reduction of the degree of polymerization (DP) (14). Milling also causes shearing of the biomass and simultaneous saccharification which was already proven successful (15). In the present study, an attempt was made to optimize culture conditions particularly physical pretreatment (particle size), that influences the enzyme production by *Cladosporium* sp. NCIM 901 in submerged fermentation process in a laboratory scale.

Material and Methods

Microorganism: The fungal culture *Cladosporium* sp. NCIM 901 was obtained from NCIM, Pune, India, and cultured on Potato Dextrose Agar (PDA) slants at 4°C. It was maintained by the periodic transfer on to fresh medium at every 3 weeks.

Preparation of inoculum: Fungal cultures were inoculated onto fresh PDA agar slants and spores were collected from 5 days grown PDA agar slant by using 10 ml of sterile water containing tween-80 (1% v/v).

Lignocellulosic substrates: Locally available cheap raw materials like sugarcane bagasse, saw dust, sorghum straw and rice straw were used in this study. These were collected, dried, milled and sieved through mesh and used in the fermentation.

Fermentation medium: The basal fermentation medium used was Mary Mandel's mineral salts solution which was supplemented with different carbon and nitrogen sources. It contained (per L) Cellulose, 10g; $(NH_4)_2SO_4$, 1.4g; KH_2PO_4, 2g; $CaCl_2$, 0.3g ; $MgSO_4.7\ H_2O$, 0.3 g; Urea, 0.3g; trace metal solution (2.5 g $FeSO_4$; 0.98g $MnSO_4.H_2O$; 1.76 g $ZnSO_4.H_2O$; 1.83g $CoCl_2.6H_2O$ dissolved in 495 ml of distilled water and 5 ml of conc. HCl), 1 ml. The pH was adjusted to 4.8 (16).

Pretreatment of sugarcane bagasse: The sugarcane bagasse used in this study was obtained from S.V.Sugar Industries, Tirupati. Three physical pretreatment methods are applied for bagasse. In the primary treatment bagasse was dried in an oven at 60°C for 6 h, milled and sieved using 1 mm mesh. In the secondary pretreatment, bagasse from the primary treatment was milled once again and sieved using 0.5 mm mesh. In the final pretreatment the bagasse was made into powdery form by using mixer grinder. The fermentation was carried out by using the three above resultant substrates.

Enzyme assay: Filter paper activity (FPA) for total cellulase activity was determined according to Mandels and Andreotii (17). Aliquots of appropriately diluted culture filtrate as enzyme source was added to whatman No.1 filter paper strip (1×6 cm; 50 mg) immersed in 1 ml of 0.05 M sodium citrate buffer of pH 5.0. After incubation at 50°C for 1 h, the reducing sugar released was estimated by dinitrosalicylic acid (DNS) method (18). One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 µM of reducing sugar from filter paper per ml per min. Carboxymethylcellulase (CMCase) activity was measured as described previously (19) using a reaction mixture containing 1 ml of 1% carboxymethylcellulose (CMC) in 0.05 M citrate acetate buffer (pH 5.0) and aliquots of suitably diluted filtrate. The reaction mixture was incubated at 50°C for 1 h; the reducing sugar released was estimated by DNS method. One unit (IU) of endoglucanase activity was defined as the amount of enzyme releasing 1 µM of reducing sugar per min. The β-glucosidase activity was assayed according to Herr (20) and was measured in 1 ml of 5 mM p-nitrophenyl-β-D-glucopyranoside (PNPG) in 0.05 M citrate buffer (pH 5.0) and aliquots of appropriately diluted culture filtrate and by incubating at 50°C for 30 min. The reaction was terminated by addition of 4 ml of 0.1 M NaOH-glycine buffer solution and the released p-nitrophenol was read at 410 nm. The activity was expressed in terms of liberation of PNPG. One unit of enzyme activity was defined as the amount of enzyme that produced 1 µmole of p-nitrophenol per min. The fungal growth was expressed in terms of dry weight of mycelial mat. The content of soluble protein in the culture filtrate was estimated according to Lowry *et al*. (21) with bovine serum albumin as a standard.

Glucosamine determination: For fungal chitin hydrolysis into N-acetyl glucosamine, 20 mg dried biomass was incubated with 2 ml of H_2SO_4 (72%) in a test tube. After standing on a rotary shaker (130 rpm) for 60 min at 25ºC, it was diluted with 3 ml of distilled water and autoclaved at 121ºC for 2 h. The hydrolyzate was neutralized to pH 7.0 with 10 M and then 0.5 M NaOH using a pH meter, and diluted to 100 ml. Finally, glucosamine was assayed by the colorimetric method described by Tsuji *et al*. (22) and modified by Ride and Drysdale (23). 1 ml diluted hydrolysate was mixed with 1 ml of $NaNO_2$ (5%) and 1 ml of $KHSO_4$ (5%) in a centrifuge tube. After shaking occasionally for 15 min, it was centrifuged at 3500 rpm for 5 min; 2 ml of supernatant was mixed with 0.67 ml of $NH_4SO_3NH_2$ (12.5%) and shaken for 3 min. To the mixture 0.67 ml of 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH; 0.5%, prepared daily) was added and then the mixture was boiled for 3 min and immediately cooled to room temperature. Freshly prepared 0.67 ml of $FeCl_3$ (0.5%, prepared within 3 days) was added and incubated for 30 min and the absorbance at 650 nm was measured spectrophotometrically. The glucosamine content was calculated as milligrams per gram of fungal biomass according to the standard curve.

Culture conditions for the enzyme production: Submerged fermentation was carried out in the present study and sterile 100 ml of three different media (minimal, Czapek-Dox and mineral) each amended with 1% cellulose as carbon source was distributed in sterile 250 ml Erlenmeyer flasks. The flasks were inoculated with a density of 2×10^6 spores each and incubated at 28°C on a rotary shaker (180 rpm). Flasks were withdrawn at 7 day intervals over a

period of 4 weeks and filtered through whatman No.1 filter paper to separate mycelial mat and culture filtrate. The fungi were harvested by filtering through a weighed filter whatman No.1 filter paper and the cake was washed with distilled water. Then, the dry weight is measured after drying in an oven at 65ºC for 48 h. The mycelium was grounded in a mortar, redried at 65ºC for 24 h and stored in a desiccator until analysed. During fermentation the broth was collected under aseptic conditions and the collected sample was centrifuged (8000×*g*, 10 min, 4ºC), resultant supernatant was used for further analysis.

Results and Discussion

The fungi grown on 3 media exhibited highest cellulolytic activity at 7 days interval followed by decline in activity at lateral intervals. The highest cellulolytic activity of 1.84 FPU/ml was obtained at the 7th day with the dry weight of mycelia mat of 369 mg/flask and a total protein content of 160 μg/ml on Mary Mandel's mineral medium. Thus from the results, it is clearly evident that Mary Mandel's mineral medium is superior for growth and cellulase production by *Cladosporium* sp. than in others media used (Table 1). Cellulolytic activity exhibited by *Cladosporium* sp. is comparable to the activity exhibited by well studied *Trichoderma reesei* which showed activity ranges from 1-2 FPU/ml on different media (24, 25) and the activity does not exceed more than 1.5 FPU/ml in case of *Asperigillus niger* and *A. fumigatus* (26).

The supplementation of carbon source such as cellulose within a range of 0.5-3.0% on cellulase production was examined. The yields of fungal biomass, protein content and cellulase production by *Cladosporium* sp. on Mary Mandel's mineral medium with different cellulose concentrations at 7 days interval are presented in Table 2. Each cellulosic material employed had an optimum concentration at which the cellulase complex was produced in highest amounts. The *Cladosporium* sp. however, produced large quantities of cellulase only when the soluble substrates employed. The maximum cellulase activity was obtained at a substrate concentration of 1% in the present study. The activities of cellulase from this organism were greatly influenced by the concentration of substrate. Increased substrate concentration decreases the enzyme activity. This may be due to fermentation of ineffective complexes between enzyme and substrate at high substrate concentration. Also, since the substrate molecules, that may be bound to regions on the enzyme, which are not the active sites or alternatively, may crowd the active site as reported earlier (27). *Volvariella dysplasia* produced cellulolytic enzymes (550 U of CM-cellulase and 69 U of filter paper activity) when grown in shake flask culture at pH 5.4 and 28°C with 0.5% cellulose powder (28). This observation is well in agreement with the result of the present study.

The influence of supplementation of different carbon sources to Mary Mandel's mineral medium on cellulase production by *Cladosporium* sp. NCIM 901 was examined. The cellulase activity of fungus is shown in Table 3. Among carbon sources tested cellulose and CMC served as a good carbon sources for the production of cellulase enzyme. The activities of CMCase and β-glucosidase were high in culture medium containing CMC. Menon *et al*. (29) also demonstrated that CMC or cereal straw (1%, w/w) would be the best carbon source compared to sawdust for endoglucanase and β-glucosidase production using *C. globosum* as the cellulolytic agent. Low enzyme production on insoluble substrates might have resulted due to slow metabolism of fungus on these substrates, which are not known to be hydrolyzed easily, or due to their exclusion along with the unutilized substrates, since they are known to be more strongly adsorbed on cellulose particles (30). Although most of the substrates used were capable of inducing all three types of the cellulase components, except for glucose, the levels of enzyme produced varied greatly, depending upon the nature of the substrate and the conditions of cultivation.

Table 1. Growth, total cellulolytic activity and protein secretion by *Cladosporium* sp. NCIM 901 on different media

Incubation	Minimal media			Czepek-Dox media			Mary Mandel's mineral media		
in weeks	Glucosamine content (mg/g)	Total activity (FPU/ml)	Total protein (μg/ml)	Glucosamine content (mg/g)	Total activity (FPU/ml)	Total protein (μg/ml)	Glucosamine Content (mg/g)	Total activity (FPU/ml)	Total protein (μg/ml)
1	150±1.57	1.08±0.06	62±8.22	250±6.08	1.65±0.07	130±2.68	369±9.24	1.84±0.04	160±2.61
2	270±8.26	0.90±0.01	120±2.46	340±1.14	1.63±0.02	162±1.68	420±6.26	1.41±0.02	178±4.68
3	530±2.04	0.63±0.0	112±6.26	580±1.40	0.70±0.0	196±3.43	640±12.4	1.03±0.02	210±11.24
4	650±0.04	20.42±0.04	120±1.14	655±8.16	40.62±0.01	130±2.71	610±16.24	0.89±0.01	140±9.64

Filter paperase activity is expressed in terms of Filter paper units (FPU).One unit is the amount of enzyme in the culture filtrate that releasing 1 ìmole of reducing sugar from filter paper per min; Data represents the mean ± SEM (n=3).

Table 2. Effect of cellulose concentration on cellulase production by *Cladosporium* sp. NCIM 901

Cellulose concentration (g %)	Glucosamine (mg/g)	Total protein (μg/ml)	Cellulase activity		
			FPase (U/ml)	CMCase (U/ml)	β-glucosidase (U/ml)
0.5	590±16.42	582±4.94	1.22±0.12	0.345±0.01	0.80±0.12
1	918±8.96	923±2.68	1.80±0.08	0.924±0.04	1.14±0.16
1.5	910±6.42	904±7.42	1.74±0.06	0.912±0.06	1.08±0.06
2	905±24.26	900±6.24	1.72±0.02	0.905±0.02	1.06±0.04
2.5	900±18.42	890±9.48	1.70±0.02	0.894±0.08	1.03±0.08
3	892±11.42	876±7.26	1.68±0.06	0.891±0.04	1.01±0.02

Filter paperase activity is expressed in terms of filter paper units (FPU).One unit is the amount of enzyme in the culture filtrate that releasing 1μmole of reducing sugar from filter paper per min; Carboxy methyl cellulase activity (CMCase) is expressed in terms of units. One unit is the amount of enzyme in the culture filtrate that releasing 1 μmole of reducing sugar from CMC per min; One unit of β-glucosidase activity is defined as the amount of enzyme liberating 1 mole of p-nitrophenol per min; Data represents the mean ± SEM (n=3).

The dry weight of mycelia mat was higher in glucose amended medium than other media amended with other carbon sources including cellulose or sawdust as reported by other researchers (31). The differences in the complexity of the carbon sources could account for the variation in the growth of the organism in the different media. In a study on the growth and â-galactosidase activity of the penicillin producer industrial *P. chrysosporium* NCIM 00237 strain using different carbon sources, good growth was observed using glucose, sucrose, glycerol and galactose, while growth on lactose was substantially slower (32). Since glucose is more readily assimilated and metabolized by cells, there is greater tendency for organisms to grow very rapidly in media containing the simple sugar than that which contain cellulose or saw dust.

Cellulose is a polymer of D-glucose while saw dust is composed of complex plant cell wall polymers which include cellulose, hemicelluloses and lignin (33).

The cellulase activity and protein contents of filtrates from glucose containing media were extremely low and thus considered insignificant, this is because the organism already has the simple sugar, glucose, in its medium and hence do not need to produce hydrolytic enzymes. Cellulases and most plant cell wall hydrolyzing enzymes are inducible and also regulated by catabolite repression in most fungi (34). Presence of high concentration of glucose in the medium will thus turn off the production of the enzymes. This may account for the low protein content and insignificant cellulase activity recorded in media containing glucose. Low level of cellulolytic enzymes in this present study could be attributed to repression of synthesis of cellulolytic enzymes involved in the utilization of cellulose by easily metabolisable carbon, glucose. However, insensitization of this repression by mutations resulted in higher production of cellulase even in the presence of glucose (35). Biosynthesis of cellulase was found to be high in the medium containing carbon sources like xylose, lactose, cellulose and bagasse in case of *T. reesei* QM 9414, 97.177 and Tm3 (36). But the cellulase activity was found to be less when glucose was used as carbon source because of inhibition. In media containing multiple substrates the cellulase production was enhanced in case of *T. reesei* (37).

In the present study, the organism has different cellulase activity on different carbon sources. By using cellulose as sole carbon sources, production of cellulases may be more rapid because the glucose needed for the organism's metabolism must come from cellulose hydrolysis. There is apparent correlation between the protein content and cellulase activity of the crude enzyme obtained at the different time intervals of incubation. The organism seems to secrete the hydrolytic enzymes for the break down the polymers into the growth medium which largely accounts for the protein contents of the cell-free filtrates.

The effect of supplementation of different nitrogen sources to the medium for production of cellulolytic enzymes is represented in Table 4. The production of cellulase enzyme in medium was greatly influenced by the presence of nitrogenous source present in complexed form. Cellulase production is known to be sensitive to nitrogen source and nitrogen level in the medium (38). Different nitrogen sources are used in this present study for production of cellulase by *Cladosporium* sp. NCIM 901. The cellulase activity of the fungus is greatly varied on these nitrogenous sources. Highest activities were obtained if the medium was supplemented with nitrogen source in the form of KNO_3. Urea also acts as good nitrogen source for cellulase production. The activities of enzymes CMCase and β-glucosidase were also high in case of these nitrogen sources. The activity of three enzymes such as FPase, CMCase and β-glucosidase were 1.710, 0.935 and 1.236 U/ml, respectively in medium with KNO_3 as nitrogen source. The effectiveness of nitrogen source in supporting cellulase production along with growth, secretion of extracellular protein by *Cladosporium* sp. is presented in Table 4. Ellouz Chaabouni *et al*. (39) demonstrated that crude complex substrates used in combination with local cellulose gave high cellulase yield. Casamino acids, irrespective of carbon sources used, highly stimulated extracellular production of β-glucosidase by *Termitimyces clypeatus* (40).

The use of purified cellulose as substrate is uneconomical for large scale production of cellulase. Cheaply available agricultural lignocelluloses wastes were tested to find out whether they support to the production of cellulase by *Cladosporium* sp. at 1% (w/v) level. The maximum cellulase activity obtained in media amended with sugarcane bagasse followed by saw dust. The activity on bagasse and saw dust was 1.942 and 1.835 U/ml respectively as represented in Table 5. The CMCase and β-glucosidase activity is also high

Table 3. Effect of supplementation of different carbon source on cellulase production by *Cladosporium* sp. NCIM 901

Carbon source	Glucosamine (mg/g)	Total protein (μg/ml)	Cellulase activity		
			FPase (U/ml)	CMCase (U/ml)	β-glucosidase (U/ml)
Glucose	1214±24.42	680±6.82	0.192±0.06	0.423±0.02	0.29±0.02
Cellulose	620±14.6	940±12.4	1.645±0.12	0.752±0.04	1.10±0.12
CM cellulose	820±9.86	930±8.26	1.612±0.14	0.760±0.16	1.16±0.06
Cellobiose	740±6.42	912±8.64	1.010±0.08	0.520±0.06	0.69±0.14
Sucrose	810±6.20	978±11.2	0.332±0.12	0.654±0.08	0.46±0.02
Lactose	760±8.24	832±9.24	0.421±0.08	0.596±0.04	0.32±0.01

Data represents the mean ± SEM (n=3).

Table 4. Effect of supplementation of different nitrogen source on cellulase production by *Cladosporium* sp. NCIM 901

Nitrogen source	Glucosamine (mg/g)	Total protein (μg/ml)	Cellulase activity		
			FPase (U/ml)	CMCase (U/ml)	β-glucosidase (U/ml)
Control	540±9.24	160±2.40	1.620±0.16	0.621±0.18	1.05±0.16
KNO_3	930±16.8	300±4.60	1.710±0.24	0.935±0.08	1.236±0.02
Urea	870±12.4	292±2.16	1.512±0.10	0.762±0.04	1.092±0.01
Peptone	760±8.64	180±2.10	1.210±0.08	0.440±0.02	0.962±0.04
Yeast extract	650±4.64	120±1.42	0.610±0.12	0.324±0.06	0.932±0.02

Data represents the mean ± SEM (n=3).

Table 5. Cellulase production by *Cladosporium* sp. NCIM 901 on different lignocellulosic substrates

Lignocellulose	Glucosamine (mg/g)	Total protein (μg/ml)	Cellulase activity		
			FPase (U/ml)	CMCase (U/ml)	β-glucosidase (U/ml)
Control	540±4.28	810±8.42	1.634±0.08	0.625±0.01	1.052±0.06
Saw dust	916±9.84	1250±4.80	1.835±0.06	0.927±0.04	0.914±0.02
Bagasse	856±6.42	620±6.40	1.942±0.08	0.952±0.02	0.986±0.24
Sorghum straw	842±8.20	640±2.60	1.598±0.02	0.796±0.07	0.962±0.08
Rice straw	732±4.40	490±1.80	0.982±0.04	0.874±0.04	1.034±0.22

Data represents the mean ± SEM (n=3).

in these substrates. The rate of bioconversion of cellulosic substrates, steam pretreated spruce and solks-floc cellulose by cellulolytic enzymes derived from the growth of *Trichoderma reesei* Rut C-30 in fermentor was higher than that achieved with commercially available celluloclast and Iogen cellulase (41). The β-glucosidase activity and CMCase activity of these fungi is low compared with FPase on bagasse. However the addition of urea as nitrogen source the β-glucosidase yield was further improved by the *T. reesei* 97.177 (35). Saw dust is indicated as a good inducer of cellulase activity in the organism. Peak cellulase activity values obtained were observed to be 100.0 and 92.2 U/L respectively, obtained for the cultures containing cellulose and saw dust, by the organism *Penicillium chrysogenum* PCL501 (30). According to studies of Gonzlez *et al*. (42), higher enzymatic activities (FPase and β-glucosidase) were obtained with growth of *T. reesei* GM 9414 on wheat straw rather than on solka-floc as carbon source. In a comparative study of cellulase production by *T. reesei* Rut C-30 highest titerss of 4 U/ml on popular wood at 2% level in both shake flask and fermentor was obtained (10). Wheat straw (WS) and wheat bran (WB) combinations exhibited superior performance than those obtained when each one of these carbon sources was examined individually. The addition of WB substantially improved endoglucanase and endoxylanase activities; 58 and 71% higher values than those achieved with WS, respectively. Maximum enzyme activities were observed for the lowest WS/WB ratio tested (25 to 5 w/w), and this condition was adopted in subsequent experiments. Induction of cellulolytic and hemicellulolytic activities as a result of incorporation of WB into growth media is well documented. Both mesophilic and thermophilic fungi have been reported to produce enhanced levels of lignocellulose degrading enzymes under solid state conditions of WB and straw mixtures (42, 43). Similarly, in the present study *Cladosporium* sp. produce high cellulolytic enzymes on bagasse and saw dust. The production of cellulase by organisms on different lignocellulosic material depend on various factors such as variable cellulose content in lignocelluloses derived from different plant sources, heterogeneity of structure and cellulolytic ability of the organisms at different degree. However, the production of cellulase on the treated lignocelluloses further needs to be assessed.

Three pretreatments were given to bagasse and subjected to fermentation by *Cladosporium* sp. NCIM.901 and the results are represented in Fig. 1, Fig. 2 and Fig. 3. The maximum yield of reducing sugar and the highest cellulolytic activity was obtained when sugarcane bagasse used as a substrate is in the powdery (~0.3 mm meshed) form. The highest cellulolytic enzyme activities on powdery form substrate were observed at 7th day with the enzymatic activities 1.972, 1.103 and 1.112 U/ml of FPase, CMCase and β-glucosidase respectively. The maximum reducing sugar concentration of 0.52 mg/ml was also high in 7 days period. This is due to greater accessibility of enzyme to this powdery substrate than the granular form. Two factors most widely believed to control enzymatic digestibility are the accessible surface area and the crystallinity of the cellulosic (44) fraction. Effective pretreatment processes must therefore increases the degree to which the cellulosic fraction of the lignocellulosic material is susceptible to enzymatic attack and/or decrease the extent of cellulose microfibril crystallinity. Increased accessibility of cellulose enables more enzymes to bind to cellulose fiber surfaces; where as decreased crystallinity increases the reactivity of cellulose, i.e., the rate at which bound enzyme hydrolyzes glycosidic linkages (12). Milling can improve susceptibility to enzymatic hydrolysis by reducing the size of the materials, and degree of crystallinity of lignocelluloses, which improves enzymatic degradation of these materials toward ethanol or biogas (45). Without any pretreatment, corn stover with sizes of 53-75 ìm was 1.5 times more productive than larger corn stover particles of 425-710 μm (46). Sidiras and Koukios (47) showed that due to crystallinity reduction by ball milling, saccharification of more than 50% of

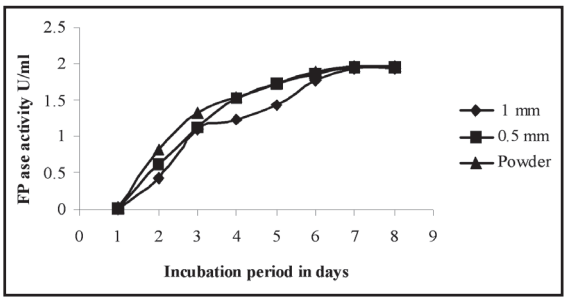

Fig. 1. FPase activity of fungi on 1 mm, 0.5 mm and bagasse powder.

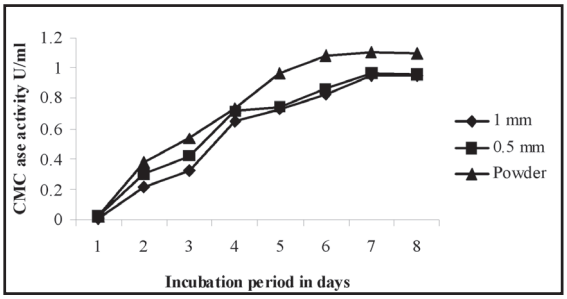

Fig. 2. CMCase activity of fungi on 1 mm, 0.5 mm and bagasse powder.

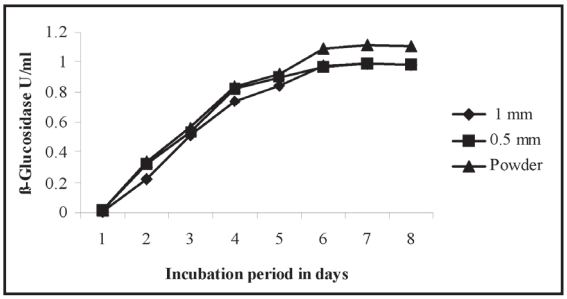

Fig. 3. β-glucosidase activity of fungi on 1 mm, 0.5 mm and bagasse powder.

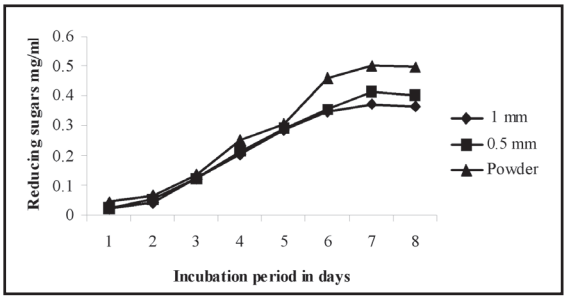

Fig. 4. Production of reducing sugars by fungi on 1 mm, 0.5 mm and bagasse powder.

straw cellulose with minimal glucose degradation becomes possible at mild hydrolytic conditions. The crystallinity index of solka floc by ball milling changed from 74.2 to 4.9% (48).

Conclusion

Among the three media tested the *Cladosprium sp*. NCIM 901 has showed high enzyme activity in Mary Mandel's mineral medium. In the present study *Cladosporium* sp. produced high cellulolytic enzymes on bagasse and saw dust. Decrease in the crystallinity of lignocellulosic substrate (powder form of ~0.3 mm bagasse) increased the enzymic activity. Further optimization of medium constituents by applied statistical designs is needed in order to increase the enzyme activities of the fungal strain.

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References

1. Perez, J., Munoz-Dorado, J., De-la-Rubia, T. and Martinez, J. (2002). Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. *Int. Microbiol*. 5:53–63.
2. Coughlam, M.P. (1990). Cellulose degradation by fungi, p1-36. in: WM Fogarty, CT Kelly (ed.), *Microbial enzymes and biotechnology*, 2nd ed. Elsevier Applied Science, London, United Kingdom.
3. Ding S.Y. and Himmel, M.E. (2006). The maize primary cell wall microfibril: a new model derived from direct visualization. *J. Agric. Food Chem*. 54:597-606.
4. Zhang, Y. P. and Lynd, L. R. (2004). Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. *Biotechnol.Bioeng*. 88:797-824

5. Himmel, M.E., Adney, W.S., Baker, J.O., Nieves, R.A. and Thomas, S.R. (1996). Cellulases: structure, function, and applications. In handbook on bioethanol-production and utilization. Wyman, C.E. (ed).Washington DC: Taylor & Francis, pp.143–161.

6. Persson, I., Tjerneld, F. and Hahn-Hagerdal, B. (1991). Fungal Cellulolytic enzyme production. *Process Biochem*. 26:65–74.

7. Wen, W., Liao. and Chen, S. (2005). Production of cellulase by *Trichoderma reesei* from dairy manure. *Bioresour. Technol*. 96: 491–499.

8. Beguin, P. and Aubert, J.P. (1994). The biological degradation of cellulose. *FEMS Microbiol Rev*. 13: 25–58.

9. Bhat, M. and Bhat, S. (1997). Cellulose degrading enzymes and their potential industrial applications. *Biotechnol. Adv*. 15:583–620.

10. Shin, C.S., Lee, J.P., Lee, P.S. and Park, S.C. (2000). Enzyme production of *Trichoderma reesei* Rut C30 on a various lingocellulosic substrates. *Appl. Biochem and Biotechnol*. 8486: 237–245.

11. Kang, S.W., Kifm, S.W. and Kimard, K. (1994). Production of cellulases and xylanases by *Aspergillus niger* KKS. *J. Microbiol. Biotechnol*. 4(1):445–743.

12. McMillon, J.D. (1992). Process for pretreating lignocellulosic biomass: A review. NREL/TP-421–4978.

13. Hendriks, A.T.W.M. and Zeeman, G. (2009). Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresour. Technol.* 100:10-18.

14. Palmowski, L. and Muller, J. (1999). Influence of the size reduction of organic waste on their anaerobic digestion. In: II International Symposium on Anaerobic Digestion of Solid Waste. Barcelona 15–17 June, 137–144.

15. Furcht, P.W. and Silla, H. (1990). Comparison of simultaneous wet milling and enzymatic hydrolysis of cellulose in ball mill and attrition mill reactors. *Biotechnol Bioeng*. 35(6): 630–645.

16. Jeffries, T. W. (1996). Production and applications of cellulase laboratory procedures. Laboratory Procedures Hand Book, US Army Meterials Laboratories. pp: 1–10.

17. Mandels, M. and Andreotii, R.C. (1976). Measurement of saccharifying cellulase. *Biotechnol. Bioeng. Symp*. 6: 21–23.

18. Miller, G.L. (1959). Use of dinitrosalicylic acid for determination of reducing sugar. *Anal. Chem*. 31: 426–429.

19. Ghosh, T.K. (1987). Measurement of cellulase activities. *Pure Appl. Chem*. 59: 257–268.

20. Herr, D. (1979). Secretion of cellulase and â-glucosidase by *Trichoderma viride* ATCC 1433 in submerged culture on different substrates. *Biotechnol. Bioeng*. 21: 1361–1371.

21. Lowry, O.H., Rosebrough, N.J., Farv, A.L. and Randall, R.J. (1951). Protein measurement with Folin Phenol reagent. *J. Biol. Chem*. 193: 265–275.

22. Tsuji, A., Kinoshita, T. and Hoshino, M. (1969). Analytical chemical studies on amino sugar. II. Determination of hexosamines using 3-methyl-2-benzothiazolone hydrazone hydrochloride. *Chem. Pharm. Bull*. 17:1505–1510.

23. Ride, J.P. and Drysdale, R.B. (1972). A rapid method for the chemical estimation of filamentous fungi in plant tissue. *Physiol. Mol. Plant Pathol*. 2:7–15.

24. Tamada, M., Kasai, N. and Kaetsu, I. (1989). Improvement of cellulase activity by

immobilization of *Sporotrichum cellulophilum*. *Biotech. Bioeng*. 33: 134–366.

25. Domingues, E.C., Queiroz, J.A., Cabral, J.M.S. and Fonseca, L.P. (2000). The influence of culture conditions on mycelial structure and cellulose production by *Trichoderma reesei* Rut C-30. *Enzyme Microbiol. Technol.* 26: 394–401.

26. Immanuel, G., Akila Bhagavath, C.M., Iyappa Raj, P., Esakkiraj, P. and Palavesam, A. (2007). Production and partial purification of cellulase by *Aspergillus niger* and *A. fumigatus* fermented in coir waste and sawdust. *The Internet J. Microbiol*.3:1.

27. Dixon, M. and Webb, E. (1971). *Enzymes*, Second ed., Longmans and co. ltd, London, Great Britain.

28. Puntambekar, U.S. (1995). Cellulase production by the edible mushroom *Volvariella diplasis*. *World J. Microbiol. Biotechnol*. 11: 695.

29. Menon, K., Rao, K. K. and Pushalkar, S. (1994). Production of â-glucosidase by *Penicillium rubram* O stall. *Indian J.Exo.Biol*. 32: 706–709.

30. Bagga, P. S. and Sandhu, D. K. (1987). Cellulase formation by *Asperigillus nidulans*. *J. Ferment. Technol*. 65: 635–642.

31. Nwodo-chinedu, S., Okachi, V.I., Smith, H. A., Okafor, U. A., Onyegeme-Okerenta, B. M. and Omidijii, O. (2007). Effect of carbon sources on cellulase production by *Penicillium chrysogenum* PCL501. *Afr. J. Biochem. Res*. 1006–1010.

32. Nagy, Z., Keresztessy, Z., Szentirmai, A. and Biro, S. (2001). Carbon source regulation of beta-galactosidase biosynthesis in *Penicillium chrysogenum. J. Basic Microbiol*. 41 (6): 321–362.

33. Grant, W.D. and Long, P.E. (1981). The carbon cycle. In Environmental Microbiology, Tertiary level Biology, Thomas Litho Ltd., Scotland. pp. 91–116.

34. Berry, D.R. and Paterson, A. (1990). Enzymes in Food Industry. In Enzyme Chemistry, Impact and applications, 2nd ed. CJ Suckling (Ed.). pp. 306–351.

35. Kotchoni, O.S. and Shonukan, O.O. (2002). Regulatory mutations affecting the synthesis of cellulase in *Bacillus pumilus*. *World J. Microbiol. Biotechnol*. 18: 487–491.

36. Deshpande, S.K., bhotmange, M.G., Chakrabarthi T. and Shastri, P.N. (2008). Production of cellulase and xylanase by *Trichoderma reesei* (QM 9414 mutant), *Aspergillus niger* and mixed culture by solid state fermentation (SSF) of water hyacinth (*Eichhornia crassipes*). *Ind J. Chem. Technol.* 15, 449–456.

37. Muthuvelayudham, R. and Viruthagiri, T. (2006). Fermentative production and kinetics of cellulase protein on *Trichoderma reesei* using sugarcane bagasse and rice straw. *Afr J. Biotechnol*. 5 (20):1873–1881.

38. Desai, J.D., Desai, A.J. and Patel, N.P. (1982). Production of cellulase and â-glucosiadase by shake flask culture of *Scytalidium lignicola*. *J. Ferment. Technol.* 60:117–124.

39. Ellouz Chaabouni, S., Hadj-Taieb, N., Mosrati, R. and Ellouz, R. (1994). Preliminary assessment of *Penicillium occitanis* cellulases a further useful system. *Enzyme Microbiol. Technol*. 16:538–542.

40. Khowala, S. and Sengupta, S. (1992). Secretion of glucosidase by *Termitomyces clypeatus*: Regulation by carbon catabolic products. *Enzyme Microb.Tehnol*.14:144–149.

41. Szengyel, Z., Zacchi, G., Varga, A. and Reczey, K. (2000). Cellulase production of *Trichoderma reesei* Rut C-30 using steam pretreated spruce. Hydrolytic potential on different substrates. *Appl.Biochem. Biotechnol*. 84-86: 679–691.

42. Gonzalez, G., Caminal, G. and Lopez, S. (1986). Cellulase production on lignocellulose materials and performance of a packed-bed reactor for enzymatic hydrolysis. *Biotechnol. Bioeng*. Symp. 17: 535–542.

43. Jecu, L. (2000). Solid-state fermentation of agricultural wastes for endoglucanase production. *Ind. Crops Prod*. 11:1–5.

44. Moxley, G., Zhu, Z. and Zhang, Y-H.P. (2008). Efficient sugar release by the cellulose solvent-based lignocellulose fractionation technology and enzymatic cellulose hydrolysis. *J. Agric. Food Chem. 56,* 7885–7890.

45. Taherzadeh, M.J. and Karimi, K. (2008). Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. *Int J Mol Sci*., 9, 1621–1651.

46. Jatinder, K., Chadha, B.S. and Saini, H.S. (2006). Optimization of culture conditions for production of cellulases and xylanases by *Scytalidium thermophilum* using response surface methodology. *World J. Microbiol. Biotechnol*. 22: 169–176.

47. Zeng, M., Mosier, N.S., Huang, C.P., Sherman, D.M. and Ladisch, M.R. (2007). Microscopic examination of changes of plant cell structure in corn stover due to hot water pretreatment and enzymatic hydrolysis. *Biotechnol. Bioeng*. 97:265–278.

48. Sidiras, D. K. and Koukios, E.G. (1989). Acid saccharification of ball milled straw. *Biomass*.19:289–306.

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Organic-inorganic Hybrids of Nano Silica and Certain Botanical Compounds for their Improved Bioactivity Against Agricultural Pests

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Abstract

Control of lepidopteran insects which are serious pests of agriculture causing major economic losses is always a challenge, particularly when the whole world is insisting to reduce the usage of harmful synthetic pesticides. In this study, we formulated two terpene compounds, Nerolidol and Carene with silica nanoparticles (SINPs) by simple immersion method and investigated the interaction between these terpenes and SINPs controlled release, suspension stability and bioactivity against two major agricultural pests. Nerolidol and Carene showed considerable antifeedant activity in their normal state which could be further enhanced significantly by formulating with silica nanoparticles. Chemical characterization of nanobiocomposites by Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) revealed the formation of additives and their enhanced dispersion on formulations. These Nano-biocomposites also showed prolonged shelf-life and bioactivity.

Keywords: Terpenes; Silica nanoparticles; nano-biocomposites; nano-bioformulations; insect antifeedant; agricultural pest control.

Introduction

Indian climate is suitable for the growth of pests and diseases, hence annually huge amounts are lost during the crop growth or even after harvesting. Among the herbivorous pests, Lepidopteran insects are important that their feeding results in severe crop losses leading to economical constrain. Sometimes it is recorded even up to more than 30% economic losses to various crops (1, 2). In a process of elimination of these pests, farmers apply high concentrations of chemical pesticides which resulted in several unwanted secondary effects such as contamination of the environment, bio magnification and affecting non target organisms. Due to the constant use of synthetic pesticides many pests are now even developed resistance (3). All these, lead to the development and usage of plant originated pest management chemicals that are biodegradable. In recent years due to the more and more awareness and concern to the environment, the botanical pesticides utilization has enhanced considerably. Till now several plants have been exploited for the pest control purpose and reports are available on their biological activity against important crop pests (4, 5). Many of the botanicals does not affect the seed germination or seedling growth on treatment (6) which as an added advantage. Compounds that affect the feeding behaviour of the insect pests, preventing them from feeding thus: leading to its starvation death are known as antifeedant compounds. These behavior modifying substances are gaining tremendous importance in recent years.

However botanical compounds have some limitations such as low bio availability, high

volatility and photo degradation that restrict their use in several occasions (7). It is essential to enhance the shelf life of the biological compounds for their better use and to prevent from faster degradation. This can be done by adopting some of the techniques in nanotechnology such as, nanoencapsulation, entrapment in polymers or dendrimers, which can reduce the degradation of botanicals and also deliver the compound in a control release pattern (8). Silica nanoparticles (SINPs) are known for their easy preparation and for their wide range of industrial as well as biological application (9). The diversified possibilities in chemical and physical modifications in silica nanoparticles increase its effectiveness, and its biocompatibility makes it suitable for biological applications. The use of silica nanoparticles are not only limited to DNA delivery, It can also be utilized in agriculture and veterinary sectors (10) as an entomotoxic agent (8) and also applied as an anti-malarial compounds in poultry farm (11). Although Silica nanoparticles are inert in nature, after formulating with botanical compounds, they enhance the bio availability of the compound as well as persistence of the active ingredient by regulating their degradation.

The present study was performed to investigate the antifeedent potential of Nerolidol and Carene, two important plant compounds and preparation of their formulations with silica nanoparticles for their improved shelf life and bioactivity. Nerolidol (3,7,11-trimethyl–1,6,10-dodecatrien-3-ol) also known as peruviol, is a naturally occurring and predominant sesquiterpene, which has been reported in many essential oils (12). Particularly, Nerolidol was main components of the *Cassia fistula* flower oil (38.0%) (13). Doskotch *et al.* (1980) isolated Nerolidol from *Melaleuca leucadendron* leaves as a feeding deterrent against gypsy moth larvae (14). Yang *et al.* (2011) reported the presence of Nerolidol in the essential oil of *Rhododendron anthopogonoides* aerial parts which possessed significant toxic action against maize weevils, *Sitophilus zeamais* (15). Nerolidol isolated from *Artemisia chamaemelifolia* Vill. has effective antibacterial activity (16). Essential oil containing Nerolidol was extracted from *Artemisia lavandulaefolia* DC. That showed insecticidal activity towards *Sitophilus zeamais* (17). Nerolidol has been documented to have significant antibacterial (18), antimalarial (19), antifungal (20) antileishmanial (21) and antiulcer activities (12).

Carene (3,7,7-trimethylbicyclo (4.1.0) hept-3-ene) also known as delta-3-carene, is a bicyclic monoterpene which occurs naturally as a constituent of turpentine oil of anethi oil (*Anethum sowa*) showed antifeedant activity against adults of *Schistocerca gregaria* (Forsk.) (22). Szczepanik *et al.* (2009) reported the enhancement of antifeedant activity of Carene via introduction of α-methylenelactone moiety against Colorado potato beetle, *Leptinotarsa decemlineata* (23). Carene was reported in essential oil of *Baccharis salicifolia*, which exhibited toxic and repellent effects of against *Tribolium castaneum* (24). Fan *et al.* (2011) demonstrated the antifeedant activity of the essential oils obtained from *Piper nigrum* which contain 5.5% of Carene (25). The mosquito larvicidal activity of Carene was also reported earlier (26).

Even though above two compounds were known for several properties, the antifeedant activity towards two of the major insect pests in Andhra Pradesh, *Spodoptera litura* (F) and *Achaea janata* (L) was not been explored. In this paper we present the results of the evaluation of natural Nerolidol and Carene as insect antifeedant compounds against two agricultural pests, *A. janata* and *S. litura* as well as the preparation of their formulations with silica nanoparticles for their enhanced antifeedent activity and improved shelf life. The effective formulations were chemically characterised to assess the interactions between the terpenes and silica nanoparticles.

Materials and Methods

Test insects: Castor semilooper, *Achaea janata* (L.) and the tobacco cutworm, *Spodoptera litura*

(F.) larvae were obtained from the laboratory colonies maintained at CSIR-Indian Institute of Chemical Technology (IICT), Hyderabad, India. The cultures have been continuously maintained on fresh castor leaves (*Ricinus communis* L) at room temperature (28±2°C), 65±5 % RH and a photoperiod of 16:8 h L: D in the laboratory.

Chemicals and compounds: Nerolidol, Carene and Silicon dioxide nanoparticles (SINPs) were purchased from Sigma Aldrich. SINPs were commercial ultra-fine amorphous powder with an average particle diameter of 10 nm and 115 m^2 g^{-1}specific surface area. Chloroform and methanol were of HPLC grade and used as received.

Preparation of nano-formulations: Nerolidol and Carene were formulated with the SINPs using a simple immersing method described by Mellaerts *et al.* (2008) (27). In this, the SINPs about 100 mg was dispersed in volumetric flask containing 6 ml chloroform. After being dispersed, an appropriate amount of terpenes were separately mixed to get final concentrations 10 and 25 percent with dispersed SINPs and stirred with a glass rod to ensure even distribution of the mixture. After sonication for 10 minutes, this suspension was agitated for another 24 hours using a rotary mixer (20 rpm, Snijders, Tilburg, The Netherlands). The chloroform was subsequently removed by evaporation and the powder was vortexed for 20s. The resultant formulations were placed in airtight, self-sealable polyethylene pouches and stored at 25°C in desiccators and used for the bioassays against two major lepidopteron pests.

Instrumental analysis: The interactions between terpene and SINPs were confirmed by analysing the Fourier transform infrared spectroscopy (FTIR) spectra obtained from Spectrophotometer (Nicolet Co., Nexus-870, USA) with a resolution of $4cm^{-1}$. The sample was made in to pellets by Potassium Bromide (KBr) powder and the peaks were compared with native SINPs. Transmission Electron Microscope (TEM) analyzer (Jeol 100CX–II JAPAN) was used to study the morphology of the formulations. Siemens/D-5000 X-ray diffractometer was used to study the XRD patterns of the nanocomposites. Particle size, diameter and zeta potential of the formulations were measured by using a dynamic light scattering analyzer (Nanopartica, SZ 100, Horiba, Japan). The measurements were taken at a temperature of 25°C by dispersing the formulations in millipore water.

The dispersion stability: The dispersion stability of formulations was assessed according to the method described by Tai *et al.* (2008) (28). Briefly, 100 mg of formulation was suspended into the 100 ml deionised water and allowed to stand at room temperature. At every one-hour time interval, 5.0 ml of dispersion liquid was taken out with a pipette, and the content of SINPs dispersed was determined. The stability of dispersion was estimated from the percentage of dispersed SINPs using the following equation.

$$\text{SINPs dispersed (\%)} = G/G_0 \times 100$$

Where G (g) and G_0 (g) are the weight of SINPs dispersed after and before standing, respectively.

Antifeedant bioassay: Antifeedant activity of the formulations was observed according to the classical no-choice leaf-disc bioassay described by Devanand and Usha Rani (2009) (29). In this method, 21 cm^2 area of castor leaves were cut and their upper surfaces were treated with different concentrations of the nanoformulations. The treated leaf discs were transferred to petri plates (15 cm dia) lined with moist filter paper. Control leaf discs were treated with the same volume of solvent alone. In each petri dish, pre-starved healthy third instar larvae of *A. janata* and *S. litura* were introduced individually and were allowed to feed on the leaf discs. The consumption of the leaf area was measured after 24 h in both treated and control leaf disks using an AM-300 leaf area meter (ADC, Bioscientific Limited, England). The antifeedant index was then calculated with the formulae [(C - T)/(C + T)] x 100, where C is the area of the leaf consumed in control discs, and T is the leaf area

consumed in treated discs. Each set of experiments consisted of 30 replicates and all the experiments were carried out in similar conditions and were repeated 3 times. The results were subjected to probit analysis (30) to determine ED_{50} representing the concentrations that caused 50% feeding deterrence along with the 95% fiducial limits.

Results

***FTIR analysis*:** Interaction between terpenes and SINPs were confirmed by FTIR spectroscopy and the FTIR spectra of formulations and native SINPs were shown in Figs. 1. The wave numbers of the terpenes and their hybrid functional groups are described in this section. Fig. 1(a) represented the FTIR spectra of pure SINPs. The corresponding bands appear at 950 and 1090 cm^{-1}, due to the vibrations of Si-OH and Si-O-Si bands respectively. These bands are very intense and correspond to the formation of the SiO_2 network. Fig. 1(b) shows the FTIR spectra of Nerolidol/Silica formulation. The peak at 2923-3000 cm^{-1} indicates the presence of C-H stretching in Nerolidol compound. The peak shift to lower wave number of OH group position in silica shows the presence of hydrogen bonding between Nerolidol and SINPs. Fig. 1(c) shows the FTIR spectra of Carene formulation. The band at 3400-3600 cm^{-1} indicates the present of O-H stretching. The peak at 2923-3000 cm^{-1} indicates the present of C-H stretching in Carene compound. The chemical interactions between Carene molecules and silica surface sites are clearly demonstrated by hydrogen bonding. The peak shift to lower wave number of OH group position in silica shows the presence of hydrogen bonding. A strong absorption band in the range 1000-1200 cm^{-1} shows the asymmetric stretching vibrations of the Si-O-Si bonds of the silica component in all the Carene-silica hybrid formulations. The band at 945-968 cm^{-1} show the Si-OH vibration, the intensity is decreased with increasing the Carene concentration.

TEM analysis: TEM results in the form of images were presented in Fig. 2. The obvious

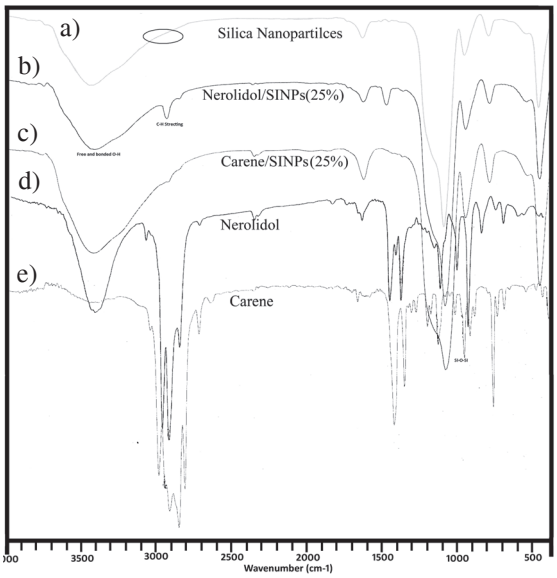

Fig. 1. FT-IR spectra of Silica nanoparticles, Nerolidol/ nanosilica composites and Carene/ nanosilica composites, Nerolidol and Carene,.

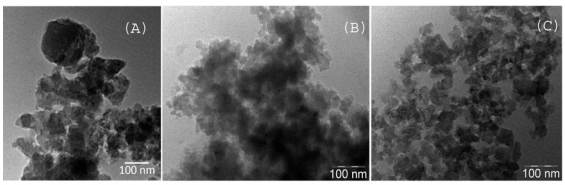

Fig. 2. TEM micrographs of (a) native silica nanoparticles (SINPs) (b) Nerolidol/nanosilica composites and (c) Carene/ nanosilica composites.

agglomeration was present in both the formulations, while the formulations exhibited much less agglutination in comparison with the native silica nanoparticles. Among the nanoformulations CSI showed well dispersed in the suspension. This suggests a bonding either physical or chemical occurs between the terpene and SINPs, which reduces the surface free energy and controls the agglomeration. The above results indicate that adsorption of terpene has the advantage that besides being less agglutination it also has got the high dispersion stability.

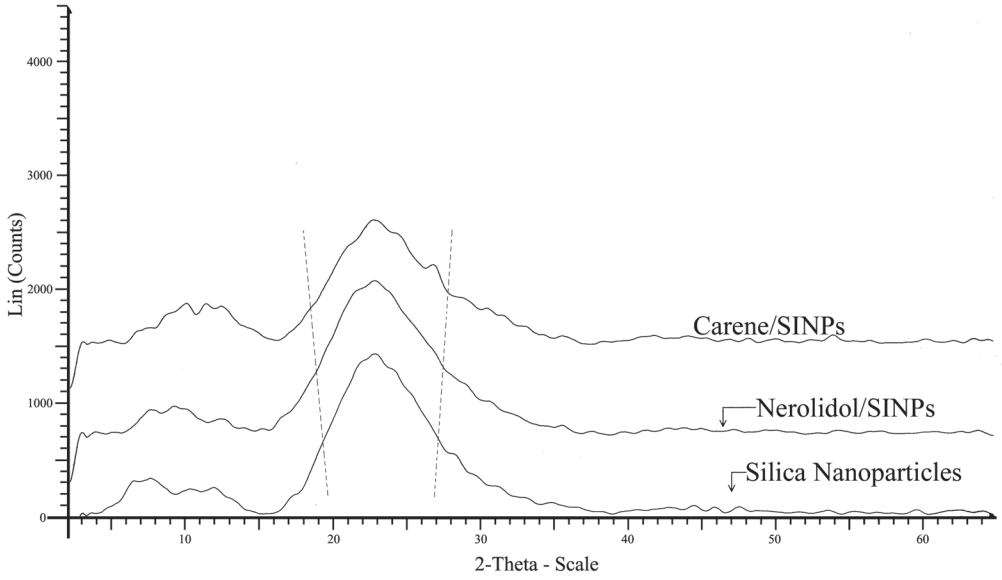

Fig. 3. XRD spectra of silica nanoparticles, Silica nanocomposites of Nerolidol, Carene

XRD analysis: X-ray diffraction images showed a broad peak in the range of 18–30° (2θ) and the broadness of peak was increased on formulating with Nerolidol and Carene confirms the formation of additive (Fig. 3). The peaks in spectrum, indicates an amorphous structure and lacks long range order. Primary building units of amorphous silica, SiO_4 tetrahedra, were connected to each other and do not exhibit a regular pattern like crystalline silica polymorphs quartz or tridymite.

Particle size and zeta potential analysis: The native silica nanoparticles dispersed in the millipore water, exhibited a hydrodynamic particle size (32.68 nm) and zeta potential value of -28.8 mV. A slight change in the particle size was observed NSI and CSI (54.3 and 68.6 nm respectively) in compared with native silica naoparticles. The formulations exhibited higher zeta potential value (CSI and NSI with -68.4 mv and -39.7 mV respectively), which is superior to that of SINPs (-28.8 mV) as shown in Fig. 4. Interaction of Nerolidol and Carene might cause easy de-protonated of hydroxide groups (Si–OH–terpene) to form Si-O which enhances the surface more negatively charged.

The dispersion stability: Formulations displayed high dispersion stability in compared with native SINPs. Complete precipitation was observed in SINPs within 2 days; while CSI and NSI have a stable colloidal dispersion even after 7 days. Interaction of hydroxyl groups (-OH) of SINPs with terpene played a vital role in reducing the agglutination of the silica nanoparticles for a longer period.

Antifeedant bioassays: The antifeedant activity of the formulations is directly proportional to the treated dosage as well as the concentration of terpenes present in the formulations. The activity of the nano-formulations was compared with that of parent compounds and presented in Table 1. We have calculated the accurate amount of terpene present in the treated dose of formulations. The parent terpenes, Nerolidol and Carene showed antifeedant activity against *A. janata* and *S. litura*, however, the *A. jantha* was

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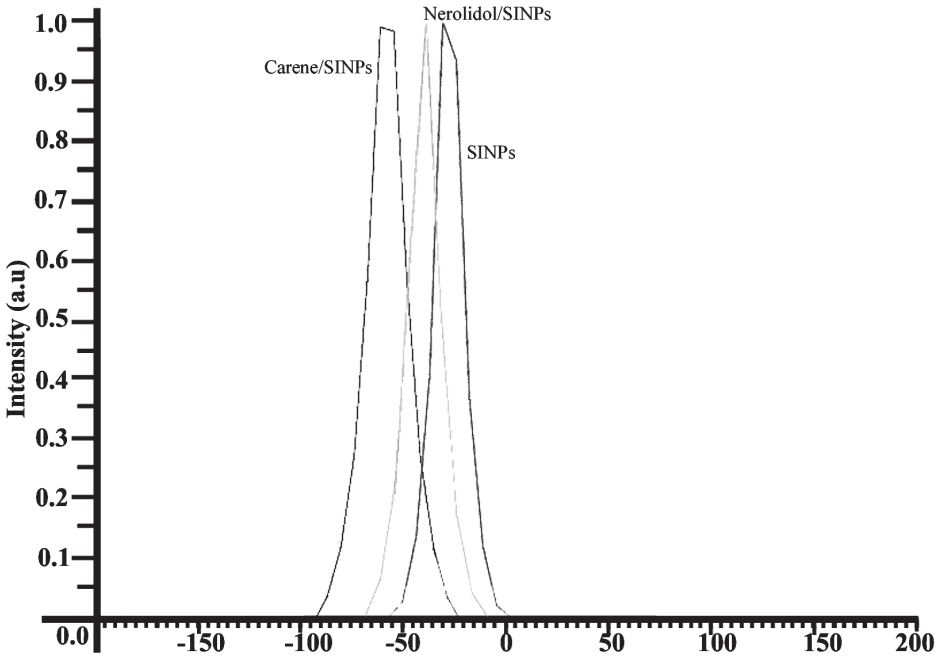

Fig. 4. The zeta potentials of the silica nanoparticles (SINPs) silica nanocomposites of Nerolidol and Carene.

more susceptible to the treatment than *S. litura* with ED_{50} values between 6-9 µl cm^{-2}. After formulating these two compounds the activity has been increased (with ED_{50} value between 2-6 µl cm^{-2}). The formulations with Nerolidol showed higher activity than the Carene formulations. The quantity of the terpene in the formulations played a very important role in executing the bioactivity and the bioactivity increased with the increase in the terpene quantity (Table 1). Interestingly, the SINPs alone did not produce any toxicity or antifeedant activity to the test insects even at higher concentration tested (15 µg cm^{-2}).

Discussion

The two terpene compounds Nerolidol and Carene were highly potent in inhibiting the feeding activity of the two Lepidopteran insects tested.

Table 1. Antifeedant activity of nano-biocomposites against *S. litura* and *A. janata.*

S.No	Formulations	ED_{50} (95% FL[a]) µl cm^{-2}	
		S. litura	***A. janata***
1	Nerolidol	7.21 (3.46 – 9.78)	6.13 (4.26 – 8.31)
2	NSI (10%)	4.12 (2.98 – 7.32)	3.68 (2.13 – 5.72)
3	NSI (25%)	3.16 (1.98 – 4.43)	2.80 (1.82 – 4.37)
4	Carene	9.83 (6.28 – 14.52)	8.96 (5.26 – 11.02)
5	CSI (10%)	7.57 (5.67 – 9.16)	6.69 (4.01 –10.36)
6.	CSI (25%)	6.03 (4.37 – 8.72)	5.11 (3.34 – 7.45)
7.	SINPs (µg cm^{-2})	> 15	>15

[a] Fiducial limits

Interestingly, their formulation with silica nanoparticles has improved this bioactivity to several folds. Moreover these two compounds form effective composites on formulating with silica nanoparticles which enhanced their shelf life for more than six months. The parent terpenes, Nerolidol and Carene are effective in restricting the feeding activity of the insects which lead to the death of the insect due to starvation. However, the shelf life and their persistence on treated leaf is limited due to their volatile nature which restricts their use in the field (31, 32). In this study we have attempted to overcome these problems by formulating the terpene compounds with silica nanoparticles. FTIR analysis reveals that, the peaks corresponding to the silica was not disturbed on formulation, suggesting the interaction as a simple adsorption and are not chemically reacted (31). Increase in peak intensity at 2964 cm^{-1} with the increase of the Nerolidol and Carene concentration in formulations resulted in quantitative increase in adsorption (33). High zeta potential of the CSI suggests that the silica nanoparticles were well stabilised on formulating with Carene which enhances its dispersion stability with higher dispersion for more than 7 days while the native silica nanoparticles were precipitated within 2 days. Similar results were reported by Tai *et al*. (2008) that, silica nanoparticles were stable after stabilization with maleic anhydride in the solution for four days (28).

Bioactivity of Nerolidol and Carene have enhanced on formulation with silica nanoparticles. Nerolidol and Carene showed 50 % antifeedant activity at a dose range from 6- 9 µl cm^{-2} while upon formulating the same compounds with nano silica particles the dose required (2- 6 µl cm^{-2}) for the same activity being reduced to 2 folds. Dispersion and TEM studies reveal the even distribution of nanoformulations which results in enhancing the bioavailability of the compounds. Previously Liu *et al.* (2008) reported the similar enhanced bioavailability of bifenthrin on-formulation with nano particles (34).

Throughout the antifeedant bioassays with terpenes and their nano-formulations, nibbling was observed on the treated leaf discs, indicating that the larvae tried to feed as there is no volatile repellence and due to antifeedant activity of the compounds the feeding rate was inhibited. When the fresh leaves were supplied to the treated larvae, they fed and moulted in to normal adults, providing evidence that neither terpenes nor nano-formulations have repellent or toxic activity and the larval death during the experimentation was merely due to starvation.

Silica nanoparticles used in the formulation is effective in enhancing the dispersion and antifeedant activity and XRD analysis confirm that all the formulations are in amorphous nature. World health organisation and US department of agriculture reported that the amorphous silica particles are safe for humans (11). Therefore, the formulations may not have ill effects on environment after treatment on plant or soil in the fields (35). The nano nature of the formulations offers the compounds to distribute evenly over the treated leaves to enhance the bio availability, activity and also protect the compounds from degradation.

We conclude that, the Nerolidol and Carene can be made effective formulations with silica nanoparticles which can enhance the bio-availability, antifeedant activity against *S. litura* and *A. janata*. The results suggest scope of utilization these nanobiocomposites as insect controlling agents in the future pest management programme as they are environmentally safer than the existing synthetic pesticides.

Abbreviations Used : SINPs: Silica nano-particles; NSI: Nerolidol adsorbed silica nanoparticles; CSI: Carene adsorbed silica nanoparticles; FTIR: Fourier Transform Infrared Spectroscopy; TEM: Transmission Electron Microscope.

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References

1. Oerke, E.C. (2006). Crop losses to pests. The Journal of Agricultural Science.144 (1): 31-43.
2. Dhaliwal1, G.S., Jindal, V. and Dhawan A.K. (2010). Insect Pest Problems and Crop Losses: Changing Trends Indian J. Ecol. 37(1): 1-7
3. Reichenberger. S., Bach. M., Skitschak. A. and Frede, H.G. (2007). Mitigation strategies to reduce pesticide inputs into ground and surface water and their effectiveness; a review. Sci Total Environ. 384(1-3):1-35.
4. Usha Rani, P., Venkateshwaramma, T. and Devanand, P. (2011). Bioactivities of *Cocos nucifera* L. (Arecales: Arecaceae) and *Terminalia catappa* L. (Myrtales: Combretaceae) leaf extracts as post-harvest grain protectants against four major stored product pests. J of Pest Science. 84(2):235-247.
5. Pavela, R. and Vrchotova, N. (2013). Insecticidal effect of furanocoumarins from fruits of *Angelica archangelica* L. against larvae *Spodoptera littoralis* Boisd. Ind. Crop. Prod.43:33-39
6. Usha Rani, P. and Madhusudhanamurthy, J. (2008). Botanical treatment for grain protection and their effects on seed germination and seedling performance of stored maize. J of biopest. 1: 118-121.
7. Dayan, F., Cantrell, C. and Duke, S. (2009). Natural products in crop protection. Bioorg Med. Chem. 17: 4022-4034.
8. Debnath, N. Das, S. Seth, D., Chandra, R. Bhattacharya, S.C. and A. Goswami, (2011). Entomotoxic effect of silica nanoparticles against *Sitophilus oryzae*. J. Pest Sci. 84: 99-105.
9. Xu, H. Yan, F. Monson, E.E. and Kopelman, R. (2003). Room-temperature preparation and characterization of poly (ethylene glycol) coated silica nanoparticles for biomedical applications, J Biomed Mater Res. 66: 870–879.
10. Debnath, N. Mitra S. Das, S. and Goswami, A. (2012). Synthesis of surface functionalized silica nanoparticles and their use as entomotoxic nanocides. Powder Technol. 221: 252–256
11. Rahman, A. Seth, D. Mukhopadhyaya, S.K. Brahmachary, R.L. Ulrichs, C. and Goswami, A. (2009). Surface functionalized amorphous nanosilica and microsilica with nanopores as promising tools in biomedicine. Naturwissenschaften. 96 :31–38.
12. Klopell, F.C., Lemos, M., Sousa, J.P., Comunello, E., Maistro, E.L., Bastos, J.K. and Andrade S.F. (2007). Nerolidol, an antiulcer constituent from the essential oil of Baccharis dracunculifolia DC (Asteraceae). Z Naturforsch C. 62:537–542.
13. Tzakou, O., Loukis, A. and Said, A. (2007) Essential Oil from the Flowers and Leaves of *Cassia fistula* L., J Essent Oil Res. 19:4, 360-361
14. Doskotch, R.W., Cheng, H.Y., Odell, T. M. and Girard, L., (1980). Nerolidol an antifeeding sesquiterpene alcohol for gypsy moth larvae from *Melaleuca leucadendron*. J Chem Ecol. (4); 845-851.
15. Yang, K., Zhou, Y.X., Wang, C.F., Du, S.S., Deng, Z.W., Liu, Q.Z. and Liu, Z.L. (2011). Toxicity of *Rhododendron anthopogonoides* essential oil and its constituent compounds towards *Sitophilus zeamais*. Molecules. 16(9):7320-30.

16. Masoudi, S., Rustaiyan, A., Vahedi, M. (2012). Volatile oil constituents of different parts of *Artemisia chamaemelifolia* and the composition and antibacterial activity of the aerial parts of *A. turcomanica* from Iran. Nat Prod Commun. 7(11):1519-22.

17. Yuan, H.B., Shang, L., Wei, C. and Ren, B. (2010) Comparison of constituents and insecticidal activities of essential oil from *Artemisia lavandulaefolia* by steam distillation and supercritical-CO_2 fluid extraction. Chem Res Chin Univ. 26(6), 888-892

18. Inoue, Y., Shiraishi, A., Hada, T., Hiros, K., Hamashima, H. and Simada, J., (2004). The antibacterial effects of terpene alcohols on *Staphylococcus aureus* and their mode of action. FEMS Microbiol Lett. 237: 325–331.

19. Lopes, N.P., Massuo, M.J., Andrade, E.H.A., Maia, J.G.S., Yoshida, M., Planchart, A.R. and Katzin, A.M. (1999). Antimalarial use of volatile oil from leaves of *Virola surinamensis* (Rol.) Warb. By Waiapi Amazon Indians. J Ethnopharmacol. 67:313–319.

20. Lee, S.J., Han, J.I., Le, G.S., Park, M.J., Ghoi, I.G., Na, K.J., and Jeung, E.B.(2007). Antifungal effect of eugenol and Nerolidol against Microsporum gypseum in a guinea pig model. Biol Pharm Bull. 30:184–188.

21. Arruda, D.C., Alexandri, F.L, Katzin, A.M., and Uliana, S.R.B. (2005). Antileishmanial activity of the terpene Nerolidol. Antimicrob Agents Chemother. 49:1679–1687.

22. Saxena, V. S. (1980). Carene ethyl ether and fractions of anethi oil as antifeedant. Indian J Entomol. 42(4): 780-782

23. Szczepanik, M., Szumny, A. and Wawrzenczyk, C. (2009). The Effect of á-Methylenelactone group on the feeding deterrent activity of natural and synthetic alkenes against colorado potato beetle, *Leptinotarsa decemlineata* Say. Pol J Environ Stud. 18(6): 1107-1112

24. Garcýa, M., Donadel, O.J., Ardanaz,C. E., Tonn, C.E. and Sosa, M.E. (2005). Toxic and repellent effects of *Baccharis salicifolia* essential oil on *Tribolium castaneum*. Pest Manag Sci 61:612–618

25. Fan, L.S., Muhamad, R. Omar D. and Rahmani, M. (2011). Insecticidal properties of *Piper nigrum* fruit extracts and essential oils against *Spodoptera litura*. Int. J. Agric. Biol. 13: 517–522

26. Cheng, S.S., Chua, M.T., Chang, E.H., Huang, C.G., Chen, W.J., and Chang, S.T., (2009) Variations in insecticidal activity and chemical compositions of leaf essential oils from *Cryptomeria japonica* at different ages. Bioresour Technol. 100(1):465-70.

27. Mellaerts, R. Jammaer, J. Van Speybroeck, M. Chen, H. Van Humbeeck, J. Augustijns, P. Van den Mooter G. and Martens, J. (2008). Physical state of poorly water soluble therapeutic molecules loaded into SBA-15 ordered mesoporous silica carriers: A case study with itraconazole and ibuprofen. Langmuir 24: 8651-8659.

28. Tai, Y. Qian, J. Zhang Y. and Huang, J. (2008). Study of surface modification of nano-SiO2 with macromolecular coupling agent (LMPB-g-MAH). Chem Eng J 141: 354-361.

29. Devanand, P. and Usha Rani P. (2011). Insect regulatory activity of crude and purified fractions of *Solanum melongena* L., *Lycopersicum esculentum* Mill. and *Capsicum annuum* L. *Journal of Biopesticides*, 4 (3): 118- 13

30. Finney D.J. (1971) Statistical methods in biological assay. In 2nd Ed.; Cambridge University Press: London, England, 1971: 68.

31. Hummelbrunner L. and Isman, M. (2001). Acute, sublethal, antifeedant, and

synergistic effects of monoterpenoid essential oil compounds on the tobacco cutworm, *Spodoptera litura* (Lep., Noctuidae). J Agric Food Chem. 49: 715-720.

32. Isman, M. B. (2002). Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. Annu Rev Entomol., 51: 45-66,

33. Liu, F. Wen, L. Li, Z. Yu, W. Sun H. and Chen, J. (2006) Porous hollow silica nanoparticles as controlled delivery system for water-soluble pesticide. Mater Res Bull. 41: 2268-2275.

34. Liu, Y., Tong Z. and Prudhomme, R. (2008). Stabilized polymeric nanoparticles for controlled and efficient release of bifenthrin. Pest Manage Sci. 64: 808-812.

35. Barik, T., Sahu B. and Swain, V. (2008). Nanosilica - from medicine to pest control. Parasitol Res. 103: 253-258.

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Induction of Somatic Embryogenesis and Long Term Maintenance of Embryogenic Lines of Litchi

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Abstract

We have been successful in the long term maintenance of embryogenic cultures for two cultivars of litchi (*Litchi chinensis* Sonn.) Purbi and Bedana. The embryogenic lines could be stably maintained through frequent sub-culturing on long term maintenance medium containing 2,4-D + IASP without loss of embryogenic competence. The combination of activated charcoal, sucrose, and polyethylene glycol in the embryo development and maturation medium provided the highest number of mature embryos that were not very rich in starch granules but had high phenolic compounds. Nevertheless the matured somatic embryos were successfully germinated on MS medium consisted of MS salts and B5 vitamins with Kinetin 1 mgl^{-1}, Gibberellic acid (GA) 5 mgl^{-1}, coconut water 50 ml l^{-1}, 3% (w/v) sucrose, 7 gl^{-1} agar and grown into normal plants on conversion medium (CM) and its efficiency was different in different cultivars i.e. in Purbi-49% and Bedana-36.1%.

Keywords: *Litchi chinensis*, litchi, embryo conversion, embryo germination, embryo regeneration, embryogenesis

Introduction

The development of an efficient regeneration system is a prerequisite for transformation and production of genetically modified crop plants. Successful embryogenesis and their conversion to litchi plants have been restricted to a few species including Indian cultivars *Litchi chinensis cv Purbi*, *Litchi chinensis cv Bedana and Litchi chinensis cv Shahi etc.* or hybrids involving one of these species (1, 2, 3, 4). Several authors have attempted to induce somatic embryogenesis from vegetative tissues of litchi, such as leaves (5, 6), zygotic embryos (1), and anthers (3). Cell and protoplast culture was reported in Longan (7). In vitro techniques, for the culture of protoplasts, anthers, microspores, ovules and embryos have been used to create new genetic variation in the breeding lines, often via haploid production. Cell culture has also produced somaclonal and gametoclonal variants with crop-improvement potential. The culture of single cells and meristems can be effectively used to eradicate pathogens from planting material and thereby dramatically improve the yield of established cultivars (8). Targeting specific trait gene is predicated upon the ability to regenerate elite selections of what are generally trees from cell and tissue cultures. The integrity of the clone would thereby remain unchanged except for the altered trait. This regeneration system can be utilized for the genetic transformation of perennial tropical and subtropical fruit crops i.e. Longan (*Dimocarpus longan* Lour) and Litchi (*Litchi chinensis* Sonn.) (9). In most of the important fruit crops, tissue culture is well established for plant regeneration either via somatic embryogenesis or organogenesis. Since somatic embryo cultures often originate from a single cell, it is an ideal system for induction of mutations as it helps in preventing chimerism. The rate of somatic embryo germination is very poor, which has become a major hurdle for large-scale plant

multiplication of desirable induced mutants. The major focus will be on the development of mutant lines of fruits with improved tolerance against abiotic and biotic stresses, seedlessness e.g. Litchi, Longan, Guava and Citrus (10). Biotechnology for longan and litchi has been researched for the last 20 years. At first, cell engineering based on tissue culture was emphasized, including: 1. Saving cultures with young and small embryos 2. Anther or pollen culture to obtain haploid plants and 3. Basic research focused on protoplast culture. Subsequently, with the rapid development of biotechnology, research was re-oriented toward genetic engineering, including mainly molecular marker techniques and new germplasm construction by protoplast fusion and transformation (11). The long term maintenance of embryogenic cell lines has not been reported in litchi. Since the reported embryogenic regeneration protocol of litchi which is highly efficient obtained from zygotic embryos is of long duration (9-12 months) and availability of explants (zygotic embryos) once a year so that development of long-term maintenance of cell lines is very important for mass propagation and crop improvement of litchi. However, the long term maintenance of cell lines is highly genotype dependence in litchi like grape which has been reported using mainly liquid medium (12, 13). Embryos produced via liquid culture, often yield morphological abnormal plantlets in litchi like grape (14). These limitations hinder the possible utilization of long-term solid or liquid embryogenic cell suspension cultures for genetic transformation in litchi like grapevines. The difficulties in induction of somatic embryogenesis, the low reproducibility of the results and the narrow temporal availability of explants mean that, once induced and established, the embryogenic cultures are very valuable material in litchi like grape (15). Under certain conditions, litchi like grape, embryogenic cultures have been maintained almost indefinitely constituting a long-term source of somatic embryos (16, 17). In order to study factors affecting the long-term maintenance of embryogenic lines cultured on solid media, we are reporting a method to induce and maintain the embryogenic cultures in two Indian genotypes i.e. Purbi and Bedana of *litchi.* We further report for the first time in litchi that these lines can be maintained for long time and that they can be readily converted into normal plants during that period.

Materials and Methods

Culture initiation: To establish embryogenic cultures, young immature Indian litchi fruits of both cultivars i.e. Purbi and Bedana were collected from Horticulture Division, Bihar Agricultural University, Sabour, Bhagalpur (India) stored in plastic bags at $4^{O}C$ for approximately 24 h. Prior to dissection, fruits were disinfected with a 1.3% sodium hypochlorite (NaOCl) solution containing 0.1 % (v/v) Tween 20 for 10 min and rinsed 4 times with sterile distilled water (5 min/ rinse). The sterilized immature fruits were excised and isolated zygotic embryos were transferred to medium for callus induction and regeneration following the protocol of (1). In the culture initiation only two litchi cultivars i.e. Purbi and Bedana were taken for development of embryogenic lines. This medium containing NN(18) salts supplemented with 30 gl^{-1} sucrose, 100 mgl^{-1} myoinocitol, 500 mgl^{-1} casein hydrolysate, 17 µM indole-3-acetyl-L-aspartic acid (IASP, dissolved in dimethyl sulphoxide), 9 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 µM 6- benzylaminopurine (BAP). The medium pH was adjusted to 5.6 prior to autoclaving and 7.0 gl^{-1} agar were added as the gelling agent. The medium was autoclaved for 20 min at $121^{0}C$. Both 2, 4-D and BAP were added before autoclaving and IASP was added after autoclaving. The medium was dispensed into 100 × 15 mm disposable petri plates (30 ml per plate). Fifteen zygotic embryos were inoculated to each plate in a total of four replicated plates per cultivar. These plates were then incubated in the dark at $25 \pm 2^{0}C$ until callus developed (ca. 45 days). All culture vessels (Petri dishes, Jars) were sealed in aseptic condition.

Long term maintenance of embryogenic lines: To obtain long-term embryogenic lines of

both genotypes i.e. Purbi and Bedana, proembryonal masses (PEMs) obtained in CIM were cultured separately in a long-term maintenance medium (LTMM) containing same constituent of salts as culture initiation medium. To develop LTMM, the effects of combinations of three growth regulators, IASP, 4 μM; 2,4-D, 2 μM; (1,2,3,-thiodiazol-5-yl)-N'-phenylurea (TDZ), 0.2 μM were tested in a randomized complete block experiment in a 2 × 2 × 2 factorial with four replications. All factors other than growth regulators were as described for CIM. Each block included eight different treatments, in a different petri plates. Five inocula consisting of PEMs, approximately 2.0 mm in diameter were plated in each petri plate. After 60 days of incubation under the culture conditions described previously, the experiment was evaluated. The efficiency of each treatment was determined by measuring diameter of the calli at the surface where the cells had been in contact with medium. Some cultures of both genotypes were kept in standard cultural conditions for maintenance of embryogenic lines for long time (approx 2 years) and were sub cultured in every two weeks interval and some cultures were transferred from LTMM to EDMM separately of both cultivars for embryo development, maturation, germination and regeneration into litchi plants. Sometimes to testify the competency of embryogenic lines for embryo development were cultured on EDMM, germinate and regenerated into litchi plants to study conversion efficiency. These embryogenic lines can be used in transformation experiments to express agronomically important trait genes to develop genetically modified litchi plants without spending more time in regeneration system.

Embryo development and maturation: The PEMs that proliferated in LTMM were regenerated in an embryo development and maturation medium (EDMM), which contained the same minerals and organic nutrients as CIM but altered growth regulators. EDMM included 17μM IASP, 10μM α-naphthoxyacetic acid (NOA), 1μM TDZ (Thidiazuron), and 1μM ascorbic acid (ABA). In addition, the effect of three different factors in the medium were tested: 2.5 gl^{-1} activated charcoal (CH), 50 gl^{-1} polyethylene glycol (PEG, MW 3,350 Sigma-Aldrich), and 20 vs, 60 gl^{-1} sucrose. The plated PEMs were incubated in a culture room maintained under 16-h day cycle at 25 ± 2 ^{O}C and light intensity of 60 μmol m^{-2} s^{-1} (cool white fluorescent light). The experimental design was a randomized complete block in a 2 × 2 × 2 factorial, with four replications. Each block contained eight different treatments and each treatment was plotted in a separate Petri dish. Five randomly selected inocula consisting of PEMs (ca.2.0 mm in diameter) were plated in each petri plate. After 45 days of incubation, the number of mature embryos in each callus was counted.

Histological studies: For histological studies, the embryos at various stages of embryogenesis were fixed in FAA for 24 h. Tissues were dehydrated by transferring embryos through an ethanol-xylol series and then were infiltrated and embedded in paraffin and sectioned longitudinally to 6 mm slices with a microtome. The sections were then stained with toluidine blue O for 10 min, washed in water, dried at room temperature, stained with Lugol reagent for starch detection, and washed in water and dried again. The slides were mounted in synthetic resin (Permount) and photographs were taken with Nikon light microscope.

Germination and conversion: The germination of mature somatic embryos were accomplished in the two Indian cultivars i.e. Purbi and Bedana on MS medium containing MS salts, B5 (19) vitamins with 1 mgl^{-1} kinetin, 5mgl^{-1} gibberellic acid, 50 ml l^{-1} Coconut water, 3% (w/v) sucrose and 7.0 gl^{-1} agar. pH was adjusted to 5.6 prior to autoclaving. The germination tests were performed in a series of 100×15 mm disposable Petri dishes in replicated experiments (four replications). Thirty completely developed embryos were harvested from EDMM and transferred to each plate. The plates were incubated in the dark for 7 days and then transferred to light in a culture room maintained

at the conditioned described above. After 14 days under light, germinated embryos were transferred to conversion medium (CM) containing MS (20) salts, vitamins, 20 gl^{-1} sucrose, 100 mgl^{-1} myo-inositol, 0.25 µM BAP, 0.1 µM NAA, agar 7.0 gl^{-1} and pH was adjusted to 5.6 prior to autoclaving and dispensed into jam bottles. The germinated plantlets were allowed to grow in this medium for 30 days and then transferred to MS medium free of growth regulators. The plantlets with fully expanded four to five leaves established on this medium were further transferred to pots in soilrite. They were acclimatized and successfully transferred to the greenhouse. The acclimatized plants exhibited normal phenotype and growth habit similar to the original plant.

Statistical analysis: The measurement of embryogenic culture and the number of embryos produced from all of the calli under different treatments were done under stereo microscope and the data recorded as the mean of diameter (mm) as well as the mean number of somatic embryos in each experiment. The statistical significance was calculated by one-way ANOVA followed by Tukey's multiple comparison tests. All data analysis was performed using the Graph Pad software (Graph Pad In Stat. Software Inc. San Diego, CA 92130, USA).

Results

Embryogenic culture establishment: Embryogenic cultures were successfully induced from zygotic embryos in CIM. Since somatic embryos are repetitive in nature, calli were again dedifferentiated from these embryos, when placed in modified CIM (culture initiation medium). The PEMs like structures were observed 45 days after initiation of culture, for Purbi and Bedana (78%, and 52% respectively). PEMs were slow growing, friable, white to dark, with nodular texture (Fig.1 A-D). During establishment stage of in vitro culture both Indian litchi cultivars i.e. Purbi and Bedana explants secrete a large amount of polyphenolics which upon oxidation with the help of polypheno--loxidase, turns brown color (melanin pigment), necroses and death of explants which checks the in vitro culture i.e. proliferation of callus, somatic embryogenesis and regeneration of litchi plants. In Chenghe Yu et al. (1) (Chinese authors worked on Chinese litchi) protocol browning problem was not mentioned but with slight modifications (control of browning, period of cultures etc.) we succeeded to get somatic embryogenesis and regeneration of Indian litchis. It seems that both Chinese and Indian litchis are more or less same.

Long term maintenance of embryogenic culture: CIM was efficiently used to induce embryogenesis in both the Indian litchi cultivars but the medium was inadequate to maintain long term embryogenic cultures as the embryos turned dark and eventually died. Based on preliminary work we lowered the concentration of auxins (IASP and 2, 4-D) and tested these together with TDZ. After a few weeks in various LTMM, the cultures developed globular pro-embryogenic masses. An analysis of 60 days old cultures showed that 2, 4-D alone was the most important growth regulator for the maintenance of long-term embryogenic cultures in both cultivars. It was observed that 2, 4-D was essential for proliferation but also prevented precocious regeneration and germination that could lead to the loss of embryogenic competence (Fig. 1D). The interaction 2, 4-D × TDZ was significantly negative ($p > 0.05$) when compared with treatments containing only 2, 4-D or 2, 4-D + IASP ($p < 0.001$) (Table 1) for Bedana. The embryogenic cultures grown on TDZ-supplemented media differentiated and sometimes lost embryogenic competence (Fig. 1D). Many abnormal embryos developed in the medium, which germinated precociously yielding aberrant plantlets that failed to survive. However, treatments which included 2, 4-D + IASP yielded larger embryogenic colonies in both the cultivars with a better response in Purbi (Table 1) (21).

Embryo development and maturation: In the present experiments, several factors were tested to induce the development and maturation of *Litchi chinensis* somatic embryos. The results showed that activated charcoal had a significant

($p < 0.001$) effect on development of embryos (Table 2). Proembryonic masses (Fig. 1E) regenerated somatic embryos (Fig. 1F) when transferred to medium containing charcoal.

The effects of PEG or sucrose alone or in combination were less ($p < 0.01$) significant in embryo development and maturation. However, a significant ($p < 0.001$) interaction between PEG and charcoal was observed in both cultivars (Table 2).The best combination was 50 gl^{-1} PEG, 2.5 gl^{-1} activated CH and 30 gl^{-1} sucrose. These interactions increased the yield of mature embryos in Purbi and Bedana cultivars to 181.5±5.54 and 148.5±4.27 per inoculated PEM respectively in comparison to yields obtained in the treatment that included only charcoal (Table 2). No significant effect of the interaction of charcoal × sucrose was observed in this experiment.

Table 1. Increase of 'Purbi' and 'Bedana' embryogenic culture diameter (mm) after 60 days of culture under different treatments, measured at surface of the media where the cells had been in contact. Mean diameter of embryogenic culture of four independent experiments ± SE.

Treatment	Basal Cross Section (mm) Meandiameter ± SE	
	Purbi	Bedana
Control (without treatment)	2.97 ± 0.19	2.95 ± 0.11
TDZ	3.05 ± 0.13	3.12 ± 0.16
IASP	3.45 ± 0.25	2.92 ± 0.09
2,4-D	7.40 ± 0.36	6.25 ± 0.19
TDZ + 2,4-D	8.22 ± 0.20	3.05 ± 0.18
TDZ + IASP	3.25 ± 0.17	2.92 ± 0.08
2,4-D + IASP	9.42 ± 0.29	9.10 ± 0.49
2,4-D + TDZ +IASP	5.30 ± 0.18	4.27 ± 0.23

Table 2. Number of 'Purbi' and 'Bedana' mature embryos developed from 2.0-mm embryogenic callus, after 40 days culture in EDMM under different treatments. Mean numbers of embryos of four independent experiment ± SE.

Treatment	Number of Embryos	
	Purbi	Bedana
Control (without treatment)	19.50 ± 1.70	20.50 ± 1.04
PEG	46.75 ± 1.93	39.50 ± 2.90
CH	125.00 ± 2.64	77.50 ± 5.95
Sucrose (S)	24.25 ± 2.78	20.00 ± 1.82
PEG +CH	155.25 ± 5.18	139.75 ± 3.32
PEG +S	73.00 ± 4.67	33.25 ± 2.42
CH +S	61.75 ± 4.32	50.50 ± 4.57
PEG +CH + S	181.50 ± 5.54	148.50 ± 4.27

Histological studies: Histology of somatic embryo-producing regions confirmed that the induction of the development process was embryogenic and not organogenic in nature. Light microscope observations of embryogenic mass revealed the presence of nodular structures containing cytoplasmic cells at the central region. Development of somatic embryos appeared to progress through typical globular-, heart-, and torpedo-shaped stage embryo development. The first sign of embryogenesis was marked by the appearance of globular (Fig. 2A) structures the heart stage embryo (Fig. 2B-C), which was bilaterally symmetrical and also showed a broad suspensor like stalk. Some of the structures also had vascular tissue with unipolar meristems which ultimately developed cotyledonary somatic embryos (Fig. 2D). The dark stained meristematic area was often surrounded by parenchymatous tissue. The studies of the embryogenic explants showed that they were not very rich in starch granules but were rich in phenolic compounds that had accumulated primarily in a superficial zone between layers of cells of the peripheral region and storage parenchyma.

Germination and conversion: Germination of somatic embryos is characterized by cotyledon expansion and chlorophyll formation, followed by radicle and hypocotyls elongation. In the present study, 65 % somatic embryos of Purbi obtained from EDMM germinated well but only 32% converted into normal plants with roots, shoots and leaves whereas only 42% somatic embryos of Bedana germinated well and 17% converted into normal plantlets (Fig. 1F-J) in MS medium. Many of the somatic embryos of these cultivars

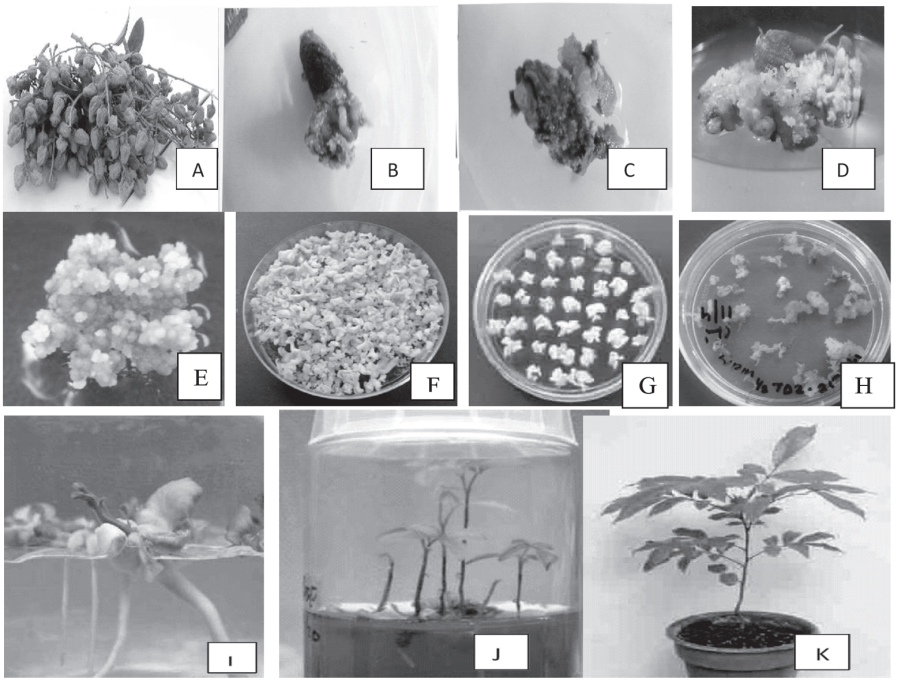

Fig.1. Regeneration of plantlets from embryonic calli from 2.0 mm diameter of somatic embryos after 40-60 days in EDMM (A) Immature litchi fruits from which zygotic embryos were excised (B-C) Proembryogenic callus developed from zygotic embryo after 45 days of culture in CIM (D) Long-term embryogenic culture grown in LTMM supplemented with 2,4-D (E-F) Viable embryogenic callus grown in LTMM supplemented with TDZ and again culture on EDMM supplemented with charcoal for controlling of browning for the development of nodular embryogenic callus which later developed a large number of somatic embryos which were isolated from embryogenic callus. (G-H) Developing plantlet from germinating somatic embryo (J) In-vitro developed litchi plantlet (K) Field grown litchi plantlet.

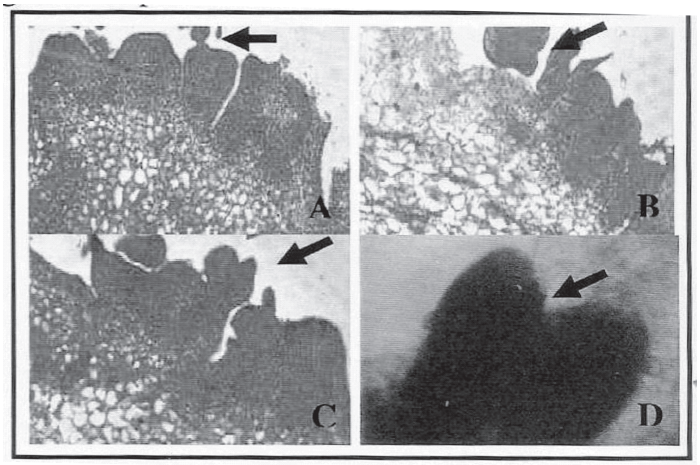

Fig.2. Different stages of somatic embryogenesis (arrow) of litchi in longitudinal sections(A Globular embryo development on the surface of explant (B) Early heart shaped embryo (C)Late heart shaped embryo (D) Cotyledonary stage somatic embryo.

had roots and cotyledons but failed to convert and some were albinos. These normal plantlets have been moved to a greenhouse where they have been growing normally (Fig 1K).

Discussion

The present study describes results obtained for the maintenance of long term cultures for initiation and development of somatic embryos from zygotic embryos of two Indian cultivars of *Litchi chinensis* for which protocols were developed earlier for regeneration (1). Since development of embryogenic calli from zygotic embryo explants takes 9-12 months and it is labour intensive, so we have developed an efficient protocol for long term maintenance of embryogenic calli from zygotic embryos which takes 3-months (unpublished data). Thus with this protocol, without compromising their phenotypic structure, we are able to reduce the time needed to get embryos for these and above mentioned applications. Most of the successful protocols reported for litchi species, have been developed for Chinese *litchi* species and for interspecific hybrids. Successful plant regeneration in litchi has been reported less frequently and in only for a few cultivars (22). In this study we have observed somatic embryogenesis and methods for long term embryogenesis as well as high frequency regeneration of normal plantlets in litchi. The establishment and maintenance of embryogenic cultures in *litchi sp*. is highly genotype dependent and thus far has been confined to a few species and their hybrids. The combination of plant growth regulators is essential for somatic embryo induction, and that explants will not develop on medium depleted of growth regulators. 2, 4-D was the main auxin giving the early results (23, 24, 25). Kikert et al. (26) reported a low frequency embryogenesis (0.1-3%) in anther cultures of *Vitis lambruscana*, after nearly one year from the initiation in various combinations of auxins (2,4-D and NOA) and cytokinin (BAP and CPPU). The higher frequency and precocity of embryogenesis in CIM represent the synergistic effect of combining several auxins. Similar results were not observed if media were supplemented with each of the phytohormone. These results may be credited to the synergistic effect of IASP and 2, 4-D in inducing embryogenesis in litchi. The synergistic effect of these two auxins was previously reported by other authors in grape (27, 21) and it was used the same combinations of growth regulators to induce recallusing in mature somatic embryos of *litchi*. IASP was always superior to free IAA. Most of the IAA in plant tissues exists as conjugated or 'bound' IAA, the latter form is relatively inactive and less sensitive to catabolism or degradation and can be stored or transported in the plant (28, 29).Various synthetic conjugates of IAA have been found to evoke responses similar to those produced by IAA (29). Moreover, some IAA conjugates were more active in inducing soybean callus growth than free IAA (30).

In transformation system, transformants are more efficiently selected from highly synchronized cultures of litchi because the sensitivity of litchi embryogenic tissues to selection agents varies in different stages of embryo development. Synchronized long-term embryogenic cultures were obtained in the treatments which included 2, 4-D alone or 2, 4-D + TDZ or 2, 4-D + TDZ + IASP. In litchi, auxins, especially 2,4-D have been very effective for inducing somatic embryogenesis (1) it is not used in embryogenic callus culture maintenance. We have observed that the embryogenic lines can be maintained for long time (approx. 2 years), under frequent subculturing in LTMM supplemented with 2,4-D with no loss of embryogenic competence and embryo development in litchi (21).

TDZ is among the most active cytokinin like substance highly effective in the induction of somatic embryogenesis on leaf callus of litchi (5). The negative interaction between 2, 4-D and TDZ in Purbi and Bedana in litchi embyogenic cultures may be due to the unsuitable ratio of these growth regulators for these cultivars.

Activated charcoal favours the growth and development of somatic embryos in litchi (21), it may also be attributed to the establishment of a darkened environment, adsorption of inhibitory substances (phenolic compound), adsorption of growth regulators and other organic compounds (31) secreted from the plant tissue during *in-vitro* culture, essential for long-term maintenance of PEMs. However, in *Myrciaria aureana,* charcoal was found to be detrimental to the process (32).

The combinations of PEG and sucrose in the medium have been more effective in the development, maturation and quality of somatic embryos (33) which also increase the deposition of storage proteins similar to zygotic embryos (34). Germination of somatic embryos is characterized by cotyledon expansion and chlorophyll formation, followed by radicle and hypocotyls elongation in suitable medium. Sometimes MS basal medium was found to be inhibitory for somatic embryos germination. This could be due to a lower requirement for a nitrogen source and calcium ions for germination (22). Good quality of embryo production is important for increased rates of germination and conversion, which are limiting steps for a practical use of somatic embryogenesis of litchi (1). With the purpose of preserving the embryogenic cultures, different strategies have been developed in litchi: recurrent secondary embryogenesis without a dedifferentiated stage (35, 36), recurrent cycles of secondary embryogenesis but alternating the differentiated and dedifferentiated stages (37, 15, 27, 38, 25) or the long term maintenance of embryogenic callus culture (36, 39, 40, 41, 42, 12, 21, 43).

This protocol presented here constitutes a variation of the long term embryogenic callus culture, with low level of plant growth regulators to minimize the occurrence of somaclonal variations and maintain the embryogenic cultures in a non differentiated state. Our results in litchi cultivars were within the range of data reported in other cultivars of grape also (36, 44, 42, 21, 17, 38, 43).

Conclusion

This is the first report of successful long term maintenance and recovery of plants from embryogenic cultures of *Litchi chinensis* of two selected Indian cultivars that could be used for cryopreservation. Proembryonic lines obtained in this research are highly synchronized and each major developmental stage (i.e. proembryo, mature embryo and germination) can be controlled by varying the constituents of media. This control should also facilitate the recovery of transformants efficiently.

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References

1. Changhe, Yu., Zhenguang, Chen., Liuxin, Lu. and Jinwen, Lin. (2000). Somatic embryogenesis and plant regeneration from litchi protoplasts isolated from embryogenic suspensions. Plant Cell Tiss Org Cult, 61: 51-58.
2. Yu, Ch. and Chen, Z.G. (1998). Embryogenic suspension culture and protoplast isolation in litchi. Chinese J Trop Crops, 19: 16-20.
3. Yu, Ch. and Chen, Z.G. (1997). Induction of litchi embryogenic calli by immature embryos and anthers culture in vitro. J Fuijian Agri Univ, 26: 168-172.
4. Zhou, L.N., Kuang, Z.S., Ma, X.J., Chen, J.Q. and Cao, J. (1996). The study of factors affecting somatic embryogenesis in young embryo culture of *Litchi chinensis*. J Agric Biotech, 4:161-165.
5. Puchooa, D. (2004). Expression of green fluorescent protein gene in litchi (Litchi chinensis Sonn) tissues. J Appl Hort, 6:11-15.
6. Raharzo, S.H.T. and Litz, R.I. (2007). Somatic embryogenesis and plant regeneration of litchi (*Litchi chinensis*

Sonn.) from leaves of mature phase trees. Plant Cell Tiss Org Cult, 89:113-119.

7. Lai, Z.X., Chen, C.L. and Chen, Z.G. (2001). Progress in Biotechnology research in Longan. ISHS Acta Horticulturae 558: I International Symposium on Litchi and Longan.
8. Brown, D.C.W. and Thorpe, T.A. (1995). Crop improvement through tissue culture. World Journal of Microbiology & Biotechnology, 11:409-415.
9. Miguel, A., Gomez, L.I.M. and Richard, E.L. (2004). Genetic transformation of perennial tropical fruits. In Vitro Cell Dev Biol – Plant, 40:442-449.
10. Mohan Jain, S. (2002). A review of induction of mutations in fruits of tropical and subtropical regions. ISHS Acta Horticulturae 575: International Symposium on Tropical and Subtropical Fruits.
11. Zheng, Q.F., Hu, G.B. and Chen DC (2001). Biotechnology in Longan and litchi breeding: Applications and prospects. ISHS Acta Horticulturae 558: I International Symposium on Litchi and Longan.
12. Perrin, M., Gertz, C. and Masson, J.E. (2004). High efficiency initiation of regenerable embryonic callus from anther filaments of 19-grapevine genotypes grown worldwide. Plant Sci, 167:1343-1349.
13. Maillot, P., Kieffer, F. and Walter, B. (2006). Somatic embryogenesis from stem nodal sections of grapevine. Vitis, 45:185-189.
14. Srinivasan, C. and Mullins, M.G. (1980) High-frequency somatic embryo production from unfertilized ovules of grapes. Sci Hortic, 13: 245-252.
15. Martinelli, L., Candioli, E., Costa, D., Poletti, V. and Rascio, N. (2001b) Morphogenic competence of *Vitis rupestris* S. secondary somatic embryos with a long culture history. Plant Cell Rep, 20:279-284.
16. Gray, D.J., Compton, M.E., Harrel, R.C. and Cantcliffe, D.J. (1995) Somatic embryogenesis and the technology of synthetic seeds.In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry, Vol.30 (pp.126-151). Spring, New York.
17. Martinelli, L., Gribaudo, I., Bertoldi, D., Candoli, E. and Poletti, V. (2001a). High efficiency somatic embryogenesis and plant regeneration in grapevine cultivars Chardonny and Brachetto a grappolo lungo. Vitis, 40:111-115.
18. Nitsch, J.P. and Nitsch, C. (1969). Haploid plants from pollen grains. Science, 163:85-87.
19. Gamborg, O.L., Miller, R.A. and Ojima, K. (1968). Plant Cell Cultures. 1. Nutrient requirements of suspension cultures of soybean root cells, Exp Cell Res 50: 151-158.
20. Murashige, T. and Skoog, F.A. (1962). A revised media for rapid growth and bioassays with tobacco tissue culture. Physiol Plant, 15:473-497.
21. Motoike, S.Y., Skirvin, R.M., Morton, M.A. and Otterbacher, A.G. (2001). Somatic embryogenesis and long-term maintenance of embryogenic lines from fox grapes. Plant Cell Tiss Org Cult 66:121-131.
22. Das, D.K., Shiva Prakash, N. and Sarin, N.B. (1999). Muliple shoot induction and plant regeneration of litchi (*Litchi chinensis* Sonn.). Plant Cell Rep, 18: 691-695.
23. Gray, D.J. and Meredith, C.P. (1992). Grape. In: Hammerschlag FA and Litz RE (eds) Biotechnology of Perennial Fruit Crops (pp.229-264). CAB International, Cambridge.
24. Reisch, B.I. and Pratt, C. (1996). Grapes. In: Janick J. and Moore J.N. (eds) Fruit Breeding: Vine and Small Fruits (pp.297-369).John Wiley & Sons, New York.
25. Das, D.K., Reddy, M.K., Upadhyaya, K.C. and Sopory, S.K. (2002). An efficient regeneration and transformation of grape (*Vitis vinifera* L.) through leaf disc method by somatic embryogenesis. Plant Cell Rep, 20: 999-1005.

26. Kikkert, J.R., Ali, G.S., Striem, M.J., Martens, M., Wallace, P.G., Molino, L. and Reisch, B.I. (1997). Genetic engineering of grapevine (*Vitis* sp.) for enhancement of disease resistance. Acta Hortic, 447:273- 279.

27. Perl, A., Saad, S., Sahar, N., Holland, D. (1995). Establishment of long term embryogenic cultures of seedless *Vitis vinifera* L. cultivar- a synergistic effect of auxins and the role of abscisic acid. Plant Sci, 104:193-200.

28. Wilson, W.J., Waren Wilson, P.M., Walker, E.S. and Hall, P.J. (1992). IAA amino acid conjugates induce differentiation of tracheary strands in lettuce pith explants. Ann Bot, 69:135-144.

29. Cohen, J.D. and Bialek, K. (1984). The biosynthesis of indole-3-acetic acid in higher plants. In: Crozier A and Hillman JR (eds), The Biosynthesis and Metabolism of Plant Hormones. University Press, Cambridge, pp.165-181.

30. Feung, C.S., Hamilton, R.H. and Mumma, R.O. (1977). Metabolism of indole3-acetic acid. IV. Biological properties of amino acid conjugates. Plant Physiol, 59: 91-93.

31. Pan, M.J. and Staden, J.V. (1998). The use of charcoal in *in vitro* culture-a review. Plant Growth Regul, 26:155-163.

32. Motoike, S.Y., Saraiva, E.S., Silva, CV, Salomao (2007). Somatic embryogenesis of *Myrciaria aureana* (Brazilian grape tree) Plant Cell Tiss Org Cult, 89:75-81

33. Etienne, H., Montoro, P., Michaux-Ferriere, N., Carron, M.P. (1993). Effects of desiccation, medium osmolarity and abscisic acid on the maturation of *Hevea brasiliensis* somatic embryos. J Exp Bot, 44: 1613-1617.

34. Mishra, S., Attree, S.M., Leal, I., Fowke, L.C. (1993). Effect of abscisic acid, osmoticum, and desiccation on synthesis of storage proteins during the development of white spruce somatic embryos. Ann Bot, 71:11-22

35. Krul, W.R. and Worley, J.F. (1977). Formation of adventitious embryos in callus cultures of 'Seyval', a French hybrid grape. J Am Soc Hortic Sci, 103: 360-363.

36. Gray, D.J. and Mortensen, J.A. (1987). Initiation and maintenance of long-term somatic embryogenesis from anthers and ovaries of *Vitis longii* 'Microsperma'. Plant Cell Tiss Org Cult, 9:73-80.

37. Martinelli, L., Bragagna, P., Poletti, V. and Scienza, A. (1993). Somatic embryogenesis from leaf and petiole derived callus of *Vitis rupestris*. Plant Cell Rep, 12:207-210.

38. Croce, P., Vannini, C. and Bracale, M. (2005). An easy and convenient method for maintenance of embryogenic culture of *Vitis vinifera* L. Vitis, 44:97-198.

39. Stamp, J.A. and Meredith, C.P. (1988). Somatic embryogenesis from leaves and anthers of grapevine. Sci Hortic, 35:1-16.

40. Matsuta, N. and Hirabayashi, T. (1989) Embryogenic cell lines from somatic embryos of grape (*V.vinifera* L.). Plant Cell Rep, 7: 684-687.

41. Popescu, C.P. (1996). Somatic embryogenesis and plant development from anthers of *Vitis vinifera* L. Plant Growth Regul, 20:75-78

42. Torregrosa, L. (1998). A simple and efficient method to obtain stable embryogenic cultures from anthers of *Vitis vinifera* L. Vitis, 37:91-92.

43. Pinto-Sintara (2007). Establishment of embryogenic cultures and plant regeneration in the Portuguese cultivar 'Touriga Nacional' of *Vitis vinifera* L. Plant Cell Tiss Org Cult, 88:253- 265.

44. Nakano, M., Sakakibara, T., Watanabe, Y., Mii, M. (1997). Establishment of embryogenic cultures in several cultivars of *Vitis vinifera* L and *V. labruscana*. Vitis, 36:141-145.

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Elucidation of Structure and Biocompatibility of Levan from *Leuconostoc Mesenteroides* NRRL B-1149

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Abstract

The water soluble polysaccharide was enzymatically synthesized from *Leuconostoc mesenteroides* NRRL B-1149 using partially purified glycosyltransferases (13.0 U/mg). The average molecular weight of the polysaccharide was found to be 7.2 x 10^6. It was structurally characterized using FTIR, NMR spectroscopy and SEM analysis. The FTIR spectrum displayed the presence of hydroxyl stretching and vibrations in polysaccharide showing the presence of glycosidic bonds. The structure of polysaccharide as revealed by ^{1}H NMR and ^{13}C NMR spectra was linear levan containing β-(2→6) linkages. The SEM analysis revealed its porous structure displaying its importance as water binding agent. The *in vitro* cytotoxicity assay of levan on cervical cancer (HeLa) cell lines revealed it as non-toxic biocompatible polysaccharide showing that it can be used in drug delivery and in other biomedical applications.

Keywords: Levan, FTIR, NMR, Cytotoxicity, HeLa.

Introduction

Fructansucrases are a group of fructosyltransferase (FTF) enzymes (EC 2.4.1.10) which possess inulin synthase or levansucrase activities *i.e.* the polymerization of fructose units of the substrate sucrose into fructan with either inulin with β-(2→1) linkage or levan with β-(2→6) linkage structures. The mode of action of levan synthesizing enzyme is:

Sucrose + (2, 6-β-D-fructosyl)$_n$ → D-glucose + (2, 6-β-D-fructosyl)$_{n+1}$

The FTFs from all the known lactic acid bacteria share >60% amino acid sequence similarity (1) but their structural features which determine various linkage types in fructan are not known. Mostly, fructans produced by bacteria are of levan type and they are synthetized from sucrose by levansucrase (2,3). Levan is a biopolymer with β-(2→6) linkage and occasional β-(2→1) branching. Levan has been reported in many plants and microbial products (2). The biosynthesis of levan has been most extensively studied in a few species like *Zymomonas mobilis* (4) and *Bacillus subtilis* (5). Levan from microbial origin and its derivatives have commercial importance with various industrial applications in agriculture, cosmetics, foods and pharmaceuticals, blood plasma enhancer and sweeteners (6, 7). Levan can also be used as an emulsifier, formulation aid, stabilizer and thickener, surface-finishing agent, encapsulating agent and carrier for flavor and fragrances (2), as an antitumor agent (8) and as an immunostimulator (9). It has been reported that the high molecular weight levan reduces total cholesterol level, however serum triglycerides remain unaffected (10). Levan can also be used in formation of ecofriendly adhesive (11). The reduction in body weight and body fat was observed by intake of levan (12). Fructan degrading enzymes are absent in the upper human digestive tract, hence fructans make a part of non-digestible fibers and act as prebiotics. In the present studies we describe the production and purification of exopolysaccharide levan from *Leuconostoc mesenteroides* NRRL B-1149. The

polysaccharide produced was structurally characterized using Fourier Transform Infrared (FTIR) spectrometry, Nuclear Magnetic Resonance (NMR) spectroscopy and Scanning Electron Microscopy (SEM). The polysaccharide was further analyzed for its biocompatibility assay.

Materials and Methods

Bacterial Strain, medium and growth conditions : The strain *Leuconostoc mesenteroides* NRRL B-1149 was obtained from ARS Culture Collection, National Centre for Agricultural Utilization Research, Peoria, USA. The culture was maintained in modified MRS agar medium (13) as a stab at 4°C and sub cultured every 15 days. A loopful culture from an agar stab was transferred to 5 ml of sterile medium described by Tsuchiya *et al.*, 1952 (14) and incubated at 28°C and 180 rpm for 6-8 h.

Production and purification of levan : The levan was enzymatically synthesized using the method as described earlier (17). To 30 ml of 20 mM sodium acetate buffer (pH 5.4) containing 0.1% (w/v) sodium azide, 10% (w/v) sucrose and 5% (w/v) maltose using 600 µl of purified enzyme (13 U/mg, 0.2 mg/ml) was added. The reaction mixture was then incubated at 28°C and 180 rpm for 24 h. After incubation, the mixture was put in a boiling water bath for 10 min and then centrifuged at 8,000 *g* for 10 min. The pellet containing insoluble dextran was purified and characterized in our study as reported earlier (15). The supernatant containing soluble polysaccharide levan was precipitated using 65% (v/v) final concentration of ethanol. This was repeated two times and finally the pellet containing soluble polysaccharide was re-suspended in distilled water and lyophilized for further studies.

Structural characterization of levan : The reducing value of purified levan was determined by copper (Cu) bicinchoninate method (16). The number-average degree of polymerization (DPn) and averagre molecular weight (MW) were determined by following equations described by Fox and Robyt, 1991 (16).

$$DPn = (Cc/Cm) \times 1.9$$

where, Cc and Cm are Concentrations of carbohydrate sample and maltose as determined by reducing value (mg/ml)

$$MW = [DPn \times 162] + 18$$

The purified levan was structurally characterized using FTIR, NMR and SEM techniques. The FTIR spectrum was recorded in spectrometer (Spectrum One FTIR spectrometer, PerkinElmer Instruments, San Jose, CA, USA) for purified fructan in a KBr pellet. Nuclear magnetic resonance (NMR) spectroscopic analysis was performed in a Varian AS400 spectrometer (Agilent Technologies, Palo Alto, CA, USA). The levan was vacuum dried and then exchanged with deuterium by successive lyophilization steps in D_2O (99.6% atom 2H, Sigma-Aldrich, St. Louis, MO, USA). 15 mg levan sample was dissolved in 0.4 ml of D_2O for ^1H NMR and 30 mg was dissolved in 0.4 ml of D_2O for ^{13}C NMR. Tetramethyl silane (TMS) was used as an internal reference. The SEM analysis of dried levan was done by fixing it to the SEM stub with a double-sided carbon tape. The sample was coated with ~10 nm Au in a sputter coater (Polaron, Model SC7620). The surface of the dry levan powder was viewed in Field Emission Scanning Electron Microscope (Carl Zeiss, Model Sigma) operated at 10.0 kV.

Cytotoxicity and biocompatibility analysis of levan : The lyophilized powder of levan from *L. mesenteroides* NRRL B-1149 was used for *in vitro* cytotoxicity assay. The effect of levan on HeLa cells was determined using the colorimetric 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) assay as reported earlier (17). HeLa cells at a density of 1.2×10^4 cells/well in 100 µl medium were seeded in 96-well plate and allowed to attach at 37°C in 5% CO_2 atmosphere for 12-16 h. After incubation the medium was removed from plate and the levan at different concentrations (1-1000 µg/ml) was added in each well. The medium without levan was used as negative control. The plate was incubated at 37°C in 5% CO_2 atmosphere for 48 h. After 48 h the medium were removed, 100 µl

MTT (500 µg/ml) was added to each well and incubated at 37°C for 4 h. The supernatant was removed and 100 µl DMSO was added to each well. Absorbance was measured at 570 nm by a 96-well microplate reader (Tecan, Infinite 200 Pro).

Results and Discussion

Production and purification of levan from L. mesenteroides NRRL B1149 : The soluble levan purified by ethanol precipitation was lyophilized and the powdered levan was structurally characterized using FTIR, NMR and SEM analysis and was used for biocompatibility studies.

Structural characterization of water soluble levan Number average molecular weight : The number average degree of polymerization (DPn) of purified levan sample as determined by Copper bicinchoninate method was 44887. The number average molecular weight of levan from *L. mesenteroides* NRRL B-1149 calculated using DPn was $7.2x10^6$. The water soluble levan from *Bacillus polymyxa* also showed similar molecular weight of about $2x10^6$ (18). The high molecular weight levans (>10^7) are reported to have applications in reduction of total cholesterol level (10) and in tumor therapy (19).

FTIR analysis of soluble levan : The FTIR data of soluble levan from *L. mesenteroides* NRRL B-1149 is shown in Fig. 1. The band in region of 3422 cm^{-1} showed the presence of hydroxyl stretching vibration of polysaccharide while the band in 2926 cm^{-1} region was due to C-H stretching vibration and the presence of bound water was confirmed by the band in region of 1640 cm^{-1}. The results were supported by Cao *et al*., 2006 (20) and Liu *et al*., 2007 (21). The strong complex absorption at 1122 and 1063 cm^{-1} signified the stretching vibrations of C-O-C

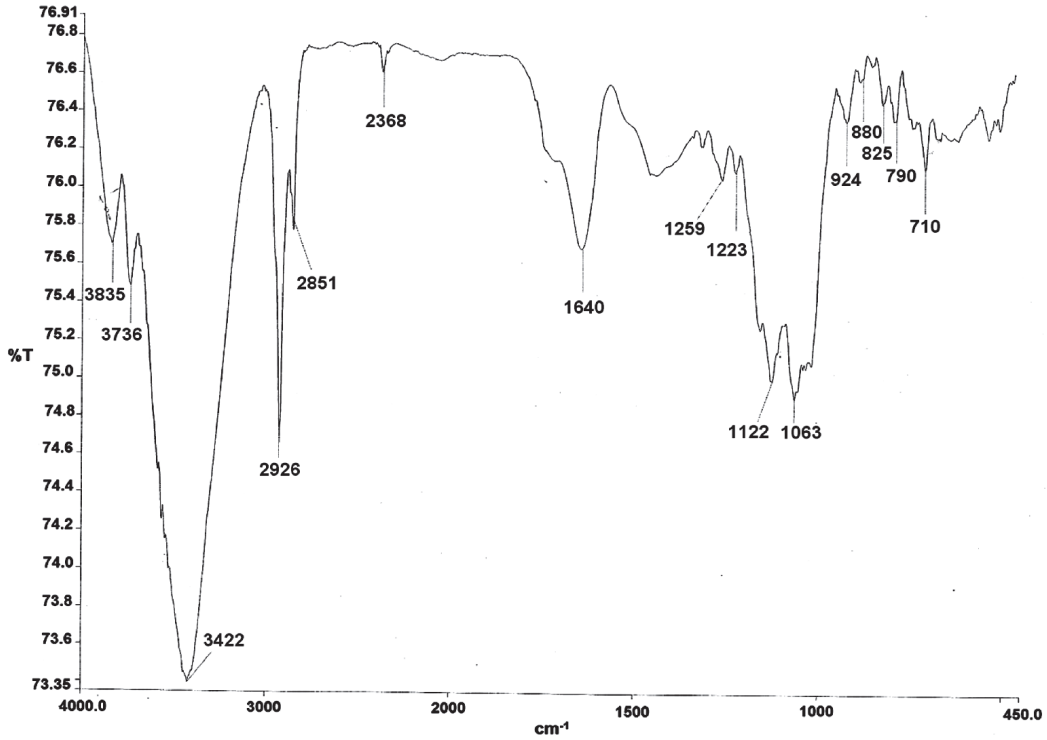

Fig. 1. FTIR spectrum of levan from *Leuconostoc mesenteroides* NRRL B-1149.

groups and ring vibrational modes in the composition of cyclic structures. The band at 1122 cm^{-1} was assigned to valent vibrations of C–O–C bond and glycosidic bridge. The presence of a peak at 1063 cm^{-1} was due to the great chain flexibility present in polysaccharide around the glycosidic bonds. The similar results were also described by Shingel, 2002 (22). These

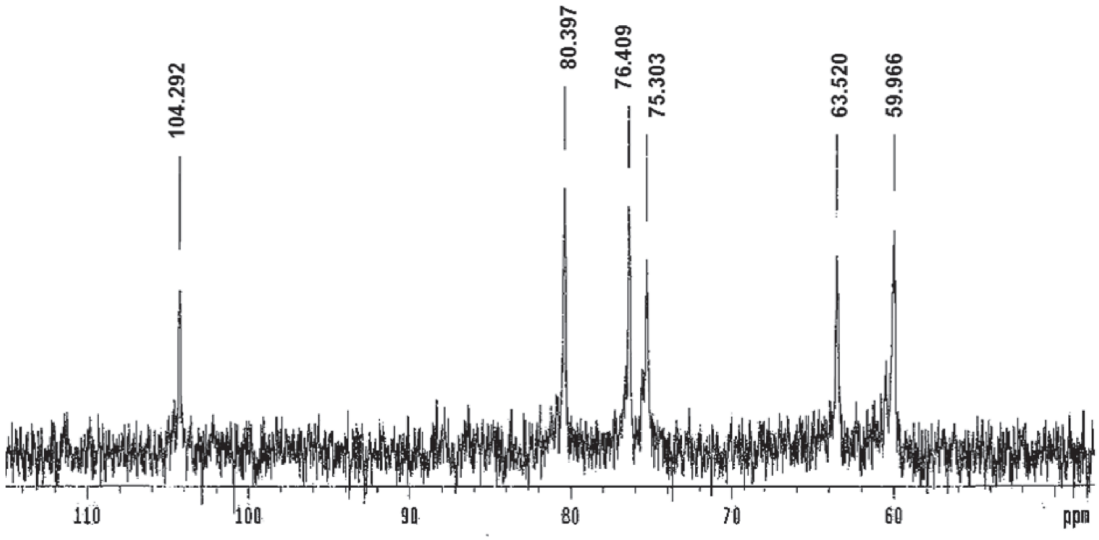

Fig. 2. ^{13}C NMR spectrum of levan from *Leuconostoc mesenteroides* NRRL B-1149.

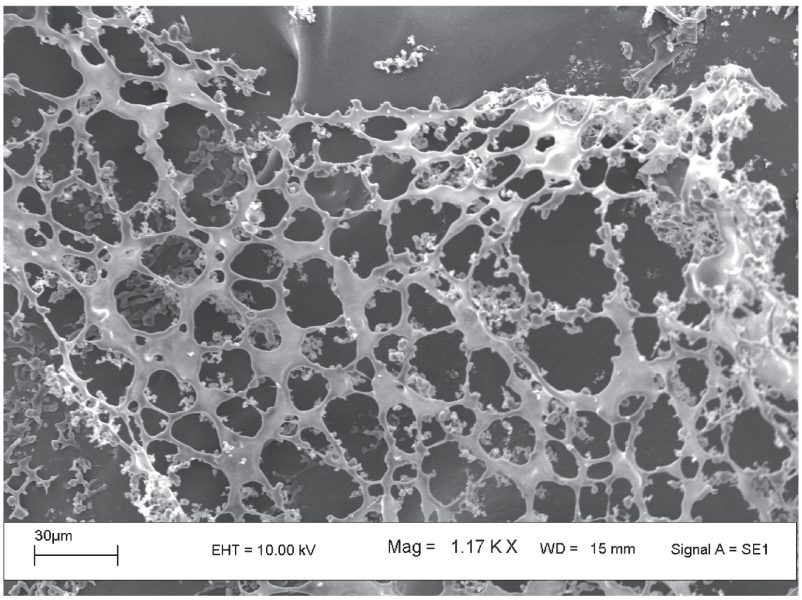

Fig. 3. Scanning electron microscopic image of levan from *Leuconostoc mesenteroides* NRRL B-1149 at 1.17 kx magnification.

Table 1. ^{1}H NMR and ^{13}C NMR chemical shifts of levan from *L. mesenteroides* NRRL B-1149

^{1}H shifts (in ppm)						
H-1a	H-1b	H-3	H-4	H-5	H-6a	H-6b
3.79	3.69	4.18	4.11	3.95	3.84	3.58
^{13}C shifts (in ppm)						
C-1	C-2	C-3	C-4	C-5	C-6	
104.29	80.39	76.40	75.30	63.52	59.96	

Table 2. Comparison of ^{13}C NMR chemical shifts of levan from *L. mesenteroides* NRRL B-1149 with other reported strains.

Carbon atom	Levan *L. mesenteroides* B-1149 (present study)	Levan Levan *S. mutans*[26]	LevS polymer *L. mesenteroides* B-512FMC[27]	*L. mesenteroides* B-512F[36]
Chemical shift (ppm)				
C-1	59.96	60.7	60.5	60.145
C-2	104.29	104.2	109.1	104.3
C-3	76.40	76.3	76.5	76.523
C-4	75.30	75.2	75.4	75.391
C-5	80.39	80.3	80.7	80.453
C-6	63.52	63.4	63.6	63.57

linkages and branching in soluble polysaccharide was analyzed by ^{1}H NMR and ^{13}C NMR spectroscopy.

^{1}H NMR analysis of soluble levan : The ^{1}H NMR spectrum of soluble levan from *L. mesenteroides* NRRL B-1149 showed seven signals in the region of skeletal protons (4.25-3.10 ppm region) due to â-fructofuranoside (bFruf) units. The signals in the regions 3.79, 3.69, 4.18, 4.11, 3.95, 3.84 and 3.58 ppm were assigned to H1a, H1b, H3, H4, H5, H6a and H6b, respectively (Table 1). The assignments for different resonances of ^{1}H and ^{13}C NMR of levan from *L. mesenteroides* NRRL B-1149 is shown in Table 1.The similar results were observed in spectra of fructans from *Lactobacillus reuteri* LB 121 (23) and *Bacillus* sp. 3B6 (24). In 1D-^{1}H NMR spectrum of levan the absence of peak in anomeric region (4.9-5.3 ppm) were found, which confirmed the absence of any anomeric proton in it (Table 1). No signals in the region downfield to 4.91 ppm confirmed the absence of branching points. The observed peak pattern fitted the fructofuranoside configuration.

^{13}C NMR analysis of soluble levan : The structure of soluble levan produced from *L. mesenteroides* NRRL B-1149 was determined by ^{13}C NMR spectroscopy. The ^{13}C NMR spectrum showed six major resonances in the region of 104.29, 80.39, 76.40, 75.30, 63.52 and 59.96 ppm (Fig. 2). The keto-anomeric signal C2 due to bFru*f* appeared at 104.29 ppm while C1 and C6 signals were detected at 59.96 and 63.52 ppm, respectively. These data were in

accordance with previous reports (25). The chemical shifts obtained for the polymer were also similar to those observed in levan from *S. mutans* (26) but differed from levan produced by *L. mesenteroides* NRRL B-512 FMC (27), where a difference in the C2 shift was found (109.1 ppm instead of 104.3 ppm) (Table 2). The ^{13}C-NMR spectrum of levan from *L. mesenteroides* NRRL B-1149 was also comparable with the spectrum of fructan from *Lactobacillus fermentum* (AKJ15) having β-(2-1) and β-(2-6) linkages (28). The absence of corresponding signal of C2 in ^{1}H NMR spectrum suggested that the glycosidic carbon (104.29 ppm) has no linkage with the hydrogen. The peaks at 80.39, 76.40, and 75.37 ppm were due to -CH carbons while those at 63.52 and 59.96 ppm corresponded to the -CH_2 carbon. The ^{1}H and ^{13}C NMR spectra of soluble polysaccharide from *L. mesenteroides* NRRL B-1149 showed its nature to be levan with (→6)-β-D-Fru*f*-(2→)$_n$ structure. This was in agreement with the previous reports (29,30).

Scanning electron microscopy of levan : The surface morphology of dried and powdered soluble levan as analyzed by scanning electron microscopy (SEM) at 1.17 kx, is shown in Fig. 3. The SEM analysis revealed its porous structure. In the present study, the levan was more porous as compared to the insoluble dextran produced by the same strain. Due to the porous structure the polysaccharides can be used in foods as thickening, gelling, stabilizing, emulsifying, and water-binding agents (31-33).

Cytotoxicity and biocompatibility analysis of levan : The effect of levan on viability of HeLa cells is shown in Fig. 4. The results showed that there was no effect of levan on the viability of cells up to 48 h even at higher concentration of 1000 µg/ml. The levan from *L. mesenteroides* NRRL B-1149 in current study was proved to be non-toxic and biocompatible and hence can be used as a biomaterial for biomedical applications. The results were in accordance with previous

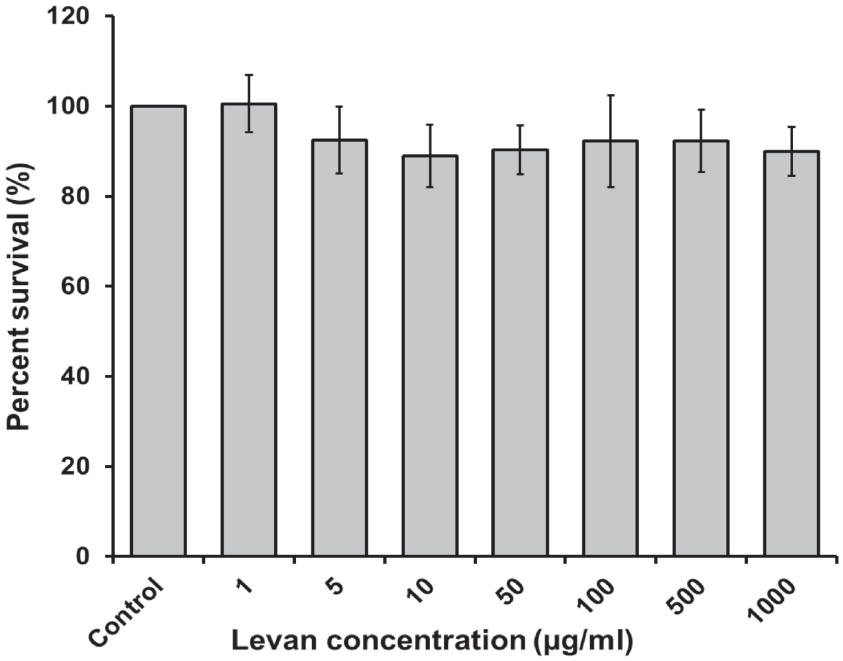

Fig. 4. *In vitro* cytotoxicity assay showing the viability of HeLa cells after treatment with different concentrations of levan (1-1000 µg/ml) from *Leuconostoc mesenteroides* NRRL B-1149 up to 48 h incubation.

reports of non-toxic and biocompatible polysaccharides where the levan from *Halomonas* sp. AAD6 showed negligible effect on cellular viability and proliferation of osteoblasts and murine macrophages (34). The *in vitro* cytotoxicity test of levan from *Halomonas* sp. also showed no effect on HeLa and L929 cell lines (35). Apart from its gelling, stabilizing and water holding capacity, the levan from *L. mesenteroides* NRRL B-1149 can be further studied for its *in vitro* and *in vivo* antitumor, antiulcer, immunostimulating, immunomodulating and cholesterol-lowering activities which will open a new horizon in various biomedical fields.

Conclusion

The water soluble polysaccharide was synthesized from *Leuconostoc mesenteroides* NRRL B-1149 using purified enzyme. The average molecular weight of the polysaccharide was 7.2 x 10^6. The soluble polysaccharide was identified as levan with β-(2→6) linkage using FTIR and NMR spectroscopic analysis. The surface morphology of levan by scanning electron microscopy revealed its porous structure hence it can be used as thickening, gelling, stabilizing, emulsifying and water-binding agent. The *in vitro* cytotoxicity assay using levan showed its non-toxic and biocompatible behavior.

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References

1. Anwar, M.A., Kralj, S., Pique, A.V., Leemhius, H., Van der Maarel, M.J.E.C. and Dijkhuizen, L. (2010). Inulin and levan synthesis by probiotic *Lactobacillus gasseri* strains: characterization of three novel fructansucrase enzymes and their fructan products. Microbiology, 156: 1264-1274.
2. Han, Y.W. (1990). Microbial levan. Advances in Applied Microbiology, 35: 171-194.
3. Meng, G. and Futterer, K. (2003). Structural framework of fructosyl transfer in *Bacillus subtilis* levansucrase. Structure and Molecular Biology, 10: 935–941.
4. Yanase, H., Iwata, M., Nakahigashi, R., Kita, K. and Tonomura, K. (1992). Purification, crystallization and properties of the extracellular levansucrase from *Zymomonas mobilis*. Bioscience Biotechnology and Biochemistry, 56: 1335-1337.
5. Chambert, R., Treboul, G. and Dedonder, R. (1974). Kinetic studies of levansucrase of *Bacillus subtilis*. European Journal of Biochemistry, 41: 285-300.
6. Clarke, M.A., Roberts, E.J. and Garegg, P.J. (1997). New compounds from microbiological products of sucrose. Carbohydrate Polymers, 34: 425.
7. Vijn, I. and Smeekens, S. (1999). Fructan: More than reserve carbohydrate. Plant Physiology, 120: 351-359.
8. Yoon, E.J., Yoo, S.H., Cha, J.H. and Lee, H.G. (2004). Effect of levan's branching structure on antitumor activity. International Journal of Biological Macromolecules, 34: 191-194.
9. Xu, Q., Yajima, T., Li, W., Saito, K., Ohshima, Y. and Yoshikai, Y. (2006). Levan (â-2, 6-fructan), a major fraction of fermented soybean mucilage, displays im-munostimulating properties via Toll-like receptor 4 signaling: induction of interleukin-12 production and suppression of T-helper type 2 response and immu-noglobulin E production. Clinical and Experimental Allergy, 36: 94-101.
10. Yamamoto, Y., Takahashi, Y., Kawano, M., Iizuka, M., Matsumoto, T., Saeki, S. and Yamaguchi, H. (1999). *In vitro* digestibility and fermentability of levan and its hypocholesterolemic effects in rats. Journal of Nutritional Biochemestry, 10: 13-18.

11. Combie, J., Steel, A. and Sweitzer, R. (2004). Adhesive designed by nature (and tested at Redstone Arsenal). Clean Technologies and Environmental Policy, 6: 258-262.

12. Kang, S.A., Jang, K.H., Lee, J.C., Chang, B.I., Lim, Y.A. and Song, B.C. (2003). The effects of fructose polymer levan on the body fat accumulation and serum lipid profiles of Korean women. Korean Journal of Community Nutrition. 8: 986-992.

13. Goyal, A. and Katiyar, S.S. (1996). Regulation of dextransucrase productivity from *Leuconostoc mesenteroides* NRRL B-512F by the maintenance media. Journal of General and Applied Microbiology, 42: 81-85.

14. Tsuchiya, H.M., Koepsell, H.J., Corman, J., Bryant, G., Bogard, M.O., Feger, V.H. and Jackson, R.W. (1952). The effect of certain cultural factors on production of dextransucrase by *Leuconostoc mesenteroides.* Journal of Bacteriology, 64: 521-527.

15. Shukla, R., Shukla, S., Bivolarski, V., Iliev, I., Ivanova, I. and Goyal, A. (2011). Production and structural characterization of insoluble dextran produced in the presence of maltose from *Leuconostoc mesenteroides* NRRL B-1149. Food Technology and Biotechnology, 49(3): 291-296.

16. Fox, J.D. and Robyt, J.F. (1991). Miniaturization of three carbohydrate analyses using a microsample plate reader. Analytical Biochemistry, 195: 93-96.

17. Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods, 65: 55-63.

18. Han, Y.W. and Clarke, M.A. (1990). Production and characterization of microbial levan. Journal of Agricultural and Food Chemistry, 38: 393-396.

19. Calazans, G.M.T., Lima, R.C., de Franca, F.P. and Lopes, C.E. (2000). Molecular weight and antitumor activity of *Zymomonas mobilis* levans. International Journal of Biological Macromolecules, 27: 245- 247.

20. Cao, W., Li, X.Q., Liu, L., Yang, T.H., Li, C., Fan, H.T., Jia, M., Lu, Z.G. and Mei, Q.B. (2006). Structure of antitumor polysaccharide from *Angelica sinensis* (Oliv.) Diels. Carbohydrate Polymers, 66: 149-159.

21. Liu, C., Lin, Q., Gao, Y., Ye, L., Xing, Y. and Xi, T. (2007). Characterization and antitumor activity of polysaccharide from *Strongylocentrotus nudus* eggs. Carbohydrate polymers, 67: 313-318.

22. Shingel, K.I. (2002). Determination of structural peculiarities of dextran, pullulan and c-irradiated pullulan by Fourier-transform IR spectroscopy. Carbohydrate Research, 337: 1445-1451.

23. Van Geel-Schutten, G.H., Faber, E.J., Smit, E., Bonting, K., Smith, M.R., Ten Brink, B., Kamerling, J.P., Vliegenthart, J.F.G. and Dijkhuizen L. (1999). Biochemical and structural characterization of the glucan and fructan exopolysaccharides synthesized by the *Lactobacillus reuteri* wild-type strain and by mutant strains. Applied and Environmental Microbiology, 65: 3008-3014

24. Matulova, M., Husarová, S., Capek P., Sancelme, M. and Delort, A.M. (2011). NMR structural study of fructans produced by *Bacillus* sp. 3B6, bacterium isolated in cloud water. Carbohydrate Research, 346: 501-507.

25. Van Hijum, S., van Greel-Schutten, H., Rahaoui, H., van der Maarel, M. and Dijkhuizen, L. (2002). Characterization of a novel fructosyltransferase from

Lactobacillus reuteri that synthesizes high molecular weight inulin and inulin oligosaccharides. Applied and Environmental Microbiology, 68: 4390-4398.

26. Shimamura, A., Tsuboi, K., Nagase, T., Ito, M., Tsumori, H. and Mukasa, H. (1987). Structural determination of D-fructans from *Streptococcus mutans*, serotype b, c, e, and f strains, by ^{13}C-N.M.R. spectroscopy. Carbohydrate Research, 165: 150-154.

27. Kang, H.K., Seo, M.Y., Seo, E.S., Kim, D., Chung, S.Y., Kimura, A., Day, D.F. and Robyt, J.F. (2005). Cloning and expression of levansucrase from *Leuconostoc mesenteroides* B-512 FMC in *Escherichia coli*. Biochimica et Biophysica Acta, 1727(1): 5-15.

28. Dutta, A., Das, D. and Goyal, A. (2012). Purification and characterization of fructan and fructansucrase from *Lactobacillus fermentum* (AKJ15) isolated from Kodo ko Jaanr, a fermented beverage from North Eastern Himalayas. International Journal of Food Science and Nutrition, 63(2): 216-224.

29. Angyal, S.J. and Bethell G.S. (1976). Conformational analysis in carbohydrate chemistry. III. The ^{13}C N.M.R. spectra of the hexuloses. Australian Journal of Chemistry, 29(6): 1249-1265.

30. Barrow, K.D., Collins, J.G., Rogers, P.L. and Smith, G.M. (1984). The structure of a novel polysaccharide isolated from *Zymomonas mobilis* determined by nuclear magnetic resonance spectroscopy. European Journal of Biochemistry, 145(1): 173-179.

31. Kirk, R.E., Othmer, D.F., Kroschwitz, J.I. and Howe-Grant, H. (1991). Kirk-Othmer Encyclopedia of Chemical Technology. Vol. 16, Wiley Inc., New York.

32. Khan, T., Park, J.K. and Kwon, J.H. (2007). Functional biopolymers produced by biochemical technology considering applications in food engineering. Korean Journal Chemical Engineering, 24: 816-826.

33. Shih, I.L., Yu, Y.T., Shieh, C.J. and Hsieh, C.Y. (2005). Selective production and characterization of levan by *Bacillus sub*tilis (Natto) Takahashi. Journal of Agricultural and Food Chemistry, 53: 8211 -8215.

34. Poli, A., Kazak, H., Gürleyendað, B., Tommonaro, G., Pieretti, G., Toksoy Öner, E. and Nicolaus, B. (2009). High level synthesis of levan by a novel *Halomonas* species growing on defined media. Carbohydrate Polymer, 78: 651-657.

35. Küçüka^{g}ik, F., Kazak, H., Güney, D., Finore, I., Poli, A., Yenigün, O., Nicolaus, B. and Toksoy Öner, E. (2011). Molasses as fermentation substrate for levan production by *Halomonas* sp. Applied Microbiology and Biotechnology, 89:1729-1740.

36. Sandra, M.A., Maria, E.R., Lorenzo S., Agustín, L.M. and Clarita, O.C. (2006). Identification and functional characterization of levS, a gene encoding for a levansucrase from *Leuconostoc mesenteroides* NRRL B-512F. Gene, 376: 59-67.

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Comparative Study of Allelopathic Effects of Green Tea and Black Tea

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Abstract

The present study focused on the evaluation and comparison of allelopathic effects of aqueous extracts of green tea and black tea leaves (*Camellia sinensis*) against germination and radicle growth of *Cicer arietinum* seeds. The extracts at different concentrations was incubated in controlled experimental conditions with the surface sterilized seeds of *C. arietinum* and observed periodically for seed germination and radicle growth to assess the allelopathic behavior. Both the tea extracts mainly at higher concentrations demonstrated promising allelopathic potential by significantly affecting seed germination and radicle elongation up to 96 h in a concentration dependent manner. Green tea was found to be more effective than black tea, plausibly due to higher flavonoid contents of green tea.

Keywords: Allelopathic, polyphenols, green tea, *Cicer arietinum.*

Introduction

The phenomenon of allelopathy, where a plant species chemically interferes with the germination, growth or development of other plant species has been known for over 2000 years. Allelopathy can be defined as any direct or indirect harmful or beneficial effect of one plant on another through the production of chemicals that it releases into the environment (1). In 1996, the International Allelopathy Society defined allelopathy as follows: "Any process involving secondary metabolites produced by plants, micro-organisms, viruses, and fungi that influence the growth and development of agricultural and biological systems (excluding animals), including positive and negative effects" (2). Chemicals released from plants and imposing allelopathic influences are termed as allelochemicals or allelochemics. Most allelochemicals are classified as secondary plant metabolites which are biosynthetically derived form the primary metabolites of the plant (3). When susceptible plants are exposed to allelochemicals, germination, growth and development may be affected. Allelochemicals are present in several parts of plants that are known to interfere in seed germination and growth of neighbouring or successional plants by releasing allelochemicals in their environment (1). The search and development of new herbicides through the identification of active compounds from allelopathic plants is an interesting research area (4). These compounds can be regarded as 'natural herbicides'.

Tea, a product made from leaf and bud of the plant, *Camellia sinensis*, is the second most consumed beverage in the world (5). *Camellia sinensis* is a large evergreen shrub indigenous to Eastern Asia where it is cultivated extensively. The dried cured leaves of *C. sinensis* have been used to prepare beverages for more than 4000 years. The method of curing determines the nature of the tea to be used for infusion. Green tea is a type of cured tea that is 'non fermented' and produced by drying and steaming the fresh leaves; whereas black tea leaves are withered,

rolled, fermented and then dried (6). Tea has been used medicinally for centuries in the Traditional Chinese Medicine (TCM). Recently there has been renewed interest on green tea in prevention of several disease risks ands other important health benefits (7). Previous researchers have reported several pharmacological and toxicological properties on green tea on animals and humans (8-11). The present study was conducted to assess and compare the possible allelopathic effects of green tea and black tea on the germination and radicle growth of *Cicer arietinum* seeds.

Materials and Methods

Plant materials: Packaged green tea and black tea leaves were procured in the month of July, 2011 from Desai & Sons, Ezra Street, Kolkata 700001, India. Just after procurement, both the type of tea leaves were ground mechanically into fine powder and kept into an air-tight container for use in the study.

Preparation of extracts: The powdered plant materials (50 g) were extracted with distilled water (350 ml) by boiling under reflux for 30 minutes. The extracts were filtered and evaporated to dryness to yield the dry extracts of green tea (AQGT, yield: 51.28%) and black tea (AQBT, yield: 17.45%). The dry extracts were kept in a vacuum desiccator until use in the study.

Test samples: The test samples for allelopathic bioassay were prepared freshly from the dry extracts. Different concentrations of both the test extracts, viz., AQGT (40, 20, 10, 5, 2.5, 1.25 mg/ml), and AQBT (40, 20, 10, 5, 2.5, 1.25 mg/ml) were prepared by dissolving in double-distilled water immediately prior to use.

***Collection and preparation of Cicer arietinum* seeds:** Healthy uniform seeds of gram (*Cicer arietinum* L., family: Fabaceae) were obtained from Agriculture Seed Store (Govt. of West Bengal) Kalyani, West Bengal, India. The seeds were soaked in distilled water for one hour. Then the seeds were surface sterilized with 70% ethanol for 2 minutes, then rinsed with double-distilled water for several times for complete removal of the sterilant.

Exposure to text samples: This procedure was performed under aseptic conditions at laminar air-flow bench. The surface sterilized seeds were placed evenly in sterilized glass Petri dishes (9 mm). Each Petri dish contained 10 seeds. Then equal volume (5 ml) of varying concentrations of the test samples were introduced into each Petri dish. Similar volume of double distilled water was used as control. Then the lids of the Petri dish were closed and incubated in dark at room temperature (24-26°C) for 96 h. Allelopathic behaviour was evaluated by recording the number of germinated seeds and measuring radicle length using a millimetre ruler, at 24 h interval for 96 h (minimum four measurements in each Petri dish). The indicating parameters viz., germination percentage and percentage inhibition of radicle growth were calculated by the following formulae:

Germination percentage = Number of germinated seeds/Total number of seeds ×100

% Inhibition of radicle growth= (X–Y)/X ×100.

Where, X= Control mean radicle length and Y= Treated mean radicle length.

The extract concentration for 50% radicle length inhibition (IC_{50}) was determined by plotting percentage inhibition of radicle growth with respect to control against treatment concentration (12, 13).

Statistical analysis: The data of radicle length were expressed as the mean ± standard error of mean (SEM). Same data were analyzed for statistical significance by one way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test using GraphPad InStat software. $P < 0.001$ was considered as statistically significant.

Results and Discussion

Screening of plant extracts and their fractions for their effects on seed germination of various plant species are routinely used to

evaluate their alleleopathic potential (4). The present findings demonstrated negative allelopathic effects of aqueous extracts of green tea (AQGT) and black tea (AQBT) on the germination and radicle growth of *C. arietinum.*

The results of allelopathic effect of AQGT are summarized in Tables 1 and 2. AQGT at all test concentrations inhibited germination of *C. arietinum* seeds in a concentration dependent way; however, as time approached to 96 h, all seeds germinated at lower concentrations (1.25 to 5 mg/ml) and a few were found to germinate at the next higher concentration of AQGT (10 mg/ml), except at 20 and 40 mg/ml where no germination was observed during 96 h (Table 1). AQGT remarkably inhibited radicle growth at the all test concentrations but at concentrations 1.25, 2.5 and 5 mg/ml, the effects were not concentration dependent up to 48 h. Concentration dependent effect was observed after 72 and 96 h. The effects were found to be prominent and significant ($p < 0.001$) right after 24 h and during the whole observation period. No radicle growth was observed at the concentration of 10 mg/ml up to 72 h, and further higher concentrations of AQGT (20 and 40 mg/ml) even up to 96 h (Table 2).

The results of allelopathic effect of AQBT are presented in Tables 3 and 4. AQBT at all test concentrations inhibited germination of *C. arietinum* seeds in a concentration dependent fashion; however, at 96 h, all seeds were found to germinate at lower concentrations (1.25 to 5 mg/ml) but no germination was observed in case of higher concentrations of AQBT (10 to 40 mg/ml) up to 96 h (Table 3). AQBT significantly ($p < 0.001$) and concentration dependently inhibited radicle growth at all the test concentrations from 24 h and during 96 h of observation. No detectable radicle growth was observed at the concentration of 5 mg/ml up to 48 h, and further higher concentrations of AQBT (10, 20 and 40 mg/ml) even up to 96 h (Table 4). The IC_{50} values of both AQGT and AQBT are summarized in Table 5. IC_{50} was not observed in case of AQBT after 24 h.

The most frequently reported allelochemical-induced gross morphological effects on plants include inhibited or retarded seed germination, effects on coleoptile elongation and on radicle, shoot and root development (14). Here, germination percentage and radicle growth were recorded to monitor the allelopathic behaviour. However, in the present study radicle growth appeared to be the most sensitive parameter and IC_{50} values based on this parameter very clearly indicated the differential allelopathic effect of AQGT and AQBT (Table 5). From these values it becomes evident that AQGT was more active than AQBT, being effective in lower concentrations.

Present findings exhibited the effect of the test tea extracts on the onset of seed germination. AQGT favoured seed germination at 24 h although inhibited radicle growth as compared with control, whereas AQBT did not at all favour germination at 24 h. This implied that AQBT was more effective than AQGT after 24 h. After 96 hours of treatment, the radicle growth inhibition by AQBT at the concentration 10 mg/ml was 100% and that of AQGT at same concentration was 88.82%. However, comparing the overall percentage radicle growth inhibitions and IC_{50} values it becomes obvious that AQGT was more effective than AQBT.

Plants exhibit allelopathic activity due to release of allelochemicals of different chemical classes mainly polyphenolic compounds (flavonoids and tannins), cyanogenic glycosides and alkaloids (3, 15). The inhibitory effect of the test extracts on seed germination and radicle length may be due to the presence of putative allelochemicals. Tea leaves contain varying amounts of polyphenols particularly flavonoids. Polyphenols are well known natural products known to possess several notable biological properties (16). Black and green teas both contain almost similar amount of flavonoids, however they differ in their chemical composition; green tea contains more catechins (simple flavonoids), while the oxidation undergone by the leaves, in order to make black tea, polymerizes

Table 1. Effect of AQGT on germination percentage of *C. arietinum.*

Concentration (mg/ml)	After 24 h (%)	After 48 h (%)	After 72 h (%)	After 96 h (%)
Control	70	80	90	100
1.25	80	80	100	100
2.5	60	80	90	100
5	40	70	80	100
10	0	0	10	30
20	0	0	0	0
40	0	0	0	0

Table 2. Effect of AQGT on radicle growth of *C. arietinum.*

Concentration	After 24 h		After 48 h		After 72 h		After 96 h	
(mg/ml)	Radicle length (mm)§	% Inhibition of radicle growth	Radicle length (mm)§	% Inhibition of radicle growth	Radicle length (mm)§	% Inhibition of radicle growth	Radicle length (mm)§	% Inhibition of radicle growth
Control	7.13±0.86	-	8.75±1.14	-	10.89±2.18	-	11.90±1.59	-
1.25	1.88±0.39*	73.63	2.56±0.50*	70.74	4.38±0.87*	59.78	6.50±0.75*	45.38
2.5	1.67±0.33*	76.58	2.38±0.53*	72.80	3.88±0.89*	64.37	5.00±0.67*	57.98
5	2.00±0.41*	71.95	2.71±0.56*	69.03	3.40±0.67*	68.78	3.80±0.76*	68.07
10	0	100	0	100	0	100	1.33±0.34*	88.82
20	0	100	0	100	0	100	0	100
40	0	100	0	100	0	100	0	100

§Data are expressed as mean ± SEM. *$p < 0.001$ compared with control.

Table 3. Effect of AQBT on germination percentage of *C. arietinum.*

Concentration (mg/ml)	After 24 h (%)	After 48 h (%)	After 72 h (%)	After 96 h (%)
Control	70	80	90	100
1.25	10	70	90	100
2.5	10	50	80	100
5	10	20	50	60
10	0	0	0	0
20	0	0	0	0
40	0	0	0	0

Table 4. Effect of AQBT on radicle growth of *C. arietinum.*

Concentration	After 24 h		After 48 h		After 72 h		After 96 h	
(mg/ml)	Radicle length (mm)§	% Inhibition of radicle growth	Radicle length (mm)§	% Inhibition of radicle growth	Radicle length (mm)§	% Inhibition of radicle growth	Radicle length (mm)§	% Inhibition of radicle growth
Control	7.13±0.86	-	8.75±1.14	-	10.89±2.18	-	11.90±1.59	-
1.25	0	100	5.34±0.49*	38.97	7.11±0.97	34.71	8.56±1.05	28.06
2.5	0	100	2.00±0.23*	77.14	5.62±0.43*	48.39	6.78±0.50*	43.02
5	0	100	0	100	4.29±0.82*	60.60	5.33±1.17*	55.21
10	0	100	0	100	0	100	0	100
20	0	100	0	100	0	100	0	100
40	0	100	0	100	0	100	0	100

§Data are expressed as mean ± SEM. *$p < 0.001$ compared with control.

Table 5. IC_{50} values of AQGT and AQBT on radicle growth of *C. arietinum.*

Treatment time	IC_{50} (mg/ml)	
	AQGT	AQBT
After 24 h	0.75	-
After 48 h	0.75	1.50
After 72 h	1.0	2.88
After 96 h	2.88	3.75

these simple flavonoids into theaflavins and thearubigins (polymerized flavonoids). The main flavonoids present in green tea include catechins (flavan-3-ols) and most importantly epigallocatechin-3-gallate (EGCG) (17). Tea leaf contains some phenolic acids such as chlorogenic acid, gallic acid, caffeic acid, etc., which may also be responsible for its allelopathic role. Phenolic acids have been shown to be toxic to germination and plant growth processes (3). In the present study, higher allelopathic effect of green tea can be attributed to its higher flavonoids (catechin) contents. Tea also contains alkaloids (namely caffeine, theobromine and theophylline) the presence of which may also be responsible for its observed allelopathic effects (18). Both black and green teas contain the principal alkaloid caffeine which has phytotoxic property (19). The effect may be due to synergistic effect rather than single constituent.

From the present preliminary investigation, it can be concluded that both green and black tea leaves exhibited remarkable negative allelopathic potential by significantly affecting the germination and radicle growth of *C. arietinum.* Green tea was found to be markedly active than black tea plausibly due to its higher flavonoid content. Further studies are necessary to determine the exact chemical constituents of tea leaf accounting for its allelopathic activity. Allelopathic effects of tea plant under field conditions also need further research in pursuit of a new effective natural herbicide.

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References

1. Rice, E.L. (1984). Allelopathy. Academic Press, New York.
2. Torres, A., Oliva, R.M., Castellano, D. and Cross, P. (1996). First World Congress on Allelopathy, a Science of the Future. SAI (University of Cadiz), Cadiz, Spain.
3. Einhellig, F.A. (1995). Mechanisms of action of allelochemicals in allelopathy. In: Inderjit, Dakshini, K.M.M., Einhellig, F.A., Eds., Allelopathy. Organisms, Processes, and Applications (ACS Symposium Series 582). American Chemical Society, Washington DC, pp: 96-116.
4. Macias, F.A. (1995). Allelopathy in the search for natural herbicides models. In: Inderjit, Dakshini, K.M.M., Einhellig, F.A., Eds., Allelopathy. Organisms, Processes, and Applications (ACS Symposium Series 582). American Chemical Society, Washington DC, pp: 310-329.
5. Rietveld, A. and Wiseman, S. (2003). Antioxidant effects of tea: evidence from human clinical trials. J Nutr, 133: 3275-3284.
6. Duke, J.A., Bogenschuz-Godwin, M.J., duCellier, J. and Duke, P.K. (2002). Handbook of Medicinal Plants. CRC Press, Boca Raton.
7. DerMarderosian, A. (2001). The Reviews of Natural Products. Facts and Comparisons, Missouri.
8. McKay, D.L. and Blumberg, J.B. (2002). The role of tea in human health: An update. J Am Coll Nutr, 21: 1-13.
9. Cabrera, C., Artacho, R. and Gime´nez, R. (2006). Beneficial effects of green tea - a review. J Am Coll Nutr, 25: 79-99.
10. Chatterjee, P., Chandra, S., Dey, P. and Bhattacharya, S. (2012). Evaluation of anti-inflammatory effects of green tea and black tea: A comparative *in vitro* study. J Adv Pharm Tech Res, 3: 136-138.
11. Chauhan, D.S., Bhattacharya, S., Al-Dhubaib, B. and Ahmed, K.K.M. (2012). Comparative *in vitro* antioxidant evaluation of different extracts of *Camellia sinensis* leaves form different geographical locations in India. Phcog J, 4 (31): 44-46.
12. Dey, P., Chandra, S., Chatterjee, P. and Bhattacharya, S. (2012). Assessment of allelopathic property of *Mikania scandens* root. Curr Trends Biotechnol Pharm, 6: 322-327.
13. Chandra, S., Chatterjee, P., Dey, P. and Bhattacharya, S. (2012). Allelopathic effect of Ashwagandha against the germination and radicle growth of *Cicer arietinum* and *Triticum aestivum*. Phcog Res, 4: 166-169.
14. Kruse, M., Strandberg, M. and Strandberg, B. (2000). Ecological Effects of Allelopathic Plants - A Review (NERI Technical Report no. 315). National Environmental Research Institute, Silkeborg, Denmark.
15. Einhellig, F.A. (1995). Allelopathy - current status and future goals. In: Inderjit, Dakshini, K.M.M., Einhellig, F.A., Eds., Allelopathy. Organisms, Processes, and Applications (ACS Symposium Series 582). American Chemical Society, Washington DC, pp: 1-24.
16. Bhattacharya, S. (2011). Are we in the polyphenols era? Phcog Res, 3: 147.
17. Graham H. (1992). Green tea consumption, consumption and polyphenol chemistry. Prev Med, 21: 334-650.
18. Kokate, C.K., Purohit, A.P. and Gokhale, S.B. (2010). Pharmacognosy. Nirali Prakashan, Pune (India).
19. Rizvi, S.J.H. and Rizvi. V. (1992). Exploitation of allelochemicals in improving crop productivity. In: Rizvi, S.J.H., Rizvi, V., Eds., Allelopathy: Basic and Applied Aspects. Chapman and Hall, London, pp: 443-473.

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Protective Role of *Coriandrum sativum* Seed Extract against Lead-Induced Oxidative Stress in Rat Liver and Kidney

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Abstract

Lead (Pb) is a well-known multi-organ toxicant and it damages liver and kidney. The objective of the present investigation was to determine the therapeutic role of *Coriandrum sativum* seed extract against lead-induced oxidative stress in rat liver and kidney. Male rats were divided into four groups: control (1000 ppm sodium acetate) and exposed (1000 ppm lead acetate) for 4 weeks; *Coriandrum sativum*-treated 1 (CST1) 250 mg/kg body weight/day and *Coriandrum sativum*-treated 2 (CST2) 500 mg/kg body weight/day (CST2) received separately the hydro-alcoholic seed extract of *Coriandrum sativum* for seven consecutive days after 4 weeks of lead exposure. After exposure and treatment periods, rats were sacrificed and the liver and kidney were isolated in all the groups. Blood was immediately stored at 4°C in heparinized vials. In liver and kidney, the reactive oxygen species (ROS), lipid peroxidation products (LPP) and total protein carbonyl content (TPCC) were estimated following standard protocols. Delta-ALAD activity (δ-ALAD), Hemoglobin (Hb) concentration, red blood cell (RBC) count, white blood cell (WBC) count, and mean cell volume (MCV) were determined in blood. The data suggested a significant (p<0.05) increase in ROS, LPP and TPCC of liver and kidney in the exposed group compared with their respective controls. ROS levels were high in kidney than in liver of lead exposed group. Though the recovery was similar in both the organs, CST2 group showed higher recovery than that of CST1 group. The maximum recovery for LPP was seen in CST2 treated kidney restoring back to normal levels. Maximum increase in TPCC levels was found in exposed kidney than in liver. Whereas recovery for TPCC was partial in liver of CST1 group but complete in liver of CST2. Delta-ALAD activity, Hb, RBC, WBC and MCV showed a significant (p<0.05) decrease in exposed group. However, upon treatment with *Coriandrum sativum*, CST1 group showed partial restoration in some hematological parameters. Whereas, CST2 group showed restoration of deranged hematological parameters back to control. In conclusion, these results suggest that the seed extract of *Coriandrum sativum* might reduce the lead-induced oxidative stress organ specifically by its antioxidant and metal chelating activity and the mechanism needs to be studied further.

Keywords: Lead, *Coriandrum sativum*, Liver, Kidney, Oxidative stress.

Introduction

Coriandrum sativum (Common name: Coriander and Chinese parsley), belongs to Umbelliferae family, is an herb and is cultivated throughout India. *Coriandrum sativum* seeds are known for carminative and cooling properties (1), hypotensive property and it is also generally used for treating abdominal problems, especially stomach ulcers (2). Isocoumarines are the major active principle component present in *Coriandrum sativum* seeds, and the most vital

molecule is Coriandrin. The seeds of *Coriandrum sativum* also contain Quercetin 3-glucuronide, isoquercitin and rutin (3). Coriander has been reported to reduce lipid peroxidation and also to restore the levels of antioxidant enzymes (4). *Coriandrum sativum* showed excretion of heavy metal in the urine of patients and also augmented the efficacy of antibiotics (5, 6).

Lead is a soft bluish-grey heavy metal and it is ubiquitous, one of the common source of heavy metal poisoning in domestic animals and cattle throughout the world (7). Lead (Pb) is a well-known multi-organ toxicant and it damages liver and kidney. Common health hazards from increased lead exposure are wide range of physiological and biochemical dysfunctions (8). Symptoms of lead toxicity are loss of appetite, weight loss, constipation, irritability, fatigue, occasional vomiting (9). Generation of ROS (Reactive Oxygen Species) and disturbance of pro-oxidant and antioxidant balance has been accepted as the most possible mechanism of lead toxicity (10, 11). Lead toxicity increases the excretion of accumulated ALA into the urine (12, 13) and decreases the production of PBG (Porphobilinogen). At present interest is gaining towards protective effects of antioxidants against chemically induced toxicities (14). Using a murine model of lead intoxication (15) *Coriandrum sativum* reduced lead accumulation in bone and protected mice from lead-induced kidney damage. Studies using *Coriandrum sativum* showed suppressive activity on lead deposition, probably resulting from the chelation of lead by some substances present in it (15). Metabolic homeostasis in the body is maintained by liver and it is susceptible to toxicity of heavy metals such as lead (16). In the present investigation an attempt has been made to study the protective effect of the hydroalcoholic extract of *Coriandrum sativum* seed against lead induced oxidative stress in liver and kidney. As the reactive oxygen species (ROS) formed during oxidative stress by lead can damage lipids and proteins forming lipid peroxidation products and protein carbonyls, these parameters were estimated in the present investigation on liver and kidney.

Materials and Methods

***Chemicals*:** Lead acetate (99.8%), Thiobarbituric acid (TBA), 2, 7 Dichlorofluorescein diacetate (DCFH-DA), Meso-2, 3-Dimercaptosuccinic acid (DMSA) and Guanidine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were purchased from SISCO Research Laboratories Private Limited (Mumbai, India).

***Coriandrum sativum seed extract*:** The hydroalcoholic extract of *Coriandrum sativum* seed was obtained as a gift sample in the form of dried powder from M/S. Laila Impex Private Limited (Vijayawada, Andhra Pradesh, India).

Animals and treatments: The male rats of Wistar strain (100-120 g) were purchased from Mahaveer Enterprises (Hyderabad, India) and they were maintained in the animal facility for four days before they were used for experimentation. They were given free access to feed (Pranav Agro Industries, India) and water *ad libitum.* The study was approved by the Institutional Animal Ethical Committee (IAEC). A total of 30 rats were treated with lead acetate (1000 ppm) through drinking water for a period of 4 weeks and parallel controls (10 rats) were maintained on sodium acetate (1000 ppm). Both the solutions were prepared daily with distilled water. Group-I (10 rats): Control received sodium acetate in water; Group-II (10 rats) exposed to lead acetate; Group-III (10 rats): *Coriandrum sativum* treated1 (CST1) received the hydroalcoholic seed extract of *Coriandrum sativum* at a dose of 250 mg/Kg body weight/day for seven consecutive days after four weeks of lead exposure. Group-IV: *Coriandrum sativum* treated2 (CST2) consisting of 10 rats received the hydroalcoholic seed extract of *Coriandrum sativum* at a dose of 500 mg/Kg body weight/day for seven consecutive days after four weeks of lead exposure. The treatments and supply of food to rats were stopped six hours before sacrifice. The rats belonging to control and exposed were sacrificed after four weeks of lead exposure, whereas the Group-III and IV were sacrificed after one week treatment with plant extract. Sacrifice was done

by cervical dislocation and the liver and kidney were isolated immediately on ice. The liver and kidney were washed in cold normal saline (0.85% NaCl) solution.

Reactive Oxygen Species (ROS): ROS levels in the tissues were determined using the method of Bondy and Guo (17). 10% homogenate was prepared in 0.32 M sucrose solution. The contents were centrifuged at 1800xg for 10 min. Pellet was discarded and the supernatant was centrifuged at 31,500xg for 10 min to obtain the pellet (P_2). The P_2 pellet was suspended in HEPES buffer (120 mM NaCl, 2.5 mM KCl, 0.1 mM $MgCl_2$, 6.0 mM glucose, 1.0 mM $CaCl_2$, 5.0 mM $NaHCO_3$, 10 mM HEPES, pH 7.4) to a concentration of 0.1g equivalent/ml. Diluted fractions were incubated with 5µM 2,7 Dichlorofluorescein diacetate (DCFH-DA) (added from the stock solution of 0.5 mM in 10% ethanol) at 37 °C for 15 min. The obtained fluorescence was read at excitation wavelength of 488 nm and emission wavelength of 525 nm in a spectroflurometer (Systronics, 152). ROS levels were expressed as nanomoles of DCFH-DA oxidized/15 min/mg protein.

Lipid Peroxidation Products (LPP): Tissue LPP levels were estimated following the spectrophotometric method of Ohkawa *et al*. (18). 10% homogenate of liver or kidney was prepared in 1.5% KCl. To 1 ml of the homogenate, 2.5 ml of TCA (Trichloro acetic acid) was added to precipitate the proteins. The contents were centrifuged at 3500 rpm for 10 min at 4 °C. The supernatant was discarded and the pellet was dissolved in 2.5 ml of 0.05 M H_2SO_4 and to this, 3 ml of 2 M thiobarbituric acid (TBA) was added. Whole contents were incubated in boiling water bath at 100 °C for 30 min. The contents were cooled and color was extracted into 4 ml of n-butanol. The color was read at 530 nm using a spectrometer (Rayleigh UV-9200) against the blank. The results were presented as micromoles of MDA (Malondialdehyde) formed/g weight of tissue.

Total Protein Carbonyl Content (TPCC): TPCC levels were quantified using a slightly modified method of Levine *et al*. (19). 10% homogenate was prepared in cold buffer (50 mM Phosphate buffer containing 1 mM EDTA, pH 6.7). The homogenate was centrifuged at 10,000xg for 15 min at 4° C and the supernatant was precipitated with equal amounts of 20% trichloroacetic acid and incubated on ice for 5 min. The tubes were centrifuged at 10,000xg for 10 min at 4° C and the supernatant was discarded. The pellet was re-suspended in 500µl of 10mM 2, 4-dinitrophenyl hydrazine (DNPH) in 2 M HCl and allowed to stand at room temperature for 1hr, with vortexing every 10 min at 4 °C and the pellet was washed three times with ethanol: ethyl acetate mixture. After the final wash, the pellets were re-suspended in 500µl of 6 M guanidine hydrochloride (pH 2.3). The contents were incubated at 37°C for 15 min and were centrifuged at 10,000x g for 10 min at 4 °C. Carbonyl content was measured in spectrophotometer (Rayleigh UV - 9200) at 360 nm against a reagent blank. The results were expressed as nanomoles of carbonyl/ml tissue.

Blood parameters: The blood samples from rats were collected by cardiac puncture and stored in heparinized vials. The blood samples were stored at 4 °C until analysis. The samples were analyzed using SYSMEX-KX 21 hematological analyzer. Hemoglobin (g/dl), Red blood cell count (10^6/mm^3), White blood cell count (10^3/mm^3) (WBC) and Mean corpuscular volume (fL) (MCV) were estimated.

Delta Amino Levulinic Acid Dehydratase (ALAD) activity: Delta ALAD activity was estimated following Berlin and Schaller (20). The blood samples from rats were collected in heparinized vials using cardiac puncture. The samples were stored at 4 °C until analysis. Blood was initially hemolyzed with water for 10min at 37 °C. To the blood sample, potassium phosphate buffer and ALA (12 mM) were added and incubated for 90 min at 37 °C. After incubation the enzyme activity was stopped with the addition of 10% TCA (10 mM $HgCl_2$). Later the samples were centrifuged at 6000 rpm for 10 min and to the supernatant, 1 ml of Erlich reagent was added

and incubated for 20min. To the incubated sample, 0.5 ml of distilled water was added and read at 555 nm in a spectrophotometer (Rayleigh, UV-9200). The results were expressed as nmol PPB (Porphobilinogen) formed/hr/ml.

Metal estimation: Metal concentration was measured following the method of Zachariadis *et al*. (21). The analysis of metal content was carried out with the wet tissue. A known quantity of the tissue was kept in muffle furnace at a temperature of 600 °C for about 4-5 hrs to make into ash. The ash obtained was digested with HNO_3 and dissolved in a known amount of 0.01N HNO_3. The final clear and colorless solution was used for metal estimation with Inductively Coupled Plasma- Mass Spectrometer (ICP-MS) (Agilent-7700S). Metal concentration was given as microgram metal/gm wet weight of tissue.

Statistical analysis: All the assays and metal estimation was done in triplicate. The mean values (n=6) with standard deviations were calculated. The significant differences between control, exposed and treated groups were determined using one-way ANOVA at p <0.05 followed by Bonferronis Multiple comparison test. One-way ANOVA was performed using Graph Pad Prism version 5.0 for Windows, Graph Pad Software, San Diego California USA, www.graphpad.com.

Results

Fig. 1 represents the data on reactive oxygen species (ROS) in liver and kidney of control, exposed, CST1 and CST2 groups. There was a significant (p<0.05) increase in the ROS in liver and kidney of the exposed group compared with their respective controls. Maximum increase in ROS was seen in kidney (38.16%) of exposed group followed by exposed liver (21.67%) with respect to their controls. The CST1 group of liver and kidney showed decrease in ROS levels. However, CST2 group showed better recovery than CST1 and the ROS levels were almost nearer to the control values of liver and kidney of CST2. The values of CST1 and

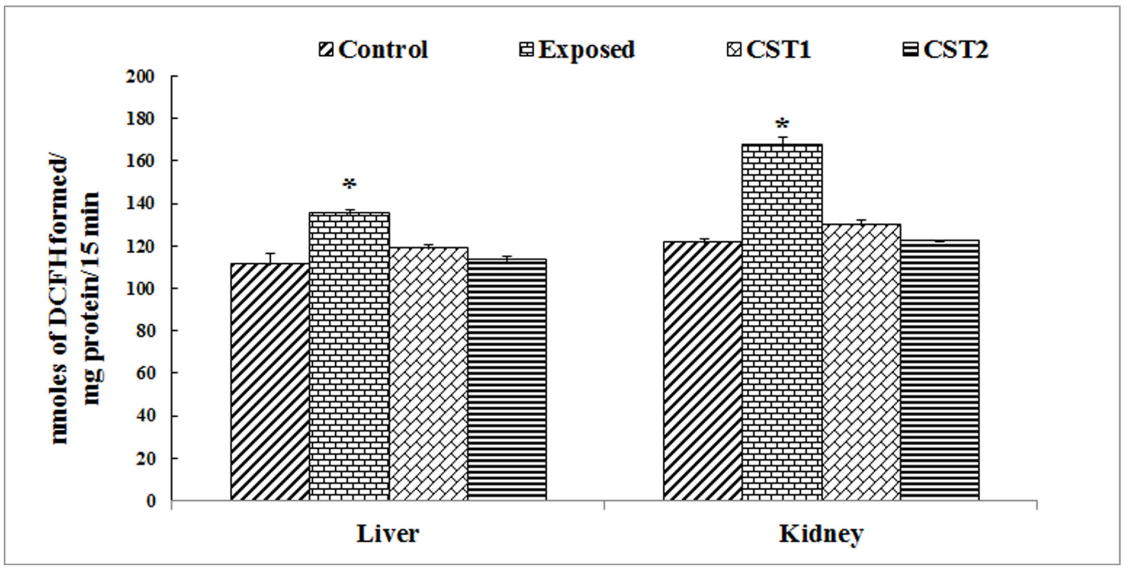

Fig. 1. Reactive Oxygen Species (ROS) in liver and kidney of control, exposed and treated rats. ROS activity was expressed as nmoles of DCFH formed/mg protein/15min. Values represent mean± S.D (n=6). Vertical lines represent standard deviation. *Significantly different from their respective controls at P<0.05.

CST2 were not significant (p>0.05) when compared to their control.

The results on lipid peroxidation products (LPP) are presented in Fig. 2 for liver and kidney of control, exposed, CST1 and CST2 groups. A significant (p<0.05) increase was observed in LPP levels of liver and kidney of the exposed group compared to their respective controls. LPP levels in control group were high in kidney (6.42 μ moles) than liver (4.64 μ moles). The treated groups showed recovery with a maximum of 97% in CST2 of kidney followed by CST1 kidney (89%) and CST2 liver (69.50%). When compared to liver, kidney showed a better recovery in LPP. The CST1 groups of liver and kidney showed decrease in LPP levels when compared to exposed but the recovery was less when compared to CST2 group of liver and kidney.

Fig. 3 depicts data on total protein carbonyl content (TPCC) in the liver and kidney of control, exposed, CST1 and CST2 treated groups. The TPCC levels increased significantly (p<0.05) in liver and kidney of exposed group. TPCC levels in control group were more for liver (0.89nmol/ml) than kidney (0.71nmol/ml). However, maximum increase in TPCC levels was observed in exposed kidney (126.19%) followed by exposed liver (48.0%) when compared to their respective controls. Liver (CST1 76%, CST2 103%) showed maximum recovery in TPCC levels when compared to kidney (CST1 38%, CST2 74%).

The data on tissue lead concentration in the liver and kidney of control, exposed, CST1 and CST2 groups are presented in Figure 4. A significant (p<0.05) increase in lead concentration was seen both in liver and kidney of exposed group. Liver of exposed group showed maximum percent increase (807%, 4.4μg/g) of lead concentration when compared to their respective control (0.48μg/g). However, treatment with *Coriandrum sativum* showed decrease in lead concentration in CST1 (1.75μg/g) and CST2 (1.47μg/g) and maximum decrease was seen in CST2 group. Kidney of exposed group showed a significant increase up to 22.01μg/g of lead when compared to the

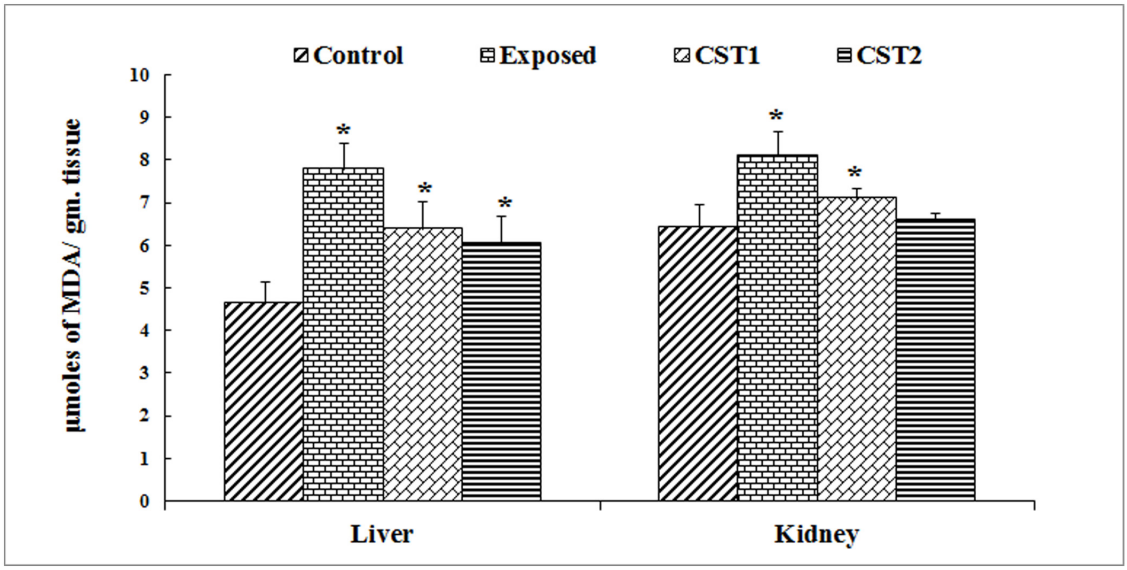

Fig. 2. Lipid Peroxidation Products (LPP) in liver and kidney of control, exposed and treated rats. Lipid peroxidation was expressed as μmoles of MDA/gm. tissue. Values represent mean± S.D (n=6). Vertical lines represent standard deviation. *Significantly different from their respective controls at P<0.05.

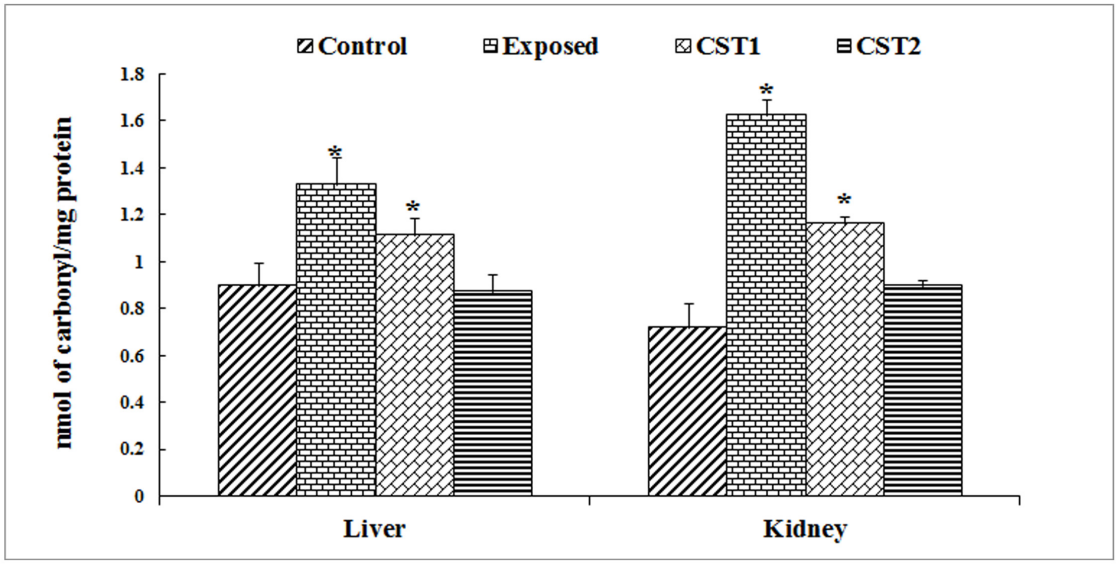

Fig. 3. Total Protein Carbonyl Content (TPCC) in liver and kidney of control, exposed and treated rats. TPCC levels were expressed as nmol of carbonyl/mg. protein. Values represent mean± S.D (n=6). Vertical lines represent standard deviation. *Significantly different from their respective controls at P<0.05.

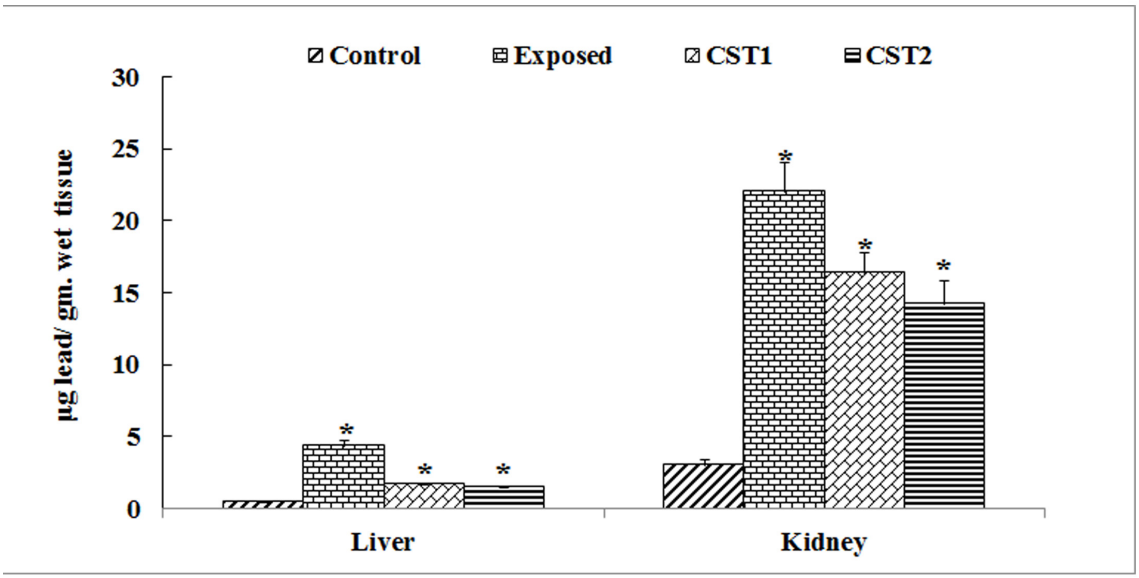

Fig. 4. Lead in liver and kidney of control, exposed and treated rats. Lead concentration was expressed as µg lead/gm. wet weight. Values represent mean± S.D (n=6). Vertical lines represent standard deviation. *Significantly different from their respective controls at P<0.05.

respective control (3.04µg/g), whereas the CST1 (16.4µg/g) kidney showed a decrease in lead concentration when compared to exposed kidney. Maximum decrease in lead concentration was seen in CST2 (14.28µg/g) group kidney.

The concentration of copper in the liver and kidney of control, exposed, CST1 and CST2 groups were presented in Fig. 5. There was a significant (p<0.05) decrease in copper concentration in the exposed liver (3.21µg/g) and kidney (4.06µg/g) when compared to their respective controls (5.35 and 10.37µg/g). Maximum decrease in copper concentration was seen in exposed kidney (60.84%) when compared to exposed liver (39.51%). However, treatment with *Coriandrum sativum* showed restoration of copper levels in the liver and kidney. The restoration is dose dependent of the extract and maximum restoration was seen in CST2 liver (74%).

Fig. 6 shows data on zinc concentration in liver and kidney of control, exposed, CST1 and CST2 groups. Zinc concentration showed a significant (p<0.05) decrease on lead exposure in liver (23.98µg/g) and kidney (14.72µg/g) when compared to their respective controls (40.18 and 21.13µg/g). CST1 (28.22µg/g) and CST2 (30.19µg/g) groups showed restoration of zinc concentration in liver, CST2 (75%) showed maximum restoration of zinc than CST1 (70%). In the kidney of CST1 (19.53µg/g) and CST2 (21.67µg/g), maximum restoration was seen and zinc concentration restored near to control values.

Concentration of iron in liver and kidney was presented in Fig. 7. Iron concentration showed a significant (p<0.05) decrease in liver of exposed (77.36µg/g) with respect to its control (100.98 µg/g). Whereas exposed kidney (71.5µg/g) showed insignificant decrease in iron concentration compared to its control (79.33µg/g). Treatment with *Coriandrum sativum* showed restoration of iron levels back to control in CST1 and CST2 of liver (93.52 and 115.18µg/g) and kidney (83.99 and 90.85µg/g). Kidney of CST2 showed maximum increase in iron concentration

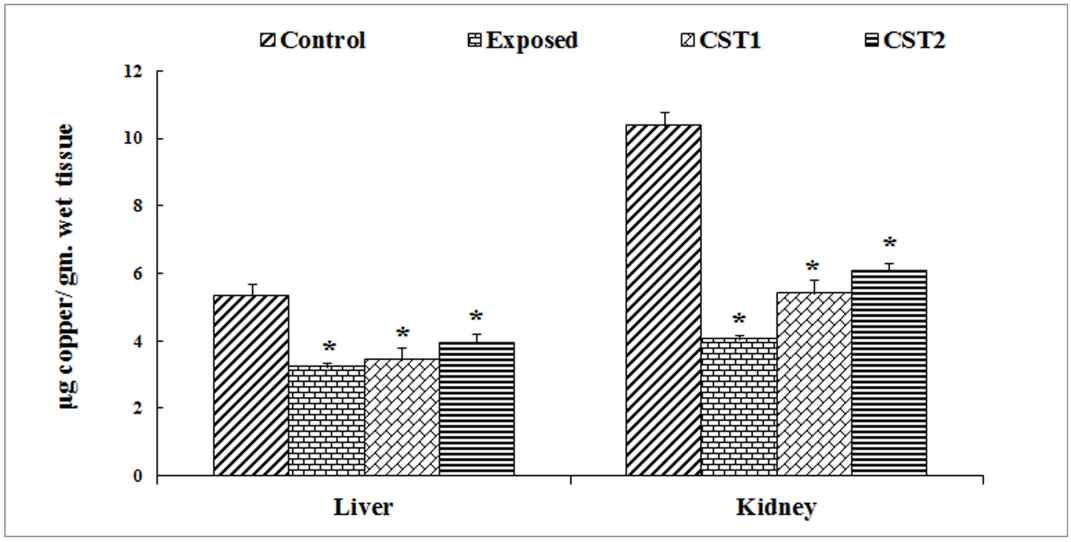

Fig. 5. Copper in liver and kidney of control, exposed and treated rats. Copper concentration was expressed as µg copper/gm. wet weight. Values represent mean± S.D (n=6). Vertical lines represent standard deviation. *Significantly different from their respective controls at P<0.05.

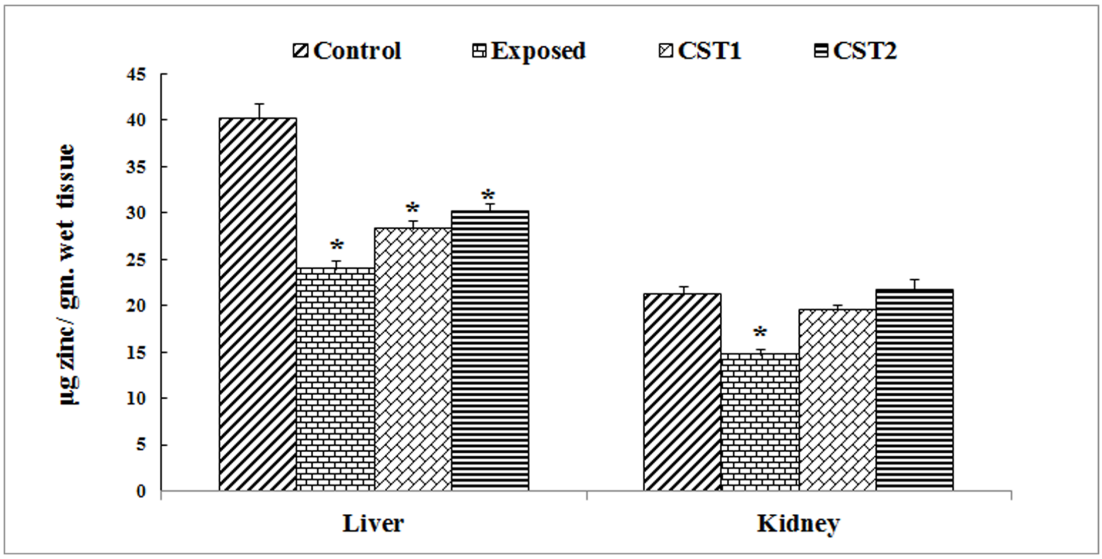

Fig. 6. Zinc in liver and kidney of control, exposed and treated rats. Zinc concentration was expressed as µg zinc/gm. wet weight. Values represent mean± S.D (n=6). Vertical lines represent standard deviation. *Significantly different from their respective controls at P<0.05.

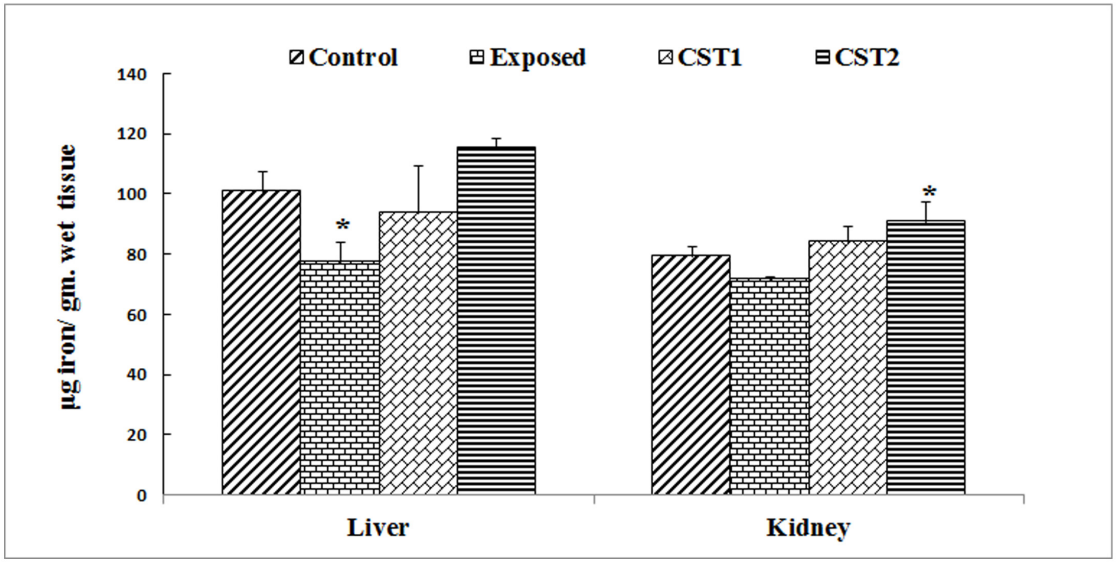

Fig. 7. Iron in liver and kidney of control, exposed and treated rats. Iron concentration was expressed as µg iron/gm. wet weight. Values represent mean± S.D (n=6). Vertical lines represent standard deviation. *Significantly different from their respective controls at P<0.05.

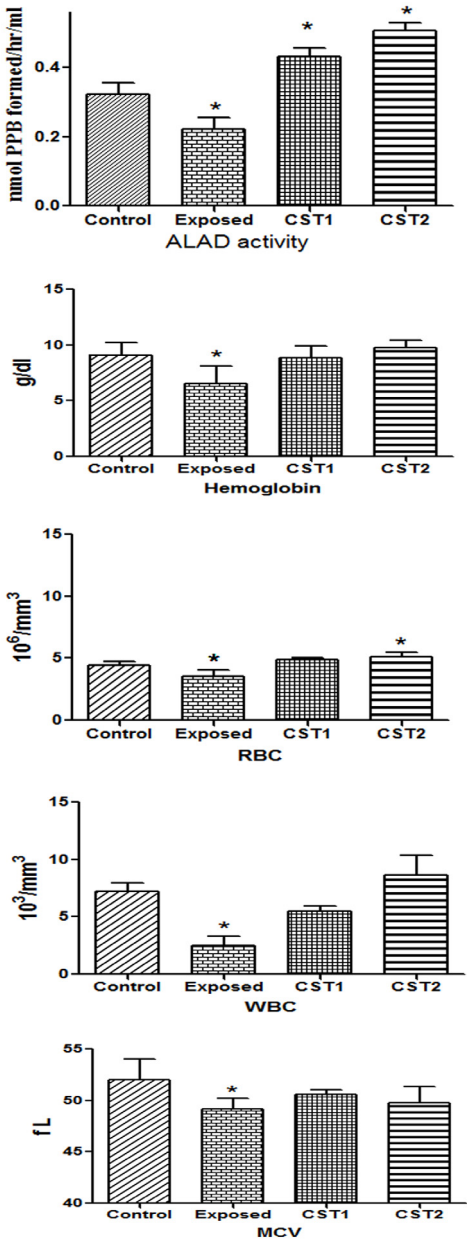

Fig. 8. Blood parameters ALAD activity, Hb, RBC, WBC and MCV in control, exposed and treated rats. ALAD-activity,Hb, RBC,WBC and MCV were expressed as nmol PPB/hr/ml, g/dL, 10^6/mm^3,10^3/mm^3 and fL. Values represent mean± S.D (n=6). Vertical lines represent standard deviation. *Significantly different from their respective controls at P<0.05.

(114.52%) than the control and the increase was significant (p<0.05).

Blood delta-ALAD activity showed a significant (p<0.05) decrease (31.77%) in exposed group when compared to the control. CST1 (33.72%) and CST2 (57.32%) groups showed significant (p<0.05) increase in delta-ALAD activity and maximum increase in enzyme activity was seen in CST2 group.

Hemoglobin (Hb) concentration in the blood of control, exposed, CST1 and CST2 groups was presented in Fig. 8. However, Hb concentration showed a significant (p<0.05) decrease in the exposed group (28.33%). Whereas CST1 (97%) and CST2 (107.35%) groups showed restoration of Hb levels, CST1 (8.84) restored Hb level near to control and CST2 (9.78) showed increase in Hb level than the control value.

RBC count showed a significant (p<0.05) decrease (19.31%) (Fig. 8) in lead exposed ($3.55x10^6$/mm^3) group when compared to the control ($4.40x10^6$/mm^3). CST1 ($4.87x10^6$/mm^3) group showed insignificant increase (10.68%) in RBC count while CST2 ($5.1x10^6$/mm^3) group showed significant increase (16.13%) in RBC count than the control group.

WBC count showed a decrease (Figure 8) in lead exposed ($2.45x10^3$/mm^3) group when compared to control ($7.17x10^3$/mm^3) and the decrease was significant (p<0.05). Whereas CST1 ($5.46x10^3$/mm^3) and CST2 ($8.66x10^3$/mm^3) group showed restoration of WBC count near to control values and maximum restoration was seen in CST2.

MCV showed a significant (p<0.05) decrease in lead exposed (49.18fL) group (Figure 8) when compared to the control (51.99fL). Whereas CST1 (50.59fL) and CST2 (49.78fL) showed insignificant increase in MCV.

Discussion

The present investigation was intended to test the efficacy of *Coriandrum sativum* seed

extract against lead induced oxidative stress in a rat model. *Coriandrum sativum* is well known as a traditional and natural medicine. The increase in lead concentration in liver and kidney following experimental exposure was associated with increased oxidative stress, which might be responsible for lead induced toxic effects indicated by the increase in ROS, LPP and TPCC.

Lead is ubiquitous and detected in all phases of the environment and it enters biological system through air, water and food. Its persistence and toxicity in human and animal tissues had often been associated with considerable health risks (22). In the present study the levels of lead in tissues of liver and kidney were significantly (p<0.05) higher in lead exposed group than controls. Lead exposed group showed increase in the levels of ROS, LPP, TPCC, metal content and decrease in the delta-ALAD enzyme activity

Metal toxicity causes mitochondrial damage, this result in excessive free radical generation (23). Lead toxicity leads to free radical damage by increasing the production of reactive oxygen species (ROS) including hydroperoxides, singlet oxygen, hydrogen peroxide and direct reduction of antioxidant reserves (24). The primary target of oxidative damage produced by xenobiotics is cellular membrane (25). Oxygen radicals attack the cellular components such as polyunsaturated fatty acids of phospholipids which are sensitive to oxidation (26). Lead produces oxidative damage by increasing the oxidation of membrane lipids (27). Flavonoids such as Quercetin 3-glucuronide, isoquercitin and rutin were separated and identified in coriander seeds (3). Studies on the antioxidant properties of flavonoids from various plant extracts showed stimulatory action on antioxidative enzymes (28) and they also showed stimulatory action on transcription and gene expression of certain antioxidant enzymes (29). The decrease in ROS levels in CST1 and CST2 can be attributed to the protective role of *Coriandrum sativum*. Administration of *Coriandrum sativum* restored the levels of ROS back to normal in both liver and kidney.

MDA (Malondialdehyde) is a major reactive aldehyde formed during the peroxidation of polyunsaturated fatty acids present in biological membrane (30). Generally the MDA levels are used as indicators of tissue oxidative stress involving a series of chain reactions (18). It is well known that lead binds to enzymes and reduces enzyme activities. It can even disturb protein synthesis in hepatocytes (31). Lead induces the free radical formation indirectly and influences the processes of lipid peroxidation through damage of protective antioxidant barrier (16). Lead possesses a strong affinity to thiol groups of amino acids, especially cysteine. Lead may affect the antioxidant barrier via inhibiting the functional thiol groups of enzymes such as SOD and GST (32, 33). Therapy with sodium molybdate showed significant protection from lead to liver and kidney and also reduced the formation of LPO (lipid peroxide) (34). Human lymphocytes pretreated with polyphenols isolated from *Coriandrum sativum* showed protection from oxidative damage induced by hydrogen peroxide. Polyphenols extracted from coriander seeds showed significant decrease in concentration of TBARS in human lymphocytes compared with the H_2O_2 control (35). Similarly in the present investigation the flavonoids present in *Coriandrum sativum* may have protected the liver and kidney from the oxidative damage induced by lead. Volatile compounds of coriander seed oil showed inhibition of lipid peroxidation (36, 37). Studies using coriander seed extract showed positive correlation between phenolic content and antioxidant activity. The seed extract showed concentration dependent inhibitory activity towards 15-lipoxygenase and DPPH radical scavenging activity (38). Treatment with *Coriandrum sativum* resulted in normalization of TBARS (Thiobarbituric acid reactive species) and glutathione of CCl_4 exposed rats. Pretreatment with *Coriandrum sativum* increased activity of antioxidant enzymes compared to CCl_4 animals

indicating the efficacy of *Coriandrum sativum* to act as an antioxidant (39). Administration of *Coriandrum sativum* (200mg/kg) significantly reduced the increased serum enzymes (SGOT and SGPT) induced by CCl_4, indicating improvement of the functional status of the liver, which was also supported by the histopathological findings (40). Quercetin a flavonoid effectively reduced lipid peroxidation and restored the activities of antioxidant enzymes and inhibited apoptotic damage (41). The seeds of *Coriandrum sativum* contain Quercetin(3) and this may be a possible mechanism of protection from lead-induced lipid peroxidation.

Similarly in the present investigation along with ROS and LPP there was decrease in TPCC levels after administration of *Coriandrum sativum*. This shows the protective role of *Coriandrum sativum* in reducing TPCC levels.

Lead toxicity inhibits delta aminolevulinate dehydratase activity. Delta-ALAD an indicator enzyme for lead toxicity and its activity is inhibited when lead binds to its active center (42). This enzyme catalyzes the asymmetric condensation of two molecules of delta-aminolevulinic acid to porphobilinogen in the initial steps of heme biosynthesis. Lead toxicity increases the excretion of accumulated ALA into the urine (12, 13) and decreases the production of PBG (Porphobilinogen). Administration of *Coriandrum sativum* decreased the concentration of urinary ALA (15). This suggests that Chinese parsley has efficacy against lead poisoning. Inhibition of Δ-ALAD activity may lead to accumulation of ALA (43), which may auto-oxidize to form reactive oxygen species. Inhibition of delta-ALAD activity leads to accumulation of delta-aminolevulinic acid (Δ-ALA) which undergoes auto-oxidation inducing free radicals and in this way induces lipid peroxidation (42). Similarly in the present study there was a decrease in Δ-ALAD activity and increase in the levels of ROS and LPP. The group treated with *Coriandrum sativum* showed reduction in the levels of ROS, LPP and TPCC. In contrast to this, there was increase in Δ-ALAD activity in CST1 and CST2. The decrease in haematological parameters (RBC, WBC, Hb and MCV) was in agreement with previous reports (44) that metal toxicity causes changes in the blood indices of rats. The significant reduction in RBCs indicated that lead adversely affected the hemoglobin synthesis by binding to delta-ALAD, and increased rate of destruction of erythrocytes. Treatment with *Coriandrum sativum* decreased the toxic effects of lead on the hematological values and had a protective role in anemia induced by lead. A similar decrease in Delta-ALAD activity was noticed by Adonaylo and Oteiza (10) and Pande et al (45) in rats exposed to lead. They also reported decrease in hematocrit on exposure to lead acetate. In the present investigation treatment with *Coriandrum sativum* restored the delta-ALAD enzyme activity in a dose dependent manner.

It is clear from the results of the present investigation that administration of the hydroalcoholic extract of *Coriandrum sativum* protected rat liver and kidney against lead induced oxidative stress. The results of the present study corroborate well with the early reports that showed the protective effects of *Coriandrum sativum* on localized lead deposition in male ICR mice (15). A similar prophylactic efficacy of *Coriandrum sativum* (Coriander) was reported by Sharma et al (46) on testis of lead-exposed mice.

Lead concentration significantly increased in the exposed rat liver and kidney, upon treatment with *Coriandrum sativum* there was decrease in lead levels in the CST1 and CST2 treated rats. Micronutrients such as zinc, copper and iron levels decreased in the lead exposed group but treatment with *Coriandrum sativum* showed restoration near to control in CST1 and CST2 treated rats. The concentration of zinc and copper in liver and kidney in the present study was similar to that of previous reports (45). Flavonoids and phenolic compounds potentiate the removal of lead from the body by their metal chelating ability (47). Administration of (Chinese parsley) *Coriandrum sativum* also prevented the accumulation of Cd in fish *Oncorhynchus mykiss*

(48). Treatment with chelating agents such as EDTA showed reduction in the levels of lead in the liver of rats exposed to lead (49, 31). The chelating agents form an insoluble complex with lead to remove it from lead burdened tissues (33). But natural chelating agent such as phitic acid has strong ability to chelate multivalent metal ions (50). There may be a possibility for the seeds of *Coriandrum sativum* that contain chelating agents similar to phitic acid. The results of the present study corroborate with earlier reports that suggest the preventive effects of *Coriandrum sativum* on lead induced oxidative stress in male rats. Using a murine model of lead intoxication (15) *Coriandrum sativum* reduced lead accumulation in bone and protected mice from lead-induced kidney damage. However, it appears that the protection shown by *Coriandrum* was organ-specific as well as parameter-specific in both liver and kidney.

Conclusion

Overall the results of the present investigation support the hypothesis that the hydro-alcoholic seed extract of *Coriandrum sativum* protects rat liver and kidney from lead induced-oxidative stress by its antioxidant and metal chelating properties. Tissue oxidative stress parameters and lead concentration in liver and kidney showed increase on exposure to lead and they restored back to control on treatment with *Coriandrum sativum.* Blood parameters, delta-ALAD enzyme activity, copper, zinc and iron levels showed inhibition on lead exposure and treatment with *Coriandrum sativum* showed protection against lead toxicity. Maximum protection from lead toxicity was seen in CST2 group. Further studies are needed to analyze the exact mechanism by which *Coriandrum sativum* protects rat liver and kidney from lead toxicity. Overall, these results suggest that the seed extract of *Coriandrum sativum* might reduce the lead-induced oxidative stress organ specifically by its antioxidant and metal chelating activity.

References

1. Sairam, T.V. (1998). Home remedies: a handbook of herbal cures for common ailments. Penguin Books India, New Delhi, pp. 75.
2. Taniguchi, M., Yanai, M., Xiao, Y.Q., Kido, T. and Baba, K. (1996). Three isocoumarines from *Coriandrum sativum*. Phytochem, 42: 843.
3. Kunzemann, J. and Herrmann, K. (1977). Isolation and identification of flavon(ol)-Oglycosides in caraway (*Carum carvi* L.), fennel (*Foeniculum vulgare* Mill.), anise *(Pimpinella anisum* L.), and coriander (*Coriandrum sativum* L.), and of flavon-Cglycosides in anise. I. Phenolics of spices. Zeitschrift fur Lebensmitteluntersuchung und forschung A, 164 (3): 194–200.
4. Chithra, V.V. and Leelamma, S. (2000). *Coriandrum sativum* effect on lipid metabolism in 1, 2-dimethyl hydrazine induced colon cancer. J. Ethnopharmacol, 17: 457.
5. Omura, Y. and Beckman, S.L. (1995). Role of mercury (Hg) in resistant infections and effective treatment of *Chlamydia trachomatis* and Herpes family viral infections (and potential treatment for cancer) by removing localized Hg deposits with Chinese parsley and delivering effective antibiotics using various drug uptake enhancement methods. Acupuncture and Electro-therapeutics Res, 20: 195–229.
6. Omura, Y., Shimotsuura, Y., Fukuoka, A., Fukuoka, H. and Nomoto,T. (1996). Significant mercury deposits in internal organs following the removal of dental amalgam, and development of pre-cancer on the gingiva and the sides of the tongue and their represented organs as a result of inadvertent exposure to strong curing light (used to solidify synthetic dental filling material) of effective treatment: a clinical case report, along with organ representation areas for each tooth. Acupuncture and Electro-therapeutics Res,

21: 133–160.

7. Khan, M.S.H., Mostafa, M.S., Hossain, M.A. and Sayed, M.A. (2008). Effect of garlic and vitamin B-complex in lead acetate induced toxicities in mice. Bang. J. Vet. Med, 6(2): 203–10.

8. Courtois, E., Marques, M. and Barrientos, A. (2003). Lead-induced downregulation of soluble guanylate cyclase in isolated rat aortic segments mediated by reactive oxygen species and cyclooxygenase-2. J. Am. Soc. Nephrol, 14: 1464–70.

9. WHO. (1977). Environmental health criteria. 3. Lead. Geneva: World Health Organization.

10. Adonaylo, V.N. and Oteiza, P.I. (1999). Lead intoxication: antioxidant defenses and oxidative stress in rat brain. Toxicology, 135(3): 77–85.

11. Hsu, P.C. and Guo, Y.L. (2002). Antioxidant nutrients and lead toxicity. Toxicology, 180(1): 33–44.

12. Tomokuni, K., Ichiba, M. and Hirai, Y. (1991a). Elevated urinary excretion of beta-aminoisobutyric acid and delta-aminolevulinic acid (ALA) and the inhibition of ALA-synthase and ALA-dehydratase activities in both liver and kidney in mice exposed to lead. Toxicol. Let., 59: 169–173.

13. Tomokuni, K., Hirai, Y. and Ichiba, M. (1991b). Fluorophotometric determination of hepatic d-aminolevulinic acid synthase activity by high-performance liquid chromatography. J. of Chromatogr., 567: 65–70.

14. Frei, B. and Higdon, J. (2003). Antioxidant activity of tea polyphenols in vivo: evidence from animal studies. J. Nutr, 133: 3275–84.

15. Aga, M., Iwaki, K., Ueda, Y., Ushio, S., Masaki, N., Fukuda, S., Kimoto, T., Ikeda, M. and Kurimoto, M. (2001). Preventive effect of *Coriandrum sativum* (Chinese parsley) on localized lead deposition in ICR mice. J. of. Ethnopharmacol, 77: 203-208.

16. Patra, R.C., Swarup, D. and Dwivedi, S.K. (2001). Antioxidant effects of alpha-tocopherol, ascorbic acid and l-methionine on lead induced oxidative stress to the liver, kidney and brain in rats. Toxicology, 162: 81–8.

17. Bondy, S.C., Guo, S.X. (1996). Lead potentiates iron-induced formation of reactive oxygen species. Toxicology. Lett. 87(2-3): 109-112.

18. Ohkawa, H., Ohisi, N., Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95(2): 351-358.

19. Levine, R.L., Garland, D., Oliver, C, N., Amici, A., Climent, I., Lenz, A., Ahn, B., Shaltiel, S., Stadtman, E.R., (1990). Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. 186: 464-478.

20. Berlin, A. and Schaller, K.H. (1974). European standardized method for the determination of delta aminolevulinic acid dehydratase activity in blood. Z. Klin. Chem. Klin. Biochem, 12: 389-390.

21. Zachariadis, G.A., Stratis, J.A., Kaniou, I., Kalligas, G. 1995. Critical comparison of wet and dry digestion procedures for trace metal analysis of meat and fish tissues. Microchim. Acta 119, 3-4.

22. Juberg, D.R., Kleiman, C.F. and Simon, C.K., (1997). Position paper of the American Council on Science and Health: lead and human health. Ecotoxicol. Environ. Saf, 38: 162–80.

23. Sanfeliu, C., Sebastia, J. and Kim, S.U. (2001). Methylmercury neurotoxicity in cultures of human neurons, astrocytes, neuroblastoma cells. Neurotoxicol, 22: 317–327.

24. Ercal, N., Gurer-Orhan, H. and Aykin-Burns, N. (2001). Toxic metals and oxidative stress. Part 1. Mechanisms involved in metal-induced oxidative damage. Curr. Top. Med. Chem, 1: 529–539.

25. Halliwell, B. and Gutteridge, J.M.C. (1989). Free Radicals in Biology and Medicine, second ed. Clarendon Press, Oxford, UK.

26. Marnett, L.J., 1999. Lipid peroxidation-DNA damage by malondialdehyde. Mutat. Res. Fund. Mol. Mech. Mutagen 424, 83–95.

27. Nyska, A. and Kohen, R. (2002). Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. Toxicol. Pathol, 30: 620–650.

28. Nagata, H., Takekoshi, S., Takagi, T., Honma, T. and Watanabe, K. (1999). Antioxidative action of flavonoids, quercetin and catechin, mediated by the activation of glutathione peroxidase. Tokai J. Expt. Clin. Med, 24: 1–11.

29. Rohrdanz, E., Ohler, S., Tran-Thi, Q.H. and Kahl, R. (2002). The phytoestrogen daidzein affects the antioxidant enzyme system of rat hepatoma H4IIE cells. J. Nutr, 132 (3): 370–375.

30. Vaca, C.E., Wilhelm, J.M., Harms-Rihsdahl, M. (1988). Interaction of lipid peroxidation product with DNA. Mutat. Res. Rev. Gen. Toxicol, 195: 137–149.

31. Shalan, M.G., Mostafa, M.S., Hassouna, M.M. and El-Nabi S.E. (2005). Amelioration of lead toxicity on rat liver with vitamin C and Silymarin supplements. Toxicol, 206(1): 1–15.

32. Jurczuk, M., Moniuszko-Jakoniuk, J. and Brz¡ýoska, M.M. (2006). Involvement of some low-molecular thiols in the peroxidative mechanisms of lead and ethanol action on rat liver and kidney. Toxicol, 219: 11–21.

33. Patrick, L. (2006). Lead toxicity part II. The role of free radical damage and the use of antioxidants in the pathology and treatment of lead toxicity. Altern. Med. Rev,11(2): 114-27.

34. Flora, S.J.S., Jeevaratnam, K. and Kumar, D. (1993). Preventive effects of sodium molybdate in lead intoxication in rats. Ecotoxicol. Environ. Saf, 26: 133–7.

35. Hashim, M.S., Lincy, S., Remya, V., Teena, M. and Anila, L. (2005). Effect of polyphenolic compounds from *Coriandrum sativum* on H_2O_2-induced oxidative stress in human lymphocytes. Food Chem., 92: 653-660.

36. Anilakumar, K. R., Nagaraj, N. S., and Santhanam, K. (2001). Effect of coriander seeds on hexachlorocyclohexane induced lipid peroxidation in rat liver. Nutr. Res., 21: 1455–1462.

37. Tanabe, H., Yoshida, M., and Tomita, N. (2002). Comparison of the antioxidant activities of 22 commonly used herbs and spices on the lipid oxidation of pork meat. Animal Science Journal, 73: 389–393.

38. Wangensteen, H., Samuelsen, A.B. and Malterud, K.E. (2004). Antioxidant activity in extracts from coriander. Food Chem., 88: 293-297.

39. Nevin, K.G. and Vijayammal, P.L. (2005). Effect of *Aerva lanata* on solid tumor induced by DLA cells in mice. Fitoterapia, 74: 578–580.

40. Sreelatha, S., Padma, P.R. and Umadevi, M. (2009). Protective effects of *Coriandrum sativum* extracts on carbon tetra chloride-induced hepatotoxicity in rats. Food and Chem. Toxicol, 47: 702-708.

41. Liu, C.M., Zheng, Y.L., Lu,J., Zhang, Zi.F., Fan, S.H., Wu,D.M. and Ma, J.Q. (2010). Quercetin protects rat liver against rat liver against lead-induced oxidative stress and apoptosis. Environ. Toxicol. and Pharmacol, 29(2): 158-166.

42. Flora, S.J.S., Mittal, M. and Mehta, A. (2008). Heavy metal induced oxidative stress & its possible reversal by chelation therapy. Indian. J. Med. Res, 128: 501–23.

43. Dreosti, I. (1991). Free radical pathology genome. In: Dreosti, I.E.(Ed.), Trace Elements, Micronutrients and Free Radicals. Humana, Totowa, NJ, pp. 149.

44. Karmakar, R., Bhattacharya, R. and Chatterjee, M. (2000). Biochemical, haematological and histopathological study in relation to time-related cadmium-induced hepatotoxicity in mice. Biometals, 13(3): 231-239.

45. Pande, M., Mehta, A., Pant, B.P. and Flora, S.J.S. (2001). Combined administration of a chelating agent and an antioxidant in the prevention and treatment of acute lead intoxication in rats. Environ. Toxicol. and Pharmacol, 9: 173-184.

46. Sharma, V., Kansal, L. and Sharma, A. (2010a). Prophylactic efficacy of *Coriandrum sativum* (Coriander) on testis of Lead-exposed mice. Biol. Trace. Elem. Res, 36: 337-354.

47. Heim, K.E., Tagliaferro, A.R. and Bobilya, D.J. (2002). Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. J. Nutr. Biochem. 13:572-584.

48. Ren, H., Jia, H., Endo, H. and Hayashi, T. (2009). Cadmium detoxification effect of chinese parsley *Coriandrum sativum* in liver and kidney of rainbow trout *Oncorhynchus mykiss*. Fish. Sci. 75: 731-741.

49. Gary, O.K. and Blair, M.J. (2007). Effect of diet on the response in rats to lead acetate given orally or in drinking water. Biol. Trac. Elem. Res, 17(1): 167–73.

50. Zhou, J.R. and Erdman, J.W. Jr. (1995). Phitic acid in health and disease. Critical reviews in Food Science and Nutrition 35, 495-508.

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Influence of Electrolytes on Controlled Release Matrix Tablets of Diphenhydramine HCl

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Abstract

Oral controlled matrix tablets of Diphenhydramine HCl were formulated by different hydrophilic polymers such as hydroxypropyl methylcellulose K15 M (HPMC K15 M), poly ethylene oxide WSR 301 (PEO WSR 301) and carbopol 940 P along with pharmaceutically acceptable electrolytes. The drug candidate selected is diphenhydramine HCl which is widely used as first generation antihistamine possessing anticholinergic, antitussive, antiemetic and sedative properties. This is mainly administered for once a day formulation for treating chronic allergic disorders and also for psychiatric disorders. Hence diphenhydramine HCl was formulated as controlled release matrix tablet formulations with several polymers by employing pharmaceutically acceptable electrolytes. Electrolytes such as sodium carbonate, magnesium carbonate and calcium carbonate were used at specific concentration (10 mg/tablet) in various formulations, while the drug to polymer concentrations were maintained at different ratios as 1:2, 1:2, 1:1 respectively. In this work a new attempt was made for *in-situ* interactions between drug and electrolytes were devised to control the release of highly water soluble drugs from oral hydrophilic monolithic systems. These electrolytes were used to monitor matrix swelling and gel properties. The results indicated that the drug released at a controlled rate were due to differential swelling rate and matrix stiffening and provides a uniform gel layer. These findings indicated that the swelling and gel formation in the presence of ionisable species within the hydrophilic matrices provide an attractive alternative for controlled drug delivery from a monolithic system.

Keywords: Diphenhydramine HCl, HPMC K15 M, PEO WSR 301, Carbopol 940 P, Sodium Carbonate, Magnesium Carbonate, Calcium Carbonate.

Introduction

Recently numerous hydrophilic polymers have been investigated and are currently used in the design of complex controlled release systems (1-3). The polymers that are most widely used in the design of controlled release of drugs include non ionic Hydroxypropyl methylcellulose (HPMC K15 M), Poly ethylene oxide's (PEO's), Carbopol 940 P. The major challenge in the development of new controlled release devices is to achieve optimal drug concentration at the site of action; liberation of drug from the device must be controlled as accurately as possible (4). The dissolution in a monolithic matrix for linear drug release over a prolonged period of time is not easily achievable and still remains a challenge. The limitation of a hydrophilic polymer may be circumvented through modification of physical and chemical infrastructure of the polymeric gel system by using electrolytes.

In the present investigation, studies were undertaken for design and development of oral

controlled release drug delivery systems of Diphenhydramine HCl by matrix diffusion technique. It is an ethanolamine derivative and H_1-receptor antagonist, thereby preventing the action of histamine on the cells. It is widely used as an anti-allergic, anti-emetic and anti-tussive in many pharmaceutical preparations. It is freely soluble in water, HCl and 7.8 p^H phosphate buffers. DPH is readily absorbed from the gastrointestinal tract. After oral doses it undergoes extensive first pass metabolism in the liver. The mean elimination half life of Diphenhydramine HCl is about 6-9 hours (5). Based on these physiochemical, biopharmaceutical properties and rationale of clinical efficacy Diphenhydramine HCl was selected as drug candidate for developing controlled release matrix tablet formulations (6).

In the present work, a reliable process has been established for inducing *in-situ* reactions between pharmaceutically acceptable electrolytes and drug which influences the intragel swelling dynamics and relative physical integrity of the swollen matrix structure. Furthermore, that may produce heterogeneous domains within the swollen gel boundary.

In the past, alkaline compounds (or) buffers have been included in solid oral formulations for several acidic drugs that undergo dissolution rate limited absorption (7).The same principle of addition of buffers, osmotically active agents, surfactants (or) combinations thereof has also been utilised to control the swelling of hydrophilic polymers with different coating and inclusion techniques(8). However more specific strategy has been employed to apply the same principle to design a simple directly compressible, monolithic controlled release system. In general the application of buffers and ionisable compounds in dosage form design has essentially been limited to the minimisation of localised GIT adverse effects and the solubility dependency of poorly soluble compounds (9).

The aim of this work was to provide and expand on a means to design, formulate and develop a novel oral monolithic, controlled release tablet dosage form of a drug that may be tailored to provide quasi steady state drug release over an extended period of time (10, 11). The rationale behind the mechanism and dynamics of electrolytes induced matrix stiffening and structural changes to the gel is the basis of controlled drug release has also been elucidated. The drug candidate selected is diphenhydramine HCl which is widely used as first generation antihistamine possessing anticholinergic, antitussive, antiemetic, and sedative properties. This is mainly administered as once a day formulation for treating chronic allergic disorders and also for psychiatric disorders. Hence diphenhydramine HCl was formulated as controlled release matrix tablet formulations with several polymers by employing pharmaceutically acceptable electrolytes for extending the drug release upto 24 hours.

Materials and Methods

Diphenhydramine HCl was commercially procured from Yarrow Chem Laboratories, Mumbai. Hydroxypropyl methylcellulose K15 M (HPMC K15 M) was a gift sample from Dow Chemical's Asia Pvt., Ltd., Mumbai. Poly ethylene oxide WSR 301 (PEO WSR 301), Carbopol 940 P and Microcrystalline cellulose were commercially procured from Yarrow Chem Laboratories, Mumbai. Sodium carbonate, Calcium carbonate and Magnesium carbonate were commercially procured from Qualigens Fine Chemicals, Mumbai.

Evaluation of powder flow properties: The powder blends of diphenhydramine HCl were evaluated for flow properties such as angle of repose and carr's index to find whether the blends are suitable for compression as matrix tablets by direct compression process.

Preparation of matrix tablets : Diphenhydramine HCl controlled release matrix tablets were prepared by direct compression method. The controlled release matrix tablet formulations consisted of a drug, polymer and electrolytes. The weights of all formulations were maintained

uniformly by using MCC as diluent. The materials were weighed and individually passed through sieve no: 60 and blended for 15 min in double cone blender. The powder mixture was lubricated with 1% talc and compressed into tablets by using a 10 station rotary punching machine (Minipress). To minimize processing variables, all bacthes of tablets were compressed under identical conditions. The compositions of various matrix tablet formulations were given in Table 1.

Evaluation of physical parameters : The physical parameters such as hardness, friability, weight uniformity and drug content were evaluated for the prepared matrix tablets as per the standards of official compendium (12).

Determination of swelling Index : The swelling behaviour of a dosage unit was measured by studying its weight gain. The swelling index of tablets were determined by placing the tablets in the basket of dissolution apparatus using distilled water as dissolution medium at 37 ± 0.5^{0}C. After 1, 2, 4, 6 and 8 hrs each dissolution basket containing tablet was withdrawn and blotted with tissue paper to remove the excess water and weighed on an analytical balance (Shimadzu, Ax 120). The experiment was performed in triplicate for each time point. Swelling index was calculated by using the following formula (13). The swelling index for various selected formulations of Diphenhydramine HCl matrix tablets were given in Table 3.

Swelling index =

$$\frac{(\text{Wet weight of tablet} - \text{Dry weight of tablet})}{\text{Dry weight of tablet}}$$

In vitro dissolution studies: Dissolution studies for all the matrix tablet formulations were performed in a calibrated 8 station dissolution test apparatus (LABINDIA DS 8000), equipped with paddles (USP apparatus II method) employing 900ml of distilled water as dissolution medium(14). Samples were withdrawn at regular intervals up to 16 hrs. Fresh volume of the medium was replaced with the same volume to maintain sink conditions and constant volume was maintained throughout the experiment. Samples withdrawn were suitably diluted with same dissolution medium and the amount of drug released was estimated by ELICO double beam spectrophotometer at 221nm and subsequently analyzed based on the equation, first order constant, Higuchi constant, and the Koresmeyer Peppas constant respectively. The following are the equations used:

$$\ln Q = k.\ t \qquad 1$$
$$Q = k.\ t \qquad 2$$
$$M_t / M_\infty = kt^n \qquad 3$$

Where Q in the equation 1 is cumulative percent drug remained, while Q in the equation 2 is cumulative amount of drug released, t is the release time and k is the constant incorporating the structural and geometrical characteristics of the release device. If the value of n =0.45 in equation 3 it indicates case I (Fickian) diffusion or square root of time kinetics, 0.45<n<0.89 indicates anomalous (non Fickian, drug diffusion in the hydrated matrix and the polymer relaxation) diffusion, n=0.89 indicates case II transport and n>0.89 indicates super case II transport. Linear regression analysis was performed for all these equations and regression coefficients (r) are determined.

Results and Discussion

The present study was under taken for design and evaluation of the controlled release matrix tablets of Diphenhydramine hydrochloride with HPMC K15 M, PEO WSR 301 and Carbopol 940 P by employing electrolytes as drug release retardants. All batches of tablets were produced under similar conditions to avoid processing variables. Before compression process the powder blends were evaluated for flow properties such as angle of repose and carr's index. The angle of repose values and the Carr's index values of all the powder blends were in the range of 13.2 - 20.2^0 and 9.4 - 15.1% respectively. The values for all the powder blends prepared established good and free flowing characteristics.

The compositions of various matrix tablets were given in Table 1.These tablets were

preliminarily evaluated for various physical parameters such as weight uniformity, hardness, friability and drug content. All batches of tablets with electrolytes were within the weight range of 250 ± 3 mg. The hardness of all the tablet formulations was in the range of 5.5 to 6 kg/cm^2. Friability loss of the tablet formulations were found to be negligible and were in the range of 0.1 to 0.2% w/w. Drug content estimated for all the tablet formulations were highly uniform with less than 2.5% variation. Drug content was also the same in case of matrix tablets containing electrolytes. All the matrix tablets were prepared under identical conditions and were found to be stable. Thus the results of physical parameters evaluated for various matrix tablets were within the limits.

The *in vitro* dissolution studies, showed greater inhibition of drug release rate of Diphenhydramine from the matrix tablets. The dissolution profiles of various matrix tablets were shown in Fig. 1-3. The dissolution parameters for all the matrix tablets were given in Table 2. The drug release from the matrix tablet formulations were extended up to 12hrs in majority of the formulations. By the incorporation of electrolytes into hydrophilic monolithic tablet matrices, it was possible to reduce the release rate of drug over an extended period of time. F1, F2 and F4 formulations containing drug and polymer in the ratio of 1:2 retarded the drug release up to 12hrs. It was found that increase in the concentration of polymers results in delay in the drug release. HPMC K15M, PEO WSR 301 and Carbopol 940 P are hydrophilic in nature and hence the formulations containing these polymers showed more swelling characteristics and drug release was extended up to 12hrs. Formulations prepared by employing electrolytes were found to extend the drug release over 16hrs (15).

The influence of retardation of drug release by various electrolytes were in the order of Sodium Carbonate > Calcium Carbonate > Magnesium Carbonate. Formulations F5, F7, F9 and F10 containing HPMC K15 M and PEO WSR 301 with electrolytes extended the drug release upto 16 hrs. Formulations F12 and F13 containing Carbopol 940 P with electrolytes have released the drug upto 75% in 16 hrs.

The SEM images of various matrix tablet formulations indicated that the formulations F1, F2 and F3 exhibited high swelling with wide pores on its surface. The formulations F5 and F9 containing electrolytes exhibited matrix stiffening with narrow or least pores on the tablet matrix. The SEM images of various formulations were shown from 4_a to 4_e. Thus the inclusion of electrolytes with in a swollen matrix for controlling

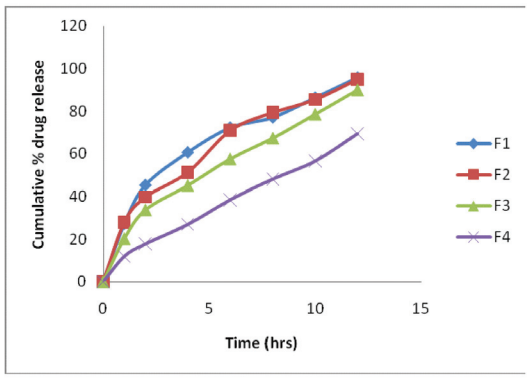

Fig. 1. Dissolution Profiles of Diphenhydramine HCl Matrix Tablets with HPMC K15M, PEO WSR301 and Carbopol 940 P as polymers.

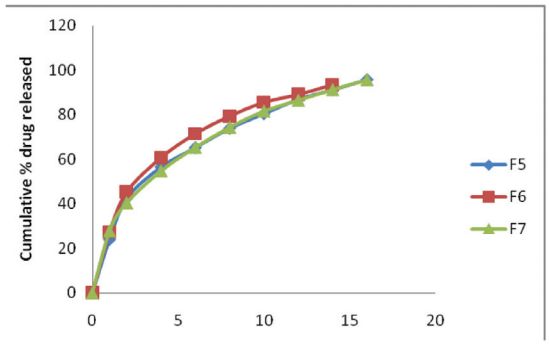

Fig. 2. Dissolution Profiles of Diphenhydramine HCl Matrix Tablets with HPMC K15 M along with Various Electrolytes

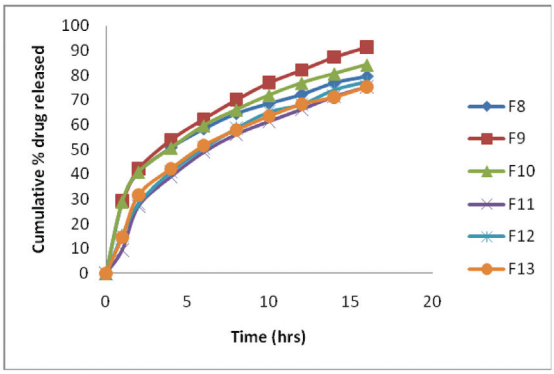

Fig. 3. Dissolution Profiles of Diphenhydramine HCl Matrix Tablets with PEO WSR 301 and Carbopol 940 P along with Various Electrolytes.

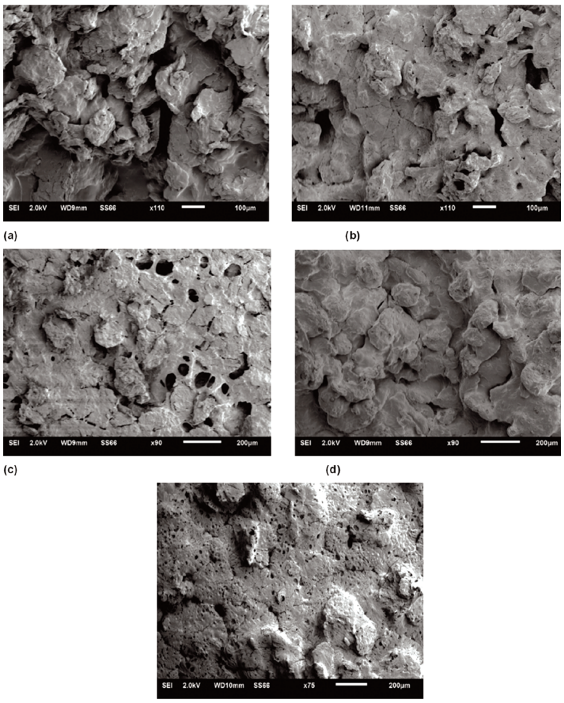

Fig. 4. SEM Photographs of Diphenhydramine HCl matrix tablets after dissolution for 6hrs a) F1 b) F2 c) F3 d) F5 e) F9

the release rate of Diphenhydramine may lead to the formation of free base of Diphenhydramine and fundamental structural changes in gel boundary, thus including the texural variations in the swollen matrix. It appears that electrolyte induced buffer threshold within the matrix place an essential role in effective interaction with drug and textural changes. Further it may be due to higher p^{Ka} values of electrolytes, which can display higher buffer threshold for maintaining suitable p^{H} values greater than 7.0 might exert better and desired control on drug release from matrix tablets (15). The following mechanism may prevail during the period of drug release from the swollen intragel structure. As the dissolution medium enters the periphery of the tablet, there is a rapid electrolyte water interaction with significant chemical reaction through electrolyte solubilization and subsequent events that may lead to both initial suppression and later enhancement of polymer swelling. During this infiltration process, the electrolyte present in the gel boundary could have been converted to chloride form (for example sodium carbonate and sodium chloride) due to which the hydrochloride form of Diphenhydramine HCl lead to the formation of free base of Diphenhydramine .The formation of free base might cause matrix stiffening. The passive and actively formed electrolytes within the gel matrix would compete for water leading to dehydration of polymer molecules, thus leading to suppression of initial swelling which was seen up to 2 to 3 hours with formulations containing electrolytes. After 3 hours the water attracted by electrolytes in to the polymer matrix could result in solubilizing the drug molecules which would diffuse by penetration of water leading to enhancement of swelling. The swelling index characteristics of various matrix tablets were given in Table 3. From these alterations and mechanisms of intragel changes, it appears possibility to inhibit drug dissolution rate. This inhibition in dissolution rate appears to be a time- dependent phenomenon. Since as more water enters the gel matrix layer- by- layer, the electrolytes and their by products are diluted and any drug base may revert to its hydrochloride form, which is subsequently released.

Conclusion

This work may provide a novel and simple approach to formulate an oral monolithic

Table 1. Composition of various diphenhydramine HCl matrix tablets

Ingredients (mg/tab)	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13
Diphenhydramine HCl	50	50	50	50	50	50	50	50	50	50	50	50	50
HPMC K15 M	100	-	-	-	100	100	100	-	-	-	-	-	-
PEO WSR 301	-	100	-	-	-	-	-	100	100	100	-	-	-
Carbopol940 P	-	-	50	100	-	-	-	-	-	-	50	50	50
Sodium carbonate	-	-	-	-	10	-	-	10	-	-	10	-	-
Magnesium carbonate	-	-	-	-	-	10	-	-	10	-	-	10	-
Calcium carbonate	-	-	-	-	-	-	10	-	-	10	-	-	10
MCC	95	95	95	145	85	85	85	85	85	85	135	135	135
Talc	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Magnesium Stearate	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Total tablet weight (mg)	250	250	250	250	250	250	250	250	250	250	250	250	250

Table 2. Dissolution parameters of diphenhydramine HCl matrix tablet formulations

Formulation Code	Zero Order		First Order		Higuchi		Peppas	
	K	R^2	K(hr^{-1})	R^2	K($mg.h^{1/2}$)	R^2	N	R^2
F1	6.924	0.708	0.226	0.986	13.054	0.984	0.477	0.990
F2	7.092	0.785	0.220	0.995	13.682	0.991	0.494	0.992
F3	6.769	0.883	0.168	0.984	13.559	0.992	0.574	0.993
F4	5.397	0.974	0.090	0.977	11.395	0.987	0.703	0.991
F5	5.193	0.645	0.216	0.998	11.402	0.983	0.469	0.984
F6	5.803	0.599	0.181	0.992	11.687	0.981	0.459	0.983
F7	5.174	0.643	0.176	0.980	11.274	0.992	0.452	0.996
F8	3.993	0.429	0.088	0.982	8.146	0.985	0.555	0.991
F9	4.779	0.580	0.213	0.997	10.134	0.995	0.460	0.996
F10	4.327	0.514	0.104	0.987	9.009	0.993	0.475	0.996
F11	4.408	0.803	0.084	0.983	10.351	0.989	0.673	0.987
F12	4.424	0.770	0.088	0.986	10.144	0.989	0.561	0.989
F13	4.238	0.710	0.083	0.983	9.586	0.986	0.542	0.984

Table 3: Swelling index of diphenhydramine HCl matrix tablets

S. No	Time (hrs)	Percent Swelling Index				
		F1	F2	F3	F5	F9
1	1	86.63	89.19	79.29	72.84	71.91
2	2	130.17	103.36	101.35	102.93	95.28
3	4	160.46	148.75	139.16	135.14	135.74
4	6	181.93	179.92	163.51	159.48	149.36
5	8	193.28	190.61	188.66	172.02	162.58

controlled release drug delivery system designed for delivery of Diphenhydramine HCl over an extended time period. An important feature of this system is the potential for generating constant drug release. The matrix tablet formulations prepared with polymers at drug and polymer ratio 1:2 in F1, F2 and 1:1 in F4 could be suitable for extending the drug release upto 12hrs. Whereas formulations F5, F7, F9 and F10 were suitable to extend the drug release upto 16 hrs due to presence of electrolytes. Hence the formulations can be further evaluated for *In vivo* pharmacokinetic and pharmacodynamic studies can be performed on a suitable animal model. Their physical parameters were within IP specified limits.

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References

1. Hayashida, T., Kikumaru, K.and Tomoyuki, O., US Patent No., US 5 593 694, 1997.
2. Conte, U., La Manna, A. and Maggi, L. US Patent No., US 5 626 874, 1997.
3. Fassihis, R. and Yang, L. US Patent No., US 5 783 212, 1998.
4. Siepmann, J., Ainaouli, A., Vergnaud, J.M., Bomeler, R.(1998) Formulation and Evaluation of Bilayered Tablets of Propanolol HCl By Using Gums. Journal of Pharmaceutical Sciences; 87:827.
5. Smith, S.G. and Davis, W.M. (1984). Non medical use of butorphanol and Diphenhydramine. Journal of American Medical Association, 25 (2):1010.
6. Reynolds, J.E. (2005) The Extra Pharmacopeia. 30th ed.In: Martindale. London: The Pharmaceutical press; 354.
7. Levina, M., Vuong, H. and Rajabi-Siahboomi, A.R. (2007). The influence of hydro-alcoholic media on hypromellose matrix systems. Drug Development and Industrial Pharmacy, 33:1125-34.
8. McClelland, G.A. and Zentner, G.M. US Patent No, US 5 120 548, 1992.
9. Vidyadhara, S. *et al.* (2004) Formulation and evaluation of propanolol hydrochloride oral controlled release matrix tablets. Indian Journal pharmaceutical Sciences, 66(2):188-192.
10. Vidyadhara, S., Babu, J.R. and Krishna, T.B. (2011) Influence of electrolytes on the controlled release of verapamil HCl from HPMC K15 M matrix tablets. Asian Journal of Pharmaceutics, 5:28-32.
11. Pillay, V. and Fassihi, R.US Patent Appl, 09/037 096, 1998.

12. United states pharmacopeia (2007) 25. Rockville, MD; United States Pharmacopeial Convention Inc; p. 1475.

13. Vendruscolo, C.W., Andreazza, I.F., Ganter, J.I., Ferrero, C. and Bresolin, T.M. (2005) Xanthan and galactomann from M. Scabrella matrix tablets based for oral controlled delivery of theophylline. International Journal of Pharmaceutics, 286: 1-11.

14. United States Pharmacopeia National Formulary 2007, Asian edition, Vol 2, p.1965.

15. Vidyadhara, S., Rama Rao, P. and Prasad, J.A.(2003). Electrolyte Induced Controlled Release of Diltiazem Hydrochloride From Methocel Matrix Tablet Formulations. International Journal of Pharmaceutical Excipients, 83-86.

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Development of Ayurvedic and Siddha Medicine through the Application of Modern Pharmaceutical Technique

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Abstract

Ayurveda and siddha are the ancient medicinal system that uses herbs as potential therapeutic agent but mainly suffer with limitations of inaccuracy in dose, less quality control standards, unacceptable odour and taste, type of dosage form, difficulty in administration and poor patient acceptance. The present paper involves the amendment of a new dosage form for improving the therapy of poly herbal formulation, and in this work Thirikadugu Chooranam and Nilavembu Kudineer Chooranam has been selected as models. These choornas are usually prescribed in the form of kashayam and kudineer (1 – 2 gm as hot water decoction twice a day), respectively in Siddha system of medicine. The inappropriate dose and dosing schedule, difficulty in preparation of the kashayam, bitter taste necessitates the development of a solid dosage form. But the direct conversion of choorana powder into tablets / capsules reduces the solubility of the active components, which actually need to be given as a solubilised extract. So, Modified Liquisolid Compaction technique is adapted her where the aqueous or hydro-alcoholic extract (kashayam or decoction) is prepared from the powder and concentrated to low liquid content, which is then converted into free flowing powder by the addition of inert carriers such as microcrystalline cellulose, silicon dioxide and lactose, followed by compression of the mass as compact discs. The liquisolid compact of the formulated Chooranam was studied for different quality control parameters.

Keywords: Thirikadugu Chooranam, Nilavembu Kudineer Chooranam, Modified Liquisolid Compaction Technique, Piperine.

Introduction

In classical ayurvedic and siddha formulation polyherbal drugs are used in the form of kashayam, choornam, etc., which has in recent years become difficult for the patient acceptance in the robotic life. Considering the minimum patient acceptance in making kashayam in day-to-day life, this work aims in a novel technique to convert the aqueous and hydro alcoholic extract of polyherbal drug into solid unit dosage form. Kashayam is the liquid containing water-soluble active principles of herbs. It is also called as herbal decoction or herbal tea (1). So taking into account the bitter taste of kashayam and the problem faced by the patients in maintaining the dosage regime an alternative source of medicine is needed that will be better for patient acceptance (2). So a conventional method of drug delivery is adopted for easy patient acceptance.

The liquisolid technique has been widely used for enhancing solubility and bioavailability of various categories of poorly soluble drugs (3). Usually, a poorly soluble drug is dissolved in a non-volatile solvent to its saturation limit, and then converted into non-adhering, dry looking, free flowing and readily compressible powder mass by the addition of inert carriers, which is then compressed into tablets (4). The conversion of kashayam into liquisolid tablets can improve the

patient compliance, increases the bioavailability and therapeutic efficacy of the drug.

Therefore a modified liquisolid technique was adopted for herbal powders containing multiple active constituents. The extract or kashayam is prepared from the poly herbal powder formulation using aqueous or hydro alcoholic solvents and the volume is reduced to the specified level as per Ayurvedic formulary i.e., concentrating the kashayam. The concentrated liquid is then converted into a free flowing powder by the addition of natural excipients and then compressed into tablets by direct compression method. This kashayam tablet formulation is expected to give immediate release of the active components. The present study deals with the making of Kashayam tablets from Thirukadugu choornam and Nilavembu choornam.

Thirikadugu Chooranam is used in the treatment of digestive disorders such as indigestion, dyspepsia, flatulence and intermittent fever (5).

Nilavembu Kudineer played an important role in the treatment of chikungunya fever and the Government of Tamil Nadu announced the nilavaembu kudineer as one of the medicine to control and treat dengue that has affected Tamil Nadu recently. In addition to this Nilavembu chooranam also exhibits anti-inflammatory, antimicrobial, antioxidant, antispasmodic, antidiabetic, nematocidal, anticarcinogenic, hepatoprotective and various other activities (6).

Methodology

***Materials*:** Thirikadugu Chooranam (Ginger (Zingiber officinale Roac.), Long pepper (Piper longum Linn.), Black pepper (Piper magnum Linn.) in the ratio of 1:1:1) and Nilavembu Kudineer Chooranam (Nilavembu (Andrographis paniculata), Peiputal camolam (Trichosanthes cucumerina), Candana tul (Santalam album), Vetiver (Chrysopogon zizanioides), Vilamiccam ver (Vetivera zizanioides), Korai kilanku (Cyperus rotundus), Milaku (Piper nigrum), Parpatakam (Mollugo cerviana), Cukku (Zingiber officinalis) are bought from SKM Siddha and Ayurvedic Manufacturer, Erode. Microcrystalline cellulose, Silicon dioxide and Talc are obtained from SD fine Chem., Mumbai. Ethanol is purchased from Hayman Ltd., UK. Piperine standard is procured from Sigma Aldrich, Mumbai.

***Preparation of extracts*:** Three different extracts of Thirikadugu Chooranam wherein an aqueous extract is made with 100% water and two hydroalcoholic extract with 50:50 and 75:25 percentage of ethanol and water, respectively is prepared with 10g of Thirikadugu Choornam. The mixture is heated at 50-60º C for 30 min. This procedure involves simple decoction process to obtain the soluble materials being extracted from the crude raw plants.

Similarly three different extracts of Nilavembu Kudineer Chooranam of 100 aqueous extract and two hydroalcoholic extract (50:50 and 75:25 respectively) is prepared with 10 g of Nilavembu Kudineer Chooranam. The mixture was heated at 50-60º C for 30 min. This procedure involves simple decoction process to obtain the soluble materials being extracted from the crude raw plants.

***Concentration of extract*:** The extract that obtained by the decoction process of both Thirukadugu Choornam and Nilavembu Kudineer Choornam is further heated separately at higher temperature of 80-85ºC till the solvent gets evaporated completely. The maximum time limit maintained for the evaporation is 3 hours. This is done with care to avoid the charring of the product. The extract is kept in dessicator for stabilising the trace amounts of moisture. The weight of the yielded product is noted (7).

***Preparation of liquisolid granules*:** The drug extract of Thirukadugu Choornam and Nilavembu Kudineer Choornam obtained is treated with suitable excipients like microcrystalline cellulose and silicon dioxide, to increase its bulkiness and converting into powder mass with passable flow property, which is then passed through sieve no: 10 to break the lumps and form uniform granules. Finally talc is added for lubrication, and again it

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is passed through sieve no: 22 and the weight is noted, as shown in table 1 (8, 9).

***Preparation of tablet*:** The different extract's (100% aqueous, two hydroalcoholic 50:50 and 75:25) liquisolid granules are compacted as tablets by direct compression method with uniform average weight using the manual single punch tablet machine (Khera Instruments, NewDelhi). The machine is adjusted to get the desired size and weight. The uniformity of weights of individual tablets are noted in table 2 (10, 11).

***Scale up study*:** To confirm the accuracy and suitability of the protocol for large scale industrial production of these two products, we adapted a scale up process, by carrying out the same experiment with higher quantity of the raw materials. So here we repeated the process with 50g of the polyherbal choornas (Thirukadugu Choornam and Nilavembu Kudineer Choornam). Here the decoction process for both Choornas followed the time duration of 1 hour and evaporation procedure with a maximum of 6 hours.

Evaluation of pre-compression properties for liquisolid powder

Compressibility: This test is to evaluate the efficiency and compatibility of the powder for suitable compression process, by comparing the bulk density and tapped density. The known amount of powder mixture taken in the measuring cylinder of tapped density apparatus (LabIndia) and volume is noted, from which the density can be calculated as the bulk density. The measuring cylinder is allowed for tapping 500 times such that the particles will be packed up to a constant volume and now the volume is noted, from which tapped density is calculated. The compressibility index is calculated using the formula (12, 13, 14), Compressibility index = (Tapped density - Bulk density / Tapped density) x 100

Flowability: Angle of repose is a conventional method used to determine the flowability of the powder. This is done by fixed height cone or funnel method, where the angle of repose can be determined from the height (h) and radius (r) of the heap that formed on the horizontal plane, which is calculated using the formula (15), θ = tan-1 (h/r)

***Evaluation of post-compression parameters for liquisolid compacts*:** are evaluated for quality control standards limits such as diameter, thickness, weight, hardness, friability and disintegration time.

Weight and dimensions: The uniformity in weight of the compacts is checked with electronic weighing balance (BL-220H, Shimadzu) by weighing 20 tablets together and also the individual tablets and its average is noted. Diameter and thickness of 20 tablets is determined using vernier calliper (532-120, Mitutoyo corporation) and the average is noted (16).

Mechanical strength: The hardness test is done using Monsanto hardness tester (Dolphin), to check its ability to withstand the pressure exerted during packaging and transport. The force needed to break the tablets is noted from the tester reading. Friability is estimated using Roche friabilator (FT 1020, labindia), to determine the incipient capping because of attrition. Randomly selected 10 tablets are weighed and run in the friabilator at 100 rpm for 4 minutes (25 rpm per minute), where the tablets fall from a height of 6 inches for its each rotation. The weight of 10 tablets is noted again. The calculation for percentage friability is done using formula (17), Percentage Friability = (Initial weight of 10 tablets before friabilating - Final weight of 10 tablets after friabilating / Initial weight of 10 tablets before friabilating) x 100

Disintegration test: Tablet disintegration test apparatus is used for experimenting the disintegration time, that is responsible for the absolute and valuable drug absorption. Disintegration of tablet into primary particles ensures large effective surface area which facilitates better absorption. Six tablets are placed

in the disintegration test apparatus basket, which is inserted into the beaker containing 900 ml of 0.1 N HCl and maintained at 37 °C ± 2ºC. The set of tubes are allowed to rise and fall in the media continuously at the rate of 25-32 cycles per minute (18). The time taken for the complete disintegration of compacts, converting into granular particles and passing through the mesh no: 10 present at the bottom of the sieve set is noted.

Estimation of piperine content in thirikadugu choornam khasayam : Piperine is the alkaloid responsible for the pungency of black pepper and long pepper. It considerably involves in altering the bioavailability of the drug and also causes poor patient compliance due to heavy bitterness. So standard for limits are prescribed for the piperine content in the poly herbal formulations. Piperine content in the Thirikadugu Choornam extract can be determined using UV-Visible spectroscopy. The stock solution of 100 µg/mL is prepared by dissolving pure piperine with ethanol. From this different concentrated solutions of 1µg/mL, 2µg/mL, 4 µg/mL, 6µg/mL, 8µg/mL are prepared for estimation of standard curve. Similarly sample solution of 5 µg/mL is prepared with ethanol and the absorbance of the solutions is measured at 342.5 nm (19).

Results and Discussion

Formulation of liquisolid compacts: The polyherbal Thirikadugu Chooranam and Nilavembu choornam tablets prepared by direct compression technique, the process and formula are optimized by experimental method. The pre-formulation studies performed for the pilot study on the granules indicated a good compressibility index and flow property, and so the scale-up batch is done with respective higher quantity of the chooranam.

The weight of Thirikadugu Chooranam and Nilavembu choornam taken for preparing the extract is 10 g for trial studies, which on treatment with aqueous, 50:50 ethanol-water and 75:25 ethanol-water have given the extract yield weight of more than 1 g and around 0.2g respectively. This trial batch experimented for the decoction concentrating time showed the duration of 5 – 6 hours for complete evaporation of solvent, leaving a concentrate of active components. The addition of excipients is optimized for the better flow of mixture, as shown in table 1. In the scale up study, as the weight of chooranam is increased to 50 g in each batch, the quantity of the solvents used for extraction is also increased proportionately. The optimized quantity of excipients added to convert the concentrated liquid into free flowing powder is shown in table 1. The yield of number of tablets and its average weight are also noted. In the scale up study, the yield of extract is found to be more than 2.5 g and 0.5g respectively which required approximately 10 - 12 hours for complete evaporation of the solvent to concentrate the content. The yield of the drug extract, liquisolid granules and the tablets obtained for 10 g and 50 g powder are compared and found to be proportionate in their results as shown in table 3.

Pre- compression parameters : The pre-compression evaluation of both the polyherbal liquisolid granules of Thirikadugu choornam showed a suitable compressibility (Carr's Index 10-16) and flowability (Angle of repose 15 - 31p) nature for the effective compression of the compacts. The estimation of piperine content in the aqueous and alcoholic extract of Thirikadugu Chooranam is found to be 20.01% and 20.04% respectively, which proved the content within the specified limits (20.02 ± 0.15%) as shown in table 3.

In the similar fashion, the Nilavembu Kudineer chooranam is also processed and its pre-compression parameters were found to be as shown in table 3. The 50:50 ethanol-water extract granules gives an excellent flow with angle of repose below 20º and also the percentage compressibility is found to be fair with the Carr's index in the range of 18-21%. The compressed tablets from different extracts (100% aqueous, 50:50 ethanol-water, and 75:25

Table 1. Excipients used in Pilot study and scale up study of Thirikadugu Chooranam (TK) and Nilavembu Kudineer Choornam (NK) Liquisolid compacts

Pilot study of liquisolid compacts						
Excipients	**Weight to be added (g)**					
	100 % water		**50:50 (Ethanol-Water)**		**75:25 (Ethanol-Water)**	
	TK	**N K**	**TK**	**N K**	**TK**	**N K**
Microcrystalline cellulose	0.7	0.2	0.5	0.2	0.9	0.3
Silicon dioxide	0.35	0.05	0.15	0.05	0.10	0.01
Talc (%)	0.01	1	0.01	1	0.01	1
Scale up study of liquisolid compacts						
Excipients	**Weight to be added (g)**					
	100 % water		**50:50 (Ethanol-Water)**		**75:25 (Ethanol-Water)**	
	TK	**N K**	**TK**	**N K**	**TK**	**N K**
Microcrystalline cellulose	2.0	0.36	2.5	0.36	2.5	0.50
Silicon dioxide	0.8	0.12	0.6	0.11	0.7	0.16
Talc (%)	0.02	1	0.05	1	0.05	1

ethanol-water) showed differences in their properties, due to variation in the liquid content and ratio of the excipients added.

Evaluation of liquisolid compacts: The data obtained for the compressed compacts of Thirikadugu chooranam are shown in table 4. The granular mass compressed into tablets resulted in average weight of 250 mg $\pm$ 5 % deviation as per the pharmacopoeial limits. The dimensions of the compacts are 8 mm in diameter and 4 – 5 mm in thickness. The hardness of 2 – 2.5 kg/cm^2 (Limit NMT 5 kg/cm^2) and percentage friability less than 0.5% (Limit NMT 1%) is observed for all the batches of compacts. The disintegration time of the compressed mass is less than 15 minutes (Limit NMT 15 minutes for uncoated tablets) for the scale up batch of liquisolid compacts. The tablets prepared using 100 % aqueous solution extracts is found to be hard, it is due to the solubility of starch from plant materials compared to the alcohol containing batches.

Correspondingly, the parameters evaluated for the Nilavembu Kudineer Chooranam liquisolid tablets are shown in the table 4, wherein the similar results were obtained, as with an average weight of 250 mg ± 5 % deviation and with 8 mm in diameter and 3-5 mm in thickness. The hardness of 2 – 2.5 kg/cm^2 (Limit NMT 5 kg/cm^2) and percentage friability less than 0.5% (Limit NMT 1%) is observed for all the batches of compacts. The disintegration time of the compressed tablet is less than 15 minutes (Limit NMT 15 minutes for uncoated tablets) for the

Table 2. Pilot study and scale up study of Thirikadugu Chooranam (TK) and Nilavembu Kudineer Choornam (NK) Liquisolid compacts

Pilot study of liquisolid compacts						
Parameters	**100 % water**		**50:50 (Ethanol-Water)**		**75:25 (Ethanol-Water)**	
	TK	**N K**	**TK**	**N K**	**TK**	**N K**
Yield of extract (g)	1.419	0.221	1.199	0.252	1.113	0.213
Yield of powder(g)	2.108	0.509	2.034	0.522	2.340	0.542
Yield of tablets(nos)	4	2	4	2	4	2
Scale up study of liquisolid compacts						
Parameters	**100 % water**		**50:50 (Ethanol-Water)**		**75:25 (Ethanol-Water)**	
	TK	**N K**	**TK**	**N K**	**TK**	**N K**
Yield of extract (g)	2.50	0.753	3.973	0.772	3.66	0.612
Yield of powder(g)	5.35	1.236	7.123	1.252	6.91	1.186
Yield of tablets(nos)	18	4	23	4	22	4

Table 3. Pre-compresssion parameters for Thirikadugu Chooranam (TK) and Nilavembu Kudineer (NK) Granules

Pre-compression Parameters	**Herbal Tablets Granules**					
	100 % water		**50:50 Ethanol-Water**		**75:25 Ethanol-Water**	
	TK	**N K**	**TK**	**N K**	**TK**	**N K**
Bulk density (g/ml)	7.1	15	8.5	15	6.4	15
Tapped density (g/ml)	6.2	13.5	7.3	13	5.8	12.5
Compressibility index	14.51	10	16.43	13	10.34	16
Angle of repose (p)	21.36	25	16.21	28	24.9	31
Piperine content of extract	20.01%	-	20.04%	-	20.04%	-

scale up batch of liquisolid compacts and it was observed that all the results were all as per the standard limit specifications.

Thirukadugu choornam during the study was observed to be very hygroscopic than nilavembu kudineer choornam because of higher amount of *Zingiber officinale rosc*. (ginger) in it as it has the property of absorbing moisture from atmosphere. In Thirukadugu choornam the piperine amount was checked to be within the specified limits as the presence of Piper longum and Piper nigrum is higher.

Table 4. Quality Control Parameters of Liquisolid Compacts of Thirikadugu (TK) and Nilavmebu Kudineer (NK) Choornam

S. No. Quality Control Tests	100 % aqueous extract		50:50 Ethanol-Water		75:25 Ethanol-Water	
	TK	N K	TK	N K	TK	N K
1. Diameter (mm)	8	8	8	8	8	8
2. Thickness (mm)	4.2	3.8 ± 0.06	4.5	3.1 ± 0.01	4.9	4.5 ± 0.02
3. Hardness (kg/cm2)	2	2.3 ± 0.01	2.5	2.2 ± 0.02	2.5	2.5 ± 0.05
4. Friability (%)	0.398	0.001	0.328	0.231	0.411	0.462
5. Disintegration Time (min)	13.35	10	10.04	13	14.08	11

Conclusion

The formulation of Thirikadugu Chooranam and Nilavembu choornam into single unit solid dosage form by liquisolid compaction technique is found to have agreeable pre-compression and post compression parameters. The results of uniformity in weight, hardness test and friability test indicates its aptness for effective packing, transport and marketing. The disintegration time within the standard limits ensures perfect release of the active components *in-vivo*. The scale up batch process also signifies positive attribute for industrial production of the tablets.

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References

1. Margret Chandira and Jayakar, B. (2010). Formulation and evaluation of herbal tablets containing Ipomoea Digitata Linn extract. Int J of Pharm Sci Rev Res, 3(1):101-110.
2. Bindu, M.B., Kusum, B. and David Banji. (2010). Novel strategies for poorly water soluble drugs. Int J of Pharm Sci Rev Res, 4 (3):76-84.
3. Sanjeev Raghavendra, G. and Ravindar Jarag. (2009). Liquisolid technique for enhancement of dissolution properties of Bromhexine Hydrochloride. Research J. Pharm. and Tech., 2(2): 382-386.
4. Yadav, V.B. and Yadav, A.V. (2009). Improvement of solubility and dissolution of Indomethacin by liquisolid and compaction granulation technique. J. Pharm. Sci. & Res., 1(2): 44-51.
5. The Siddha Formulary of India, Part-I (1st Edition). (1992). Ministry of Health and Family Welfare, Department of Health, Government of India.
6. Abhishek Niranjan., Tewari, S.K. and Alok Lehri (2010). Biological activities of Kalmegh (Andrographis Paniculata Nees) and its active principles – A review. Indian J Nat Prod Resour, 1 (2): 125-135.
7. Javadzadeh, Y., Siahi, M.R., Asnaashari, S. and Nokhodchi, A. (2005). Liquisolid technique as a tool for enhancement of poorly water soluble drugs and evaluation of their physicochemical properties. Acta pharm, 57: 99-109.
8. Javadzadeh, Y., Siahi-shadbad, M.R., Barzegar-Jalali, M. and Nokhodchi, A. (2005). Enhancement of dissolution rate of

Piroxicam using liquisolid compacts. IL Farmaco, 60: 361-368.

9. Majekodunmi, S.O., Adegoke, O.A. and Odeku, O.A. (2008). Formulation of the extract of the stem bark of Alstonia boonei as tablet dosage form. Trop J Pharm Res, 7(2): 987-995.

10. Jivraj, M., Martini, L.G. and Thomson, C.M. (2003). An overview of different excipients useful for direct compression of tablet (Controlled Release Tablet and Capsule – 17). Pharm Science Technology Today. B. F. Goodrich Bulletin, 3: 58-63.

11. Raymond, C.R., Paul, J. S. and Sian, C.O. (2006). Handbook of Pharmaceutical Excipients (5th Edition), Pharmaceutical Press, London.

12. Khalid, M. E., Ahmed, M. S. and Mohamed, I.F. (2010). Formulation and evaluation of Rofecoxib liquisolid compacts. Int J of Pharm Sci Rev Res, 3(1): 135-142.

13. Herbert, A.L., Leon Lackman. and Joseph, B. S. (1989). Pharmaceutical dosage forms, Tablets (2nd edition), CRC Press, USA.

14. Leon Lachman, Herbert, A. L. and Joseph, L. K. (1991). The theory and practice of industrial pharmacy. Varghese publishing house.

15. Sanjeev Raghavendra, G. and Ravindar Jarag (2010). Formulation and characterization of atorvastatin calcium liquisolid compacts. Asian J pharm, 5(2): 50-60.

16. Ramya devi, D., Gopinath, V. and Vedha Hari, B.N. (2010). A new approach for the design and development of poly herbal tablets of Nilavembu Kudineer Churanam. Indian Journal of natural sciences, 1(1): 35-40.

17. Indian Pharmacopeia - Vol. I and II. (2007). Indian Pharmacopeia Commission, Ghaziabad, Ministry of Health and Family Welfare. Government of India.

18. United States Pharmacopoeia- Vol. I and II. USP NF (2009). (USP 32 Revision and NF 27th edition).

19. Khalid Hussain., Zhari Ismail., Amirin Sadikum. and Pazilab Ibrahim.(2008). Analysis of proteins, polysaccharides, glycosaponins contents of Piper Sarmentosum Roxb. and anti-TB evaluation for bioenhancing / interaction effects of leaf extracts with Isoniazid (INH). Nat Prod Rad, 7(5): 402-408.

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Current and Prospective Insights on Food and Pharmaceutical Applications of Spirulina

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Abstract

The blue green algae spirulina has been used by mankind as food and drug since ages. However, the last few decades have witnessed the unprecedented momentum in research on nutritional and medicinal potency of this unicellular alga. It has emerged as an undisputed medical food with the discovery and validation of a litany of health benefits ranging from antioxidant, anti-inflammation, hypolipemic, antithrombotic, anti-diabetic, anticancer, immunestimulatory, antimicrobial, cardio-protective, hepatoprotective, antianaemic, neuroprotective, tissue engineering to aquaculture and livestock feed. Many hitherto unknown pharmacological properties are coming forth and myriad research projects are revolving around this miraculous cyanobacterium. Safety regulations recommend its inclusion in nutritional regimen for proofing body against ailments and augmenting vitality. Advances in effective cultivation, drying, extraction and purification techniques have been summarized. This review outlines the recent progresses and therapeutic possibilities of this spirulina.

Keywords: Spirulina, antioxidant, anticancer, immunomodulation, antodiabetic, neuroprotection

Introduction

Spirulina is a free-floating, microscopic, filamentous blue-green alga, thriving in alkaline fresh as well as salt water bodies (1) (Fig. 1). This cynaobacteria, belonging to the class cyanophyceae and order oscillatoriales is a storehouse of bioactive molecules *viz.* proteins (60-65% dry weight) with essential amino acids, polyunsaturated fatty acids, such as ã-linoleic acid, vitamins (B_{12} and E), polysaccharides (calcium spirulan, immulan), minerals (Na, K, Ca, Fe, Mn and Se), pigments (chlorophyll, c-phycocyanin, allophycocyanin, β-carotene, lutein, zeaxanthin etc.). The c-phycocyanin content was determined to be 12.6% in dried spirulina (2). High content of dietary zeaxanthin was reported in its biomass (3). Also, an appreciable quantity of vitamin E was estimated in *S. platensis* (4). Gallic, chlorogenic, cinnamic, pinostrobin and hydroxybenzoic acids have been found to be the key constituents in various extracts of *S. maxima* (5). The importance of its high-molecular weight polysaccharides in mitigating several maladies has been well documented (6, 7, 8, 9).

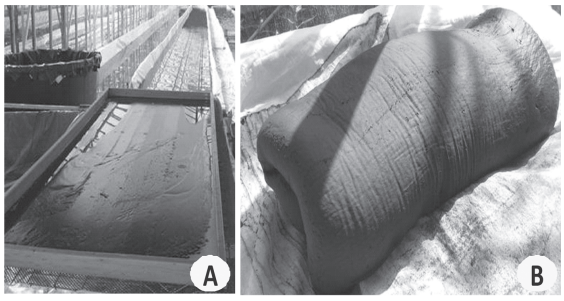

Fig. 1. (A) Spirulina harvested on a filter (B) The biomass after squeezing out water (Pictures courtesy: http://www.spirulina-vera.com)

Uses of spirulina in healthcare

Several species of spirulina have attracted commercial importance *viz. Spirulina platensis, Spirulina maxima* and *Spirulina fusiformis.* It has been consumed since centuries in various parts of the globes, ranging from Aztec civilization in Latin America to tribes inhabiting Central Africa's Lake Chad. At the United Nations World Food Conference in 1974, this alga was decorated with the epithet of 'best food for the future'. Spirulina is deemed safe for human consumption evident by its long history of food use and contemporary scientific findings (1). Now, it has become a major ingredient in many nutraceutical formulations. Current times have seen rigorous investigation on its biological effects against many health problems. Findings have reported the functional food, antioxidant, immunstimulatory, anticancer, antiinflamamtory, hypoliemic, cardioprotective, antidiabetic, hepatoprotective, neuroprotective and antimicrobial potentials of this novel alga. Also, it has made strides into aquaculture and poultry feed.

A slew of spirulina-based herbal, vitamin and mineral supplements have been released to the market. These nutraceuticals marketed as tablets, capsules, dry flakes or in powder form have garnered enormous consumer interest. '*Spirulina Pacifica'* is the trademark of a Hawaiian strain manufactured by Cyanotech Inc. 'Earthrise Spirulina' is the most popular brand in the USA. For a growing number of customers spirulina products have assumed panacea status. These products have secured good market in South America, Eastern Europe, large parts of Asia and Africa. In light of the recent surge in demonstrated clinical properties of spirulina, this review attempts to furnish the scattered data as a holistically informative piece.

Uses of spirulina : Spirulina has demonstrated a bewildering array of food and therapeutic properties.. The key implications backed by scientific findings are as functional food and additives, antioxidant, anti-inflammatory, hypolipemic and antihypertensive, antidiabetic, anticancer, immunestimulant, antimicrobial, hepatoprotective, neuroprotection, *antianaemic and antileucopenic and tissue engineering. Spirulina biomass has also proved suitable as a nutritive* aquaculture feed. The food applications and health benefits are illustrated in Fig. 2.

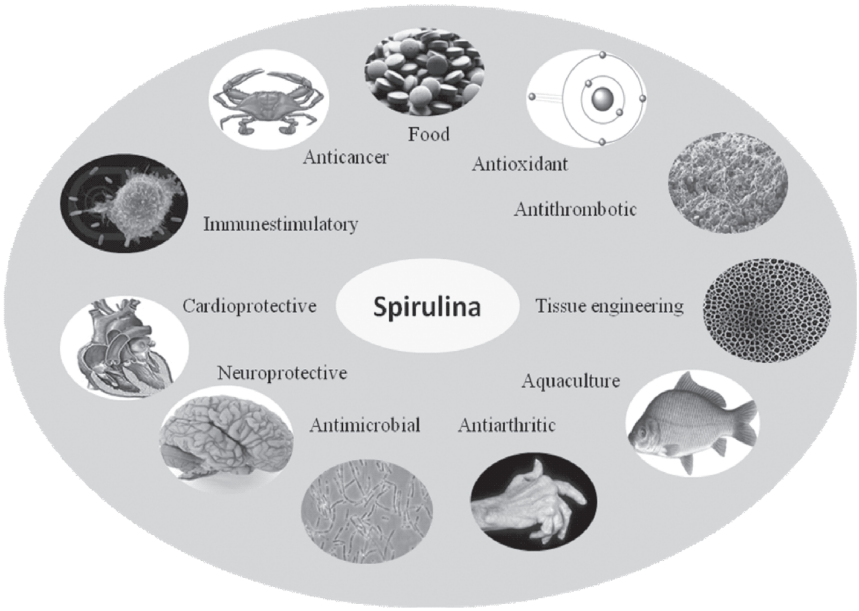

Fig 2. The food and pharmaceutical applications of spirulina

As functional food, additive and prebiotic : Spirulina has earned scientific validation regarding its role as healthy food component. This nutritious and easily digestible food can be consumed in several forms. It was demonstrated that the whole spirulina or its phycocyanin-rich fraction could be a suitable functional ingredient in soy milk, fruit juices and whole fruits (10). It was suggested that the ingestion of cocoa and spirulina powder mix can promote antioxidant status and vascular health (11). The flavonol-rich cocoa and phycocyanin-rich spirulina are assumed to work in synergy to increase the endothelial production of nitric oxide and act as a potent inhibitor of NADPH oxidase.

Spirulina is expected to enhance the nutritional content of conventional foods when incorporated as colorant, texturizing agent, gelling agent and prebiotic. The pigments phycocyanin and allophycocyanin are used in the food and beverage industry as a natural colorant. This blue colorant finds use in ice cream, sweets, chewing gum, candy, jelly, cake decorations as well as soft drinks, alcoholic drinks (12). It was observed that the incorporation of *S. platensis* increases raw pasta firmness and imparts it a stable color. Sensory analysis also showed better acceptance scores (13). The effect of *S. platensis* enrichment in semolina was observed. Addition of 2g spirulina in 100g semolina resulted in higher swelling index, lower cooking loss and increase in pasta firmness (14). It was discovered that date and spirulina powder-based food tablets are suitable for consumption by those having dysphagia (difficulty in swallowing food), to fulfil the nutritional requirement. Also, these tablets are expected to act as natural and cheap drug delivery carriers (15). It was observed that at lower heating or cooling rates, *S. maxima* gel exerted viscoelastic functions akin to that of pea protein, κ-carrageenan and starch. This finding may lead to the use of spirulina as thickener in food industry like the above hydrocolloids (16). It was observed that *S. platensis* stimulates proliferation of probiotic lactic acid bacteria. The addition of dry algal biomass at 10 mg/ml promoted growth of *Lactobacillus acidophilus* to 186%, suggesting the prebiotic potential of the microalga (17). A protective medium with spirulina as an ingredient was optimized for enhancing viability of *Lactobacillus rahamnosus* during lyophilisation. It was observed that the algal additive at 1.3%, along with lactulose and sucrose promotes viability of the microbe (18). The dermoprotective potential of raw spirulina and its lactic acid bacteria-fermented product was compared. The results showed that though both forms exert skin ameliorative functions; the fermented product performed better in terms of radical scavenging, anti-inflammation and UV protection. It was inferred that the fermentation process, released unidentified polyphenols and converted phycocyanin to phycocyanobilin. Based on the results, it was suggested that fermented spirulina can be a potent supplement for skin health (19).

Spirulina is an incredibly rich source of proteins that could efficiently fight against food deficiency in developing countries (20). The safety profile of spirulina was investigated and its microcystin toxin-free status was suggested. So, it is clear that the long-term dietary supplementation of this alga does not pose any health risks if consumed in moderation (21).

Antioxidant : Lipid peroxidation is the degradation of lipids due to free radicals, generated by toxins. If not checked, the oxidative stress causes membrane rupture and mutagenic end-product malondialdehyde (MDA) are produced. The efficacy of spirulina in eliminating mercuric chloride-induced oxidative stress was investigated in mice. Its oral supplementation (800 mg/kg body weight, in olive oil, along with the toxin) for 40 days led to decline in lipid peroxidation and the activities of antioxidants enzymes *viz.* superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GSH) were restored to normalcy (22). The supercritical fluid-extracted fraction of *S. platensis* was subjected to β-carotene bleaching method and DPPH assay to determine the optimal extraction conditions for antioxidants. The

pressure of 220-320 bar, temperature 55°C with 10% ethanol-blended CO_2 or 320 bar pressure at 75°C temperature with pure CO_2, proved optimal for the extraction (23). The protective effect of *S. platensis* on gentamicin sulphate-induced changes in the levels of lipid peroxidation and antioxidants in the kidney was investigated in murine models. *The algae when consumed at a dose of* 1g/kg, elicited significant nephro-protective activity by decreasing lipid peroxidation and elevating the levels of GSH, SOD, GPX, NO, creatinine and urea. Biochemical as well as histological results corroborated the findings (24). From a battery of *in vitro* tests, it was concluded that *S. platensis* and phycocyanin have radical-scavenging and metal chelation properties. The inhibition of hydroxyl and peroxyl radicals and the lipid peroxidation were attributed to the antioxidants (25). The possible anti-teratogenic effect (prevention of congenital abnormalities) of *S. maxima* aqueous extract against hydroxyurea abuse in mouse embryos was determined. The phycobiliprotein-rich extract showed a protective effect in a dose-dependent manner without any side effects. (26). Valproic acid is a teratogen causing neural tube defects in mammals. The effect of *S. platenisis* was investigated in conteracting the oxidative stress imposed by valproic acid. On the gestation day 8, sodium valproate was injected, while spirulina was orally administered at 125, 250, and 500 mg/kg daily from day 0 to 18. Spirulina decreased the incidence exencephaly and other genetic aberrations. Increased level of SOD, CAT and GPx was determined (27). It was reported that spirulina can decrease the frequency of cadmium-induced teratogenicity as exencephaly (a condition in which brain is located outside the skull), micrognathia (a condition when the jaw is undersized) and skeletal abnormalities. At a dose of 500mg/kg, the algal antioxidants could attenuate the toxicity of cadmium on mice foetus (28). Also, the aqueous extracts of spirulina showed protection against t-butyl hydroperoxide-induced cytotoxicity and reactive oxygen species in cultured C_6 glial cells (29). The antioxidant activity of the aqueous extract of *S. platensis* was assessed using both chemical and cell-based assays. In the cell-based assay, mouse fibroblast cells (3T3) cells were incubated for 1 h in a medium containing the algal aqueous extract or positive controls vitamin C and E prior to the addition of 50 μM DPPH or ABTS. After 24 h incubation, DPPH and ABTS assays were conducted. The extract did not elicit any cytotoxicity and reduced significantly apoptotic cell death due to by 4 to 5-fold. The radical scavenging activity of the extract was measured about 50% of vitamin C and E (30). Spirulina has emerged as a novel and affordable source of antioxidant ergothioneine (a naturally occurring aminoacid) (31). The antioxidant, radical scavenging, and metal-chelating activities of spirulina were evaluated alone and in combination with whey protein concentrate. The *in vitro* results showed the dose-dependent activity of spirulina; whereas the *in vivo* study revealed its protection against CCl_4-induced liver damage. The free radical scavenging properties and antioxidant activity effect were more pronounced in rats receiving the combination of spirulina and whey protein concentrate (32).

Anti-inflammatory and antiarthritic effect : The anti-inflammatory effect of spirulina was studied in zymosan-induced arthritis in mice (33). After 8 days of administration, the abnormal level of β-glucuronidase in synovial fluid was measured to have fallen down. Inhibition of the inflammatory reaction, without any damage to the chondrocytes was observed. Phycocyanin was assumed to be the component exerting the antiarthritic effect. The effect of polysaccharide extract from *S. platensis* was assessed on corneal neovascularisation both *in vivo* and *in vitro*. Topical application of the polysaccharide significantly inhibited the new vessel formation in alkali burn model of cornea. The results suggested that the polysaccharide may be effective in the therapy of corneal opacities involving neovascularization and inflammation (33).

Antihyperlipemic, antithrombotic and antihypertensive :The modern sedentary

lifestyle and greasy food habits have resulted in the overwhelming surge in deadly maladies like atherosclerosis and hypertension. To counteract the cholesterol deposition and to protect the heart, spirulina has been studied vigorously. The fibrinolytic effect of *c*-phycocyanin from *S. fusiformis* was investigated against vascular endothelial cells. The pigment showed increased clot dissolving activity in dose- and time-dependent manners by inducing urokinase-type plasminogen activator in the cells (34). The effect of oral administration of *S. maxima* was evaluated on serum lipids and blood pressure (35). When consumed at a dose of 4.5 g/day for 6 weeks, a pronounced hypolipemic effect was observed. Further, the potency of *S. platensis* diet at a dose of 0.5 g/day, in treating high fat diet-induced hypercholesterolemia in rabbits was evaluated. Results showed that levels of serum cholesterol decreased in the spirulina-fed rabbits and high-density lipoprotein content measured higher than control (36).

Amelioration of diabetes drug side effects : The drugs prescribed to treat diabetes often lead to many side effects. Common issues as nausea, headache, weight gain, bloating, constipation, diarrhoea may arise or serious problems as liver damage, heart complication, pancreatitis, tumour, bone loss, erectile dysfunction, psychosis and muscle spasm are encountered. The protective effects of *S. fusiformis* extract against rosiglitazone **(**a standard type II diabetes drug)-induced osteoporosis was assessed in insulin resistant rats. After 45 days, the integrity of the bone surface as well as the bone strength improved. The bone restoration was assumed to be due to the high content of calcium and phosphorous in spirulina. The chromium and ã-linoleic acid content was held responsible for decline in the fasting serum glucose, HDL, LDL and triglycerides levels. These findings suggested that synergistic therapy of rosiglitazone and spirulina can be recommended for attenuating the risk of osteoporosis (37). The intake of *S. maxima* extracts, 2 weeks prior to and 4 weeks during streptozotocin administration was reported to reverse the detrimental effects of the drug on male reproductive organ. The extract significantly increased the body and testis weight, metabolic parameters, normal seminiferous tubules, Leydig cell number, testosterone levels and mRNAs for steroidogenic enzymes (38). Clinical prospects of spirulina in alleviating other side effects are worth-exploring.

Antimutagenic and anticancer effects : Several studies testify that spirulina extracts are promising chemopreventive agents. Its antimutagenic effect on mice was investigated using cyclophosphamide as a mutagen (39). The subjects were fed with spirulina, 2 weeks prior to mutagen injection. Improvement in semen quality and prevention of post-implantation losses were observed in spirulina-treated group. It was inferred that the alga offers protection to the germ cells against cyclophosphamide abuse. The selenium-enriched *S. platensis* extract inhibited the growth of human breast cancer MCF-7 cells through induction of G1 cell cycle arrest and mitochondria-mediated apoptosis. Induction of apoptosis was evident from the accumulation of sub-G1 cell population, DNA fragmentation and nuclear condensation. The up-regulation of Bax and Bad expression and down-regulation of Bcl-xl expression accounted for the mitochondrial dysfunction, leading to cancer cell death (40). The oral administration of hot-water extract of *S. platensis* was reported to enhance NK cytotoxicity in humans. Through complex immunological and molecular studies, it was inferred that spirulina can be implicated with BCG-cell wall skeleton for synergistic development of adjuvant-based antitumor immunotherapy (41). Doxorubicin is a well-established anticancer drug, but is riddled with many side effects, including reproductive aberrations. The possible role of spirulina in alleviating its testicular toxicity was investigated in albino rats (42). Rats administered with intra peritoneal doxorubicin at a dose of 3 mg/kg once in a week for 35 days, suffered a significant decrease in sperm count and body weight. Biochemical and histopathological studies showed spirulina at a dose of 250 mg/kg,

administered daily prior to doxorubicin administration restored semen quality, sperm count and body weight. The combination treatment restored testicular impairment to normalcy. The use of spirulina as complementary and alternative medicine (CAM) by breast cancer survivors in Malay was reported (43). The functionalization of selenium nanoparticles with spirulina polysaccharides could be successfully carried out. As a surface decorator, the polysaccharide enhanced the cellular uptake of the assembly and resultant cytotoxicity towards several human cancers. The chemotherapeutic potency of the assembly towards human melanoma A375 cells was mediated through apoptosis (9).

Immunestimulatory : It was reported that immulina, a high-molecular-weight polysaccharide from spirulina was a potent activator of nuclear factor kappa B (NF-kB) and induced both IL-1α and TNF-β mRNAs in THP-1 human monocytes (6). Mice fed with immulina-enriched chow for a period of for 4-5 days, exhibited changes in several immune parameters. The *ex vivo* production of IgA and IL-6 from Peyer's patch cells was enhanced 2-fold and interferon- α production from spleen cells was increased 4-fold. The enhanced production of these immune indicate that immulina bolsters innate immunity by stimulating both mucosal and systemic immune systems. It was demonstrated that immulina activates human acute monocytic leukemia (THP-1) cells at a dose dependent manner, stimulating leukocytes response to inflammatory and infectious signals (44). The immunomodulatory effect of hot-water extract, phycocyanin and cell-wall component extract of spirulina was evaluated in mice models. The spirulina extracts enhanced proliferation of bone-marrow cells and induced colony-forming activity in the spleen-cell culture supernatant. Granulocyte macrophage-colony stimulating factor and interleukin-3 were detected in the culture supernatant (45). The immune response elicited on consumption of immulina was investigated. As a measure of the adaptive immunity, the changes in leukocyte responsiveness to *Candida albicans* and tetanus toxoid were evaluated *in vitro* (46). Intake of immulina caused an immediate but temporary increase of *Candida*-induced CD4+ T-helper cell proliferation; whereas toxoid-induced T-helper cell proliferation was increased in individuals over 50 years of age. The *Candida*-elicited production of the Th1 cytokines TNF-α, IL-2 and IFN- γ was increased after immunlina administration for 3 days, and the increased IL-2 production lasted up to 56 days. The immune-suppressive effect of *S. fusiformis* in mice was studied (47). The *in vivo* effect of spirulina on humoral immune response, cell-mediated immune response and tumour necrosis TNF-α was investigated in mice. When administered at a dose of 400-800mg/kg body wt, it significantly inhibited the humoral as well as cell-mediated immune response and TNF-α in a dose-dependent manner. *In vitro* tests showed that, *S. fusiformis* at dose range of 50-100μg/ml decreases the mitogen-induced T lymphocyte proliferation. The above-discussed studies are testimony to the immune-modulatory aspects of spirulina.

Antimicrobial : The antimicrobial activity of *S. platensis was studied against* various Gram-positive, Gram-negative bacteria and *fungal species.* The methanol extract showed maximum antimicrobial potency. GC-MS analysis identified the volatile components of *S. platensis* to be heptadecane and tetradecane (48). The supercritical fluid extraction and ethanol fractionation of *S. platensis* demonstrated some degree of activity towards *Staphylococcus aureus, Escherichia coli*, *Candida albicans* and *Aspergillus niger* (23). *S. platensis* was tested for its probiotic efficacy and inhibitory effect against several pathogens (17). The doses of 5 and 10 mg/ml promoted growth of *Lactobacillus acidophilus* up to 171.67% and 185.84%, respectively. Maximum inhibition was reported against *Proteus vulgaris*, the pathogen notorious for urinary tract and wound infections. The water extract of *S. platensis* demonstrated significant antimicrobial activity against *Klebsiella*

pneumoniae and *Proteus vulgaris* (NCIM2027); whereas, the acetone extract shows pronounced biological activity against *Klebsiella pneumoniae* followed by *Salmonella typhi*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* (49). The effect of spirulina supplement in combating HIV patients was assessed in a six month follow-up. The patients administered with spirulina at a dose of 10 g/day showed significant improvement in weight, arm girth, number of infectious episodes, CD4 count and protidemia (protein level in blood) (50). HIV-infected patients develop abnormalities of glucose metabolism due to the virus and antiretroviral drugs. The normalizing effect of *S. platensis* was assessed in HIV-infected patients. The results of the two-month long study suggested that, the insulin sensitivity in HIV patients improves significantly (about 225%) when spirulina supplement (19 g/day) is taken. Further study is needed to evaluate efficacy of spirulina in combating HIV symptoms (51).

Cardio, hepato and pulmo protective : Doxorubicin used for chemotherapy leads to cardiac toxicity characterized by decrease in myocardial contractility, mediated by reactive oxygen species-induced apoptosis. The cardioprotective efficacy of spirulina was assessed in doxorubicin administered mice (52). The animals treated with 4 mg/kg of the drug, once a week for a month were fed with the algal extract 3 days twice daily for 7 weeks. The spirulina-fed group demonstrated lower mortality (26%), less ascites (peritoneal cavity fluid), lower levels of lipid peroxidation and restoration of antioxidant enzymes.

Several studies have shown that spirulina possesses ameliorative property against stress-induced liver injuries. The hepatoprotective effect of *S. platensis* on cadmium toxicity was evaluated on rats (53). After a month-long experimental period, the liver samples of the subjects were tested for determination of MDA and cellular antioxidants. *S. platensis* treatment showed marked decrease in lipid peroxidation (lower MDA) and increase of the GSH, SOD, NO levels. The curative effect *S. maxima* on patients with non-alcoholic fatty liver diseases were reported (54). Ultrasonography and the aminotransferase data proved the hepatic amelioration by the oral supplementation of the alga. The protective power of c-phycocyanin on H_2O_2-induced liver damage was investigated (55). Viability of human hepatocyte L02 cell was determined by MTT and alanine aminotransferase (ALT) tests. It was observed that the hepatocytes incubated with c-phycocyanin were able to resist morphological changes, decrease in metabolic enzyme levels and chromatic condensation. The ameliorative effect of spirulina was investigated on non-alcoholic steatohepatitis (fatty liver disease) models of rats. Analyses of blood ans liver samples showed the increase in plasma liver enzymes and liver fibrosis, increases in productions of reactive oxygen species from liver mitochondria and from leukocytes, activation of NK-kB and change in the lymphocyte surface antigen ratio. Spirulina administration reversed these adverse changes to a significant degree. The mechanisms of action were deduced to be due to anti-oxidative and anti-inflammatory actions (56).

The effect of c-phycocyanin extracted from *S. platensis* was investigated on paraquat-induced pulmonary fibrosis in rats. The animals orally administered with c-phycocyanin (50 mg/kg) daily were subjected to histological assays on days 1, 3, 7, 14, and 28. The homogenized lung sample was measured for hydroxyproline and MDA, which showed significant decrease in c-phycocyanin-treated group. The observation showed that c-phycocyanin could alleviate pulmonary alveolitis and fibrosis in rats with paraquat poisoning (57).

Antianaemic and antileucopenic : The effect of *S. platensis* in alleviating toxic impacts of heavy metal-adulterated diet was investigated (58). The results suggested that the algal supplementation may be useful in treatment of leukaemia and anaemia caused by lead and cadmium. Based on experimental outcome, it was inferred that 12 week supplementation of spirulina may

ameliorate anaemia in senior citizens. Steady increase in the corpuscular haemoglobin content in the blood samples of the subjects was recorded (59).

Neuroprotection and tissue engineering : The effect of *S. platensis* extract and its phycocyanin was investigated on the activities of the antioxidant enzymes SOD, CAT, GPx and GR, lipid peroxidation inhibitory activity and glutathione in iron-subjected SH-SY5Y neuroblastoma cells (60). The bioactive compounds exerted antioxidant activity evident from its protection of glutathione peroxidise and glutathione reductase against oxidative stress. These results suggested that *S. platensis* extract can be implicated for therapy of iron-mediated neurodegenerative disorders as Alzheimer's or Parkinson diseases. The protective effect of spirulina in transient middle cerebral artery occlusion (MCAO)-induced focal cerebral ischemia-reperfusion injury was evaluated in rats (61). Male albino rats administered with spirulina at a dose of 180 mg/kg, per day for 7 days were subjected to arterial blockage. Spirulina pretreatment significantly reduced the histological changes and neurological deficits. Significant reversal in the elevated brain MDA content and restoration of the decreased enzymes were observed. The possible protective potential of spirulina on hippocampal progenitor cells against lipopolysaccharide (LPS) abuse was determined (62). Rats fed with 0.1% spirulina-supplemented diet were given single intra peritoneal injections of LPS (1 mg/kg). It's followed by injection of the rats with thymidine analog BrdU (50 mg/kg), in order to detect the proliferating cells. Quantification of the BrdU positive cells showed that the spirulina-enriched diet could partially check the LPS-induced decrease in progenitor cell proliferation. Spirulina showed more pronounced effect in combination with other natural antioxidants as blueberry, green tea, vitamin D and carnosine.

Like many other marine organisms, spirulina synthesizes inorganic nanoparticles and holds immense promise in nanomedicines. Recently, spirulina has made major strides in tissue engineering domains. A highly porous scaffold was fabricated by electrospinning its biomass. The nanofibers in the scaffold might act as extracellular matrices for stem cell culture (63). The electrospun polycaprolactone nanofiber containing spirulina was evaluated for its potential as extracellular matrix in the culture of glial cells (64). The extract was observed to increase growth and metabolic activity of the astrocytes. This result holds promise in treatment of central nervous system (CNS) injury. A hollow copper microspiral was synthesized using spirulina as a scaffold (65). It was suggested that an array of low cost and reproducible biomaterials could be manufactured using this filamentous alga.

Aquaculture and livestock feed : It was observed that white shrimp injected with the hot-water extract of *S. platensis* and immersed in the extract-fortified seawater could combat *Vibrio alginolyticus L. vannamei* better than the control. At the studied doses, increased phagocytic activity and the pathogen elimination was observed (66). It was observed that *S. platensis* or *S. maxima* supplementation in fish feed significantly enhanced the antioxidant ergothioneine content (31). The antimicrobial potency of ethanolic extracts of *S. platensis* was studied against fish and shellfish pathogens e.g. *Pseudomonas putida*, *Pseudomonas aeruginosa, Pseudomonas fluorescens, Aeromonas hydrophila*, *Vibrio alginolyticus, Vibrio anguillarum*, *Vibrio fluvialis*, *Vibrio parahaemolyticus, Vibrio harveyi, Vibrio fisheri*, *Edwardsiella tarda* and *Escherichia coli*. The promising result recommends the inclusion of the alga in aqua feed (67). Spirulina was evaluated as a substitute of soybean and alfalfa in the feed, on the basis of meat quality of growing rabbits (68). No significant changes in biochemical composition were observed apart from increased fatty acid content in the perirenal fat in spirulina-fed rabbits. The results of this study suggest that *S. platensis* could potentially be used in rabbit nutrition with consequent benefits on the nutritional quality of rabbit meat for consumers.

Advancement and bottlenecks in spirulina technology : Spirulina needs multiple steps of processing before reaching to market (Fig. 3). So, appropriate technology can spare manual labour, energy and expenses. Current scenario in cultivation, dehydration, extraction and purification is explored below.

The amount of phenolic compounds could be enhanced approximately eight-fold by light treatment (69). The shift in high light intensity led to increase in total amounts of carbohydrate, phycocyanin, carotenoid, malondialdehyde and antioxidant activities. A trinuclear iron (III) furoate was developed for regulating the biochemical composition of *S. platensis* biomass, which when supplemented at 5-10 mg/l increased iron, amino acid, peptide and carbohydrate contents in the alga (70). The influence of several factors on growth and protein content of *S. platensis* was determined, which showed about 60% improved productivity at aerial sparging without any additional mechanical stirring, low salinity and 1% of CO_2. Under intermittent illumination, when light/dark frequency increased from 0.01-20 Hz, specific growth rate and light efficiency were enhanced (71). It was suggested that silver coated polyester film fixed in culture racks serves as a reflector of light intensity and stimulates chrolophyll production (72). These innovative strategies may be adopted for energy-efficient and cost-effective spirulina cultivation.

Pre-dehydration treatment and drying are crucial steps for preservation of spirulina products. Antioxidants (á-tocopherol and tertiary-butyl hydroquinone or TBHQ) and two blanching methods (microwave and water bath) were employed to inactivate enzymes prior to dehydration. TBHQ proved better than á-tocopherol in minimizing the lipid peroxidation of blanched samples; whereas á-tocopherol was more suited than TBHQ in unblanched samples. Microwave blanching exerted a greater stabilizing effect than water bath blanching. The combined effect of TBHQ and microwave blanching was found to be the most effective pre-dehydration treatment for minimizing lipid peroxidation in drying spirulina. Optimization of the low-cost sun-drying method produced a dried product with comparable stability to that of spray-dried product (73). Drying of *S. platensis* on convective hot air was optimized through response surface methodology (RSM). At the optimum condition of 55°C temperature and 3.7mm sample thickness, least loss in bioactive lipids and pigments were observed (74). However, these drying techniques lead to the loss of a significant percentage of phycocyanin and carotenoid, warranting technological innovation.

The advent of effective extraction, purification, contaminant detection techniques and equipment has facilitated nutrients isolation from spirulina. The efficacy of supercritical carbon dioxide extraction and conventional solvent extraction was compared in recovery of α-linoleic acid from *S. platensis*. RSM optimization proved the suitability of the former over the latter in complete retrieval of ã-linoleic acid (75). The supercritical CO_2 extraction of antioxidants from *S. platensis* was optimized using RSM. About 10.26 g/kg of extracts, containing flavonoids, α-carotene, vitamin A and á-tocopherol, palmitic acid, linolenic acid and linoleic acid was obtained under the optimum conditions of 48°C at 20 MPa over a period of 4h (76). The supercritical fluid extraction parameters were optimized by RSM to obtain appreciable yield of vitamin E from *S. platensis* (4). The optimal conditions enhanced the tocopherol content to 12-fold as compared to the initial concentration in the crude form. The effect of ultrasonic extraction on yields of anticancer polysaccharides from *S. maxima* was studied (8). At optimal extraction condition of 60 kHz frequency and 60°C temperature applied for 30 min, extraction yield of 19.3% was reported. Human stomach cancer cells showed about 89% susceptibility to the water-soluble polysaccharides-rich extract. Microfiltration and ultrafiltration conditions proved suitable for pure, food grade c-phycocyanin extraction (77). An aqueous two-phase multi-stage countercurrent distribution technology was suggested for

effective and low cost separation of c-phycocyanin and allophycocyanin from *S. platensis* cell homogenate (12). The adulterant determining efficacy of least square support vector machines (LS-SVM) models under both full spectra and near infra red spectroscopy was advocated (78). For the optimum extraction of β-carotene from *S. platensis*, the processing parameters were optimized. The most favourable conditions were determined to be 1.5 spirulina in 50ml n-heptane at 30°C ultrasonicated for 8 min. Methanol preretreatment (2 min) raised the yield 12 times which measured 47.10% (79).

Conclusion

Being consumed since centuries, spirulina has well established itself as a superfood, an excellent weapon against an array of nutritional deficiencies. The above presented findings validate the potency of spirulina in thwarting several health issues. Moreover, there is a plethora of unexploited novel compounds and biological activities in this alga, worth-exploring. Innovative formulations are required to fortify conventional foods with spirulina. Economically feasible techniques of cultivation, drying and isolation of bioactive compounds are needed for maximum utilization. In recent years, spirulina has garnered enormous attention from research fraternity as well as industries as a thriving source of nutraceuticals and pharmaceuticals. Spirulina-based dietary habit must be promoted in the interest of the masses. This cheaply accessible functional food can sustainably combat malnutrition that eclipses the third world countries. However, quality control should be taken care of in order to ensure consumer safety. The clinical application of this microalga as complementary and alternative medicine (CAM) can reduce the cost of healthcare. This review is expected to convey contemporary scenario, kindle interest and help envision new implications of this abundantly available resource.

References

1. Deng, R. and Chow, T.J. (2010) Hypolipidemic, antioxidant and antiinflammatory activities of microalgae Spirulina. Cardiovascular Therapeutics, 28: 33-45.
2. Oliveira, E.G., Rosa, G.S., Moraes, M.A. and Pinto, L.A.A. (2009) Characterization of thin layer drying of *Spirulina platensis* utilizing perpendicular air flow. Bioresource Technology, 100: 1297-1303.
3. Yu, B., Wang, J., Suter, P.M., Russel., R.M., Grusak, M.A., Wang, Y., Wang, Z., Yin, S. and Tang, G. (2012) Spirulina is an effective dietary source of zeaxanthin to humans. British Journal of Nutrition, 7: 1-9.
4. Mendiola, J.A., García-Martínez, D., Rupérez, F.J., Martín-Álvarez, P.J., Guillermo, Reglero., Cifuentes, A., Barbas, C., Ibañez, E. and Señoráns, F.J. (2008) Enrichment of vitamin E from *Spirulina platensis* microalga by SFE. Journal of Supercritical Fluids, 43: 484-489.
5. El-Baky, H.H.A., El Baz, F.K. and El-Baroty, G.S. (2009) Production of phenolic compounds from *Spirulina maxima* microalgae and its protective effects. African Journal of Biotechnology, 8: 7059-7067.
6. Balachandran, P., Pugh, N.D., Ma, G. and Pasco, D.S. (2006) Toll-like receptor 2-dependent activation of monocytes by Spirulina polysaccharide and its immune enhancing action in mice. International Journal of Immunopharmacology, 5: 1808-1814.
7. Yang, L., Wang, Y., Zhou, Q., Chen, P., Wang, Y., Wang, Y., Liu, T. and Xie, L. (2009) Inhibitory effects of polysaccharide extract from *Spirulina platensis* on corneal neovascularization. Molecular Vision, 15: 1951-1961.
8. Oh, S.H., Ahn, J., Kang, D.H. and Lee, H.Y. (2010) The effect of ultrasonificated extracts of *Spirulina maxima* on the anticancer activity. Marine Biotechnology, 13: 205-214.

9. Yang, F, Tang, Q., Zhong, X., Bai Y., Chen, T., Zhang, Y., Li, Y. and Zheng, W. (2012) Surface decoration by Spirulina polysaccharide enhances the cellular uptake and anticancer efficacy of selenium nanoparticles. International Journal of Nanomedicine, 7: 835-844.

10. McCarty, M.F. (2007) Clinical potential of *Spirulina* as a source of phycocyanobilin. Journal of Medicinal Food, 10: 566-570.

11. McCarty, M.F., Barroso-Aranda, J. and Contreras, F. (2010) Potential complementarity of high-flavanol cocoa powder and spirulina for health protection. Medical Hypotheses, 74: 370-373.

12. Liu, Y., Feng, Y. and Lun, J. (2012) Aqueous two-phase countercurrent distribution for the separation of c-phycocyanin and allophycocyanin from *Spirulina platensis*. Food and Bioproducts Processing, 90: 111-117.

13. Fradique, M., Batista, A.P., Nunes, M.C., Gouveia, L., Bandarra, N.M. and Raymundo, A. (2010) Incorporation of *Chlorella vulgaris* and *Spirulina maxima* biomass in pasta products. Part 1: Preparation and evaluation. Journal of the Science of Food and Agriculture, 90: 1656-1664.

14. Zouari, N., Abid, M., Fakhfakh, N., Ayadi, M.A., Zorgui, L., Ayadi, M. and Attia, H. (2011) Blue-green algae (*Arthrospira platensis*) as an ingredient in pasta: free radical scavenging activity, sensory and cooking characteristics evaluation. International Journal of Food Science and Nutrition, 62: 811-813.

15. Adiba, B.D., Salem, B., Nabil, S. and Abdelhakim, M. (2011) Preliminary characterization of food tablets from date (*Phoenix dactylifera* L.) and spirulina (*Spirulina* sp.) powders. Powder Technology, 208: 725-730.

16. Batista, A.P., Nunes, M.C., Fradinho, P., Gouveia, L., Sousa, I., Raymundo, A. and Franco, J.M. (2011) Novel foods with microalgal ingredients-Effect of gel setting conditions on the linear viscoelasticity of *Spirulina* and *Haematococcus* gels. Journal of Food Engineering, 110: 182-189.

17. Bhowmik, D., Dubey, J. and Mehra, S. (2009) Probiotic efficiency of *Spirulina platensis* - stimulating growth of lactic acid bacteria. World Journal of Dairy and Food Sciences, 4: 160-163.

18. Kordowska-wiater, M., Wasko, A., Polak-Berecka, M., Kubik-Komar, A. and Targonski, Z (2011) Spirulina enhances the viability of *Lactobacillus rhamnosus* E/N after freeze-drying in a protective medium of sucrose and lactulose. Letters in Applied Microbiology, 53: 79-83.

19. Liu, J-G., Hou, C-W., Lee, S-Y., Chuang, Y. and Lin, C-C. (2011) Antioxidant effects and UVB protective activity of *Spirulina* (*Arthrospira platensis*) products fermented with lactic acid bacteria. Process Biochemistry, 46: 1405-1410.

20. Ravelonandro, P.H., Ratianarivo, D.H., Joannis-Cassan, C., Isambert, A. and Raherimandimby, M. (2011) Improvement of the growth of *Arthrospira* (*Spirulina*) *platensis* from Toliara (Madagascar): Effect of agitation, salinity and CO_2 addition. Food and Bioproducts Processing, 89: 209-216.

21. Yang, Y., Park, Y., Cassada, D.A., Snow, D.D., Rogers, D.G. and Lee, J. (2011) In vitro and in vivo safety assessment of edible blue-green algae, *Nostoc commune* var. *Spharoides* Kutzing and *Spirulina platensis*. Food and Chemical Toxicology, 49: 1560-1564.

22. Sharma, M.K., Sharma, A., Kumar, A. and Kumar, M. (2007) *Spirulina fusiformis* provides protection against mercuric chloride induced oxidative stress in Swiss

albino mice. Food and Chemical Toxicology, 45: 2412-2419.

23. Mendiola, J.A., Jaime, L., Santoyo, S., Reglero, G., Cifuentes, A., Ibañez, E. and Señoráns, F.J. (2007) Screening of functional compounds in supercritical fluid extracts from *Spirulina platensis.* Food Chemistry, 102: 1357-1367.

24. Karadeniz, A., Yildirim, A., Simsek, N., Kalkan, Y. and Celeb, F. (2008) *Spirulina platensis* protects against gentamicin-induced nephrotoxicity in rats. Phytotherapy Research, 22: 1506-1510.

25. Bermejo, P., Piñero, E. and Villar, A.M. (2008) Iron-chelating ability and antioxidant properties of phycocyanin isolated from a protean extract of *Spirulina platensis*. Food Chemistry, 110: 436-445.

26. Vázquez-Sánchez, J., Ramón-Gallegos, E., Mojica-Villegas, A., Madrigal-Bujaidar, E., Pérez-Pastén-Borja, R. and Chamorro-Cevallos, G. (2009) *Spirulina maxima* and its protein extract protect against hydroxyurea-teratogenic insult in mice. Food and Chemical Toxicology, 47: 2785-2789.

27. Escalona-Cardoso, G.N., Paniagua-Castro, N., Perez-Pasten, R. and Chamorro-Cevallos, G. (2012) Spirulina (arthrospira) protects against valproic acid-induced neural tube defects in mice. Journal of Medicinal Food, 15: 1103-1108.

28. Paniagua-Castro, N., Escalona-Cardoso, G., Hernandez-Navarro, D., Perez-Pasten, R. and Chamorro-Cevallos, G. (2011) Spirulina (Arthrospira) protects against cadmium-induced teratogenic damage in mice. Journal of Medicinal Food, 14: 398-404.

29. Shukla, V., Vashistha, M. and Singh, S.N. (2009) Evaluation of antioxidant profile and activity of amalaki *(Emblica officinalis)*, spirulina and wheat grass. Industrial Journal of Clinical Biochemistry, 24: 70-75.

30. Chu, W-L., Lim, Y-W., Radhakrishnan, A.K. and Lim, P-E. (2010) Protective effect of aqueous extract from *Spirulina platensis* against cell death induced by free radicals. BMC Complementary Alternative Medicine, 10: 53.

31. Pfeiffer, C., Bauer, T., Surek, B., Schömig, E. and Gründemann, D. (2011) Cyanobacteria produce high levels of ergothioneine. Food Chemistry, 129: 1766-1769.

32. Gad, A.S., Khadrawy, Y.A., El-Nekeety, A.A., Mohamed, S.R., Hassan, N.S. and Abdel-Wahhab, M.A. (2011) Antioxidant activity and hepatoprotective effects of whey protein and Spirulina in rats. Nutrition, 27: 582-589.

33. Remirez, D., Gonzalez, R., Merino, N., Rodriguez, S. and Ancheta, O. (2002) Inhibitory effects of Spirulina in zymosan-induced arthritis in mice. Mediators of Inflammation, 11: 75-79.

34. Madhyastha, H.K., Radha, K.S., Sugiki, M., Omura, S. and Maruyama, M. (2006) Purification of *c*-phycocyanin from *Spirulina fusiformis* and its effect on the induction of urokinase-type plasminogen activator from calf pulmonary endothelial cells. Phytomedicine, 13: 564-569.

35. Torres-Duran, P.V., Ferreira-Hermosillo, A. and Juarez-Oropeza, M.A. (2007) Antihyperlipemic and antihypertensive effects of *Spirulina maxima* in an open sample of mexican population: a preliminary report. *Lipids in Health and Disease,* 6: 33.

36. Colla, L.M., Muccillo-Baisch, A.L. and Costa, J.A.V. (2009) *Spirulina platensis* effects on the levels of total cholesterol, HDL and triacylglycerols in rabbits fed with a hypercholesterolemic diet. Brazilian Archives of Biology and Technology, 51: 405-411.

37. Gupta, S., Hrishikeshvan, H.J. and Sehajpal, P.K. (2010) Spirulina protects against Rosiglitazone induced osteoporosis in insulin resistance rats. Diabetes Research and Clinical Practice, 87: 38-43.

38. Nah, W.H., Koh, I.K., Ahn, H.S., Kim, M.J., Kang, H-G., Jun, J.H. and Gye, M.C. (2012) Effect of *Spirulina maxima* on spermatogenesis and steroidogenesis in streptozotocin-induced type I diabetic male rats. Food Chemistry, 134: 173-179.

39. Chamorro-Cevallos, G., Garduño-Siciliano, L., Barrón, B.L., Madrigal-Bujaidar, E., Cruz-Vega, D.E. and Pages, N. (2008) Chemoprotective effect of *Spirulina* (*Arthrospira*) against cyclophosphamide-induced mutagenicity in mice. Food and Chemical Toxicology, 46: 567-574.

40. Chen, T., Wong, Y-S. and Zheng, W. (2009) Induction of G1 cell cycle arrest and mitochondria-mediated apoptosis in MCF-7 human breast carcinoma cells by selenium-enriched Spirulina extract. Biomedicine and Pharmacotherapy, PMID: 19926246.

41. Akao, Y., Ebihara, T., Masuda, H., Saeki, Y., Akazawa, T., Hazeki, K., Hazeki, O., Matsumoto, M. and Seya, T. (2009) Enhancement of antitumor natural killer cell activation by orally administered Spirulina extract in mice. Cancer Science, 100: 1494-1501.

42. Sudha, M. and Kavimani, S. (2011) Protective effect of spirulina on doxorubicin induced testicular toxicity. Research Journal of Pharmaceutical Science and Biotechnology, 1: 17-21.

43. Shaharudin, S.H., Sulaimans, S., Shahril, M.R. and Hussain, S.N. (2011) The use of complementary and alternative medicine among Malay breast cancer survivors. Alternative Therapies in Health and Medicine, 17: 50-56.

44. Grzanna, R., Polotsky, A., Phan, P.V., Pugh, N., Pasco, D. and Frondoza, C.G. (2006) Immolina, a high-molecular-weight polysaccharide fraction of Spirulina enhances chemokine expression in human monocytic THP-1 cells. Journal of Alternative and Complementary Medicine, 12: 429-435.

45. Hayashi, O., Ono, S., Ishii, K., Shi, Y.H., Hirahashi, T. and Katoh, T. (2006) Enhancement of proliferation and differentiation in bone marrow hematopoietic cells by *Spirulina (Arthrospira) platensis* in mice. Journal of Applied Phycology, 18: 47-56.

46. Lobner, M., Walsted, A., Larsen, R., Bendtzen, K. and Nielsen, C.H. (2008) Enhancement of human adaptive immune responses by administration of a high-molecular-weight polysaccharide extract from the cyanobacterium *Arthrospira platen*sis. Journal of Medicinal Food, 11: 313-322.

47. Rasool, M. and Sabina, E.P. (2009) Appraisal of immunomodulatory potential of *Spirulina fusiformis*: an in vivo and in vitro study. Journal of Natural Medicine, 63: 169-175.

48. Ozdemir, G., Karabay, N.U., Dalay, M.C. and Pazarbasi, B. (2004) Antibacterial activity of volatile component and various extracts of *Spirulina platensis.* Phytotherapy Research, 18: 754-757.

49. Mala, R., Sarojini, M., Saravanababu, S. and Umadevi, G. (2009) Screening for antimicrobial activity of crude extracts of *Spirulina platensis.* Journal of Cell and Tissue Research, 9: 1951-1955.

50. Yamani, E., Kaba-Mebri, J., Mouala, C., Gresenguet, G. and Rey, J.L. (2009) Use of spirulina supplement for nutritional management of HIV-infected patients: Study in Bangui, Central African Republic. Medecine Tropicale (Mars), 69: 66-70.

51. Marcel, A-K., Ekali, L.G., Eugene, S., Arnold, O.E., Sandrine, E.D., von der Weid, D., Gbaguidi, E., Ngogang, J. and Mbanya, J.C. (2011) The effect of *Spirulina platensis* versus soybean on insulin resistance in HIV-infected patients: a randomized pilot study. Nutrients, 3: 712-724.

52. Khan, M., Shobha, J.C., Mohan, I.K., Naidu, M.U.R., Sundaram, C., Singh, S., Kuppusamy, P. and Kutala, V.K. (2005) Protective effect of *Spirulina* against doxorubicin-induced cardiotoxicity. Phytotherapy Research, 19: 1030-1037.

53. Karadeniz, A., Cemek, M., and Simsek, N. (2009) The effects of *Panax ginseng* and *Spirulina platensis* on hepatotoxicity induced by cadmium in rats. Ecotoxicology and Environmental Safety, 72: 231-235.

54. Ferreira-Hermosillo, A., Torres-Duran, P.V. and Juarez-Oropeza, M.A. (2010) Hepatoprotective effects of *Spirulina maxima* in patients with non-alcoholic fatty liver disease: a case series. Journal of Medical Case Reports, 7: 103.

55. Ou, Y., Zheng, S., Lin, L. and Li, Q. (2011) C-phycocyanin from *Spirulina maxima* protects hepatocytes against oxidative damage induced by H_2O_2 in vitro. Biomedicine and Preventive Nutrition, 1: 8-11.

56. Pak, W., Takayama, F., Mine, M., Nakamoto, K., Kodo, Y., Mankura, M., Egashira, T., Kawasaki, H. and Mori, A. (2012) Anti-oxidative and anti-inflammatory effects of spirulina on rat model of non-alcoholic steatohepatitis. Journal of Clinical Biochemistry and Nutrition, 51: 227-234.

57. Sun, Y.X., Zhang, J., Yu, G.C., Yan, Y.J., Chen, W.W., Chi, M.F. and Qin, S. (2012) Experimental study on the therapeutic effect of C-phycocyanin against pulmonary fibrosis induced by paraquat in rats. Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi, 30: 650-655.

58. Simsek, N., Karadeniz, A., Kalkan, Y., Keles, O.N. and Unal, B. (2009) *Spirulina platensis* feeding inhibited the anemia- and leucopenia-induced lead and cadmium in rats. Journal of Hazardous Materials, 164: 1304-1309.

59. Selmi, C., Leung, P.S., Fischer, L., German, B., Yang, C.Y., Kenny, T.P., Cysewski, G.R. and Gershwin, M.E. (2011) The effects of Spirulina on anemia and immune function in senior citizens. Cellular and Molecular Immunology, 8: 248-254.

60. Bermejo-Bescós, P., Piñero-Estrada, E. and del Fresno, A.M.V. (2008) Neuroprotection by *Spirulina platensis* protean extract and phycocyanin against iron-induced toxicity in SH-SY5Y neuroblastoma cells. Toxicology in Vitro, 22: 1496-1502.

61. Thaakur, S. and Sravanthi, R. (2010) Neuroprotective effect of Spirulina in cerebral ischemia-reperfusion injury in rats. Journal of Neural Transmission, 117: 1083-1091.

62. Bachstetter, A.D., Jernberg, J., Schlunk, A., Vila, J.L., Hudson, C., Cole, M.J., Shytle, R.D., Tan, J., Sanberg, P.R., Sanberg, C.D., Borlongan, C., Kaneko, Y., Tajiri, N., Gemma, C. and Bickford, P.C. (2010) Spirulina promotes stem cell genesis and protects against LPS induced declines in neural stem cell proliferation. PLoS One, 5: 10496.

63. de Morais, M.G., Stillings, C., Dersch, R., Rudisile, M., Pranke, P., Costa, J.A.V. and Wendorff, J. (2010) Preparation of nanofibers containing the microalga *Spirulina* (*Arthrospira*). Bioresource Technology, 101: 2872-2876.

64. Kim, S.H., Shin, C., Min, S.K., Jung, S-M. and Shin, H.S. (2012) *In vitro* evaluation of the effects of electrospun PCL nanofiber mats containing the microalgae *Spirulina* (*Arthrospira*) extract on primary astrocytes.

Colloids and Surfaces B: Biointerfaces, 90: 113-118.

65. Zhang, X., Yu, M., Liu, J. and Li, S. (2012) Bioinspired synthesis of a hollow metallic microspiral based on a Spirulina bioscaffold. Langmuir, 28: 3690-3694.

66. Tayag, C.M., Lin, Y-C., Li, C-C., Liou, C-H. and Chen, J-C. (2010) Administration of the hot-water extract of *Spirulina platensis* enhanced the immune response of white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus*. Fish and Shellfish Immunology, 28: 764-773.

67. Pradhan, J., Das, B.K., Sahu, S., Marhual, N.P., Swain, A.K., Mishra, B.K. and Eknath, A.E. (2012) Traditional antibacterial activity of freshwater microalga *Spirulina platensis* to aquatic pathogens. Aquaculture Research, 43: 1287-1295.

68. Peiretti, P.G. and Meineri, G. (2011) Effects of diets with increasing levels of *Spirulina platensis* on the carcass characteristics, meat quality and fatty acid composition of growing rabbits. Livestock Science, 140: 218-224.

69. Kepekci, R.A. and Saygideger, S.D. (2012) Enhancement of phenolic compound production in *Spirulina platensis* by two-step batch mode cultivation. Journal of Applied Phycology, 24: 897-905.

70. Melnic, S., Prodius, D., Simmons, C., Zosim, L., Chiriac, T., Bulimaga, V., Rudic, V. and Turta, C. (2011) Biotechnological application of homo- and heterotrinuclear iron(III) furoates for cultivation of iron-enriched *Spirulina*. Inorganica Chimica Acta, 373: 167-172.

71. Xue, S., Su, Z. and Cong, W. (2011) Growth of *Spirulina platensis* enhanced under intermittent illumination. Journal of Biotechnology, 151: 271-277.

72. Ajayan, K.V. and Selvaraju, M. (2011) Reflector based chlorophyll production by *Spirulina platensis* through energy save mode. Bioresource Technology, 102: 7591-7594.

73. Tiburcio, P.C., Galvez, F.C.F., Cruz, L.J. and Gavino, V.C. (2007) Optimization of low-cost drying methods to minimize lipid peroxidation in *Spirulina platensis* grown in the Philippines. Journal of Applied Phycology, 19: 719-726.

74. Oliveira, E.G., Duarte, J.H., Moraes, K., Crexi, V.T. and Pinto, L.A.A. (2010) Optimisation of *Spirulina platensis* convective drying: evaluation of phycocyanin loss and lipid oxidation. International Journal of Food Science Technology, 45: 1572-1578.

75. Sajilata, M.G., Singhal, R.S. and Kamat, M.Y. (2008) Supercritical CO_2 extraction of ã-linolenic acid (GLA) from *Spirulina platensis* ARM 740 using response surface methodology. Journal of Food Engineering, 84: 321-326.

76. Wang, L., Pan, B, Sheng, J., Xu, J. and Hu, Q. (2007) Antioxidant activity of *Spirulina platensis* extracts by supercritical carbon dioxide extraction. Food Chemistry, 105: 36-41.

77. Chaiklahan, R., Chirasuwan, N., Loha, V., Tia, S. and Bunnag, B. (2011) Separation and purification of phycocyanin from *Spirulina* sp. using a membrane process. Bioresource Technology, 102: 7159-7164.

78. Wu, D., Nie, P., Cuello, J., He, Y., Wang, Z. and Wu, H. (2011) Application of visible and near infrared spectroscopy for rapid and non-invasive quantification of common adulterants in *Spirulina* powder. Journal of Food Engineering, 102: 278-286.

79. Dey, S. and Rathod, V.K. (2013) Ultrasound assisted extraction of β-carotene from *Spirulina platensis*. Ultrasonics Sonochemistry, 20: 271-276.

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Pharmacological Profile of Antithrombotic, Fibrinolytic and Anticoagulant Drugs

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Abstract

The blood is a specialized fluid connective tissue or transport system which plays an important part in the maintenance of life. the transport of gases, nutrition, excretion, acid-base equity, body defence, water balance, clotting, regulation of body temperature, blood pressure, ionic balance and as a vehicle are the main functions of the blood. The anaemia, purpura, haemophilia, thrombosis, and clotting related disorders are the major complications of the hemopoietic system. Thrombosis is one of the leading cause of thromboembolic disorders affecting million peoples worldwide. Thromboembolic disorders such as pulmonary emboli, deep vein thrombosis, strokes and heart attacks are the main causes of morbidity and mortality in developed countries. Hence, antithrombotics play a pivotal role as agents for the prevention and treatment of thromboembolic disorders. A current list of various products having antithrombotic activity incorporated and prototype agents discussed. The antithrombotic includes specific antithrombotics agents like aspirin, cilostazol, clopidogrel, prasugrel, epoprostenol, dipyridamole, indomethacin, sulfinpyrazone, thromboxane receptor antagonists, thromboxane synthesis inhibitors, ticlopidine, terutroban, abciximab, eptifibatide, tirofiban, and non-specific antithrombotics agents like Ca^{2+} antagonists, ketanserin, nafazatron, α-receptor antagonists, β-blockers. The fibrinolytics like tissue plasminogen activators-streptokinase; urokinase, alteplase, duteplase, anistreplase, reteplase, saruplase, tenecteplase, snake venom, and anticoagulants also equally effective in the treatment of thrombotic disorders are discussed. This article includes general as well as recent pharmacological information on different aspects of antithrombotic, fibrinolytic and anticoagulant drugs that may be useful for their better understanding by users and health care professionals.

Keywords : Thrombosis, Platelets-inhibitors, t-PA, Ticlopidine, Aspirin, Warfarin, Heparin

Introduction

The blood is a specialized fluid connective tissue; play a vital role in maintenance of life by performing various functions. The thrombosis is a major blood disorder and threat to life. It is one of the leading cause of thromboembolic disorders affecting million peoples worldwide. Thromboembolic disorders such as pulmonary emboli, deep vein thrombosis, strokes and heart attacks are the main causes of morbidity and mortality in developed countries (1). Hence, antithrombotics, fibrinolytics and anticoagulants play a pivotal role in prevention and treatment of thromboembolic disorders (2, 3). Basically three factors are responsible for the occurrence of thrombosis-(i) Obstruction of blood flow, (ii) Hypercoagulability and, (iii) Vessel wall injury. Blood coagulation involves complex interaction between the injured vessel wall, platelets and coagulation factors (4). Diseases related to abnormal blood coagulation continues to be the number one cause of death in the world. The

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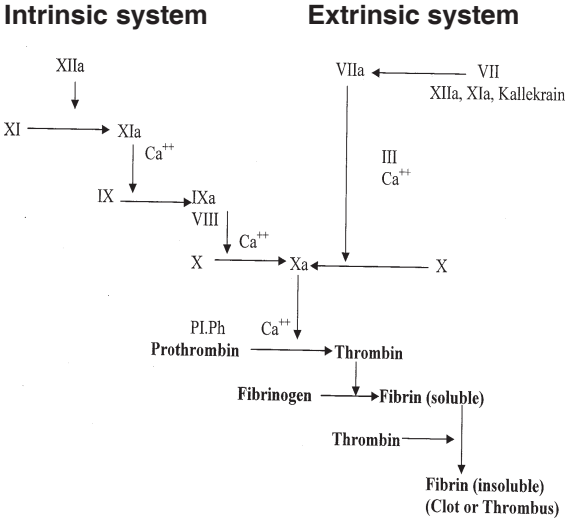
mechanism by which blood forms a stable clot has been the subject of much activity over the past 60-70 years (5). The workers in the field have been aided by two main factors, the first is fibrin clot and secondly individuals with inherited isolated deficiencies of single components. In the blood coagulation two separate but merging systems are involved i.e. the extrinsic and intrinsic pathway. The intrinsic pathway is initiated by the activation by Hageman factor (HF Factor XII). It is a plasma protein of approximately 80,000 molecular weight with associated carbohydrate (6). The coagulation cascade includes three fundamental phases-initiations, amplification, and propagation that can be amplified readily to thrombotic events occurring within veins, arteries, arterioles, and cardiac chambers. Intrinsic and extrinsic pathways converge at the point where factor X is converted to factor Xa. Factor X is a two-chain molecule of 50,000 molecular weight and activation involves splitting an arginine-isoleucine bond on the heavy chain, which liberates a peptide and also reveals an active serine site. This site is responsible for conversion of prothrombin to thrombin and then fibrin converted to insoluble fibrin. The process of coagulation occurs via a cascade of sequential reactions requiring several enzymes and other molecules known as coagulation (or clotting) factors. Factor Xa then participates in the final common pathway that results in the fibrin clot by activating prothrombin or factor II to thrombin or factor IIa. Thrombin, the final enzyme in the coagulation cascade or haemostasis, in turn converts soluble fibrinogen into insoluble fibrin monomers (1, 7). Thrombin is a 308 amino acid serine protease that cleaves peptide bonds in selective substrate including fibrinogen. Thrombin also participates in platelet, endothelial cell & leukocyte activation, increasing additional thrombin generation & the required surfaces for inflammatory & thrombotic events (8). Thrombin derives from the clearage of prothrombin after activation of the direct or indirect coagulation cascade. It acts as a catalyst for converting fibrinogen to fibrin, which subsequently cross links to form the mesh that creates a thrombus (9). Apart from its role in the conversion of fibrinogen to fibrin, it also activates factor XIII, accelerates the formation of factor V, which increases thrombin formation, and activates platelets, thereby enhancing platelet aggregation and the release of phospholipids (7). Clotting arises from a complex interaction of various mechanisms, including the activation of the coagulation and fibrinolytic systems, disruption of the vascular endothelium, and the generalised activation of the cellular mechanisms resulting in clotting on the surface of monocytes and platelets in circulation (10). This review focused on antithrombotic, fibrinolytic and anticoagulant agents, and provides general as well as recent pharmacological information that may be useful for their better understanding by users and health care professionals.

Process of blood coagulation : Formation of a blood clot is conversion of a hydrosol into a hydrogel. The blood clot is a semi-gel that retracts on standing. The extrinsic system is activated by damage to the tissue while intrinsic system is activated by blood itself. The clotting of blood involves-activation of prothrombin, conversion of prothrombin into thrombin in the presence of Ca^{2+} and numerous other clotting factors, and finally conversion of fibrinogen to fibrin (clot) by active thrombin.



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Drug therapy for thrombosis : The drugs which are used to treat the thrombosis may be classified into three main categories such as (A) antithrombotic or antiplatelet agents, (B) fibrinolytic or thrombolytic agents and (C) anticoagulants:

(A) Antithrombotic or antiplatelet agents: These agents inhibit platelet aggregation by suppressing the formation of thromboxane which promotes platelets aggregation. Platelets initiate clotting by breaking and convert the blood prothrombin into thrombin which is one of the basic substances to for the clot. They are basically useful in arterial thrombosis.

Classification of antithrombotics

(I) Specific platelet inhibitors	(1) ADP receptors/ $P2Y_{12}$ inhibitors *(a) Thienopyridines*-Clopidogrel, Prasugrel, Ticlopidine *(b) Nucleotide/nucleoside analogues*-Cangrelor, Elinogrel, Ticagrelor (2) *Glycoprotein IIb/IIIa inhibitors*-Abciximab, Eptifibatide, Tirofiban (3) *COX inhibitors*-Aspirin, Aloxiprin, Carbasalate calcium, Indobufen, Triflusal, Indomethacin, Sulfinpyrazone (4) *Prostaglandins analogues* (PGI_2)-Beraprost, Iloprost, Prostacyclin, Epoprostenol, Treprostinil (5) *Phosphodiesterase inhibitors*-Cilostazol, Dipyridamole, Triflusal (6) *Thromboxane inhibitors*- *(a) Thromboxane receptor antagonists*-Terutroban *(b) Thromboxane synthase inhibitors*-Dipyridamole, Picotamide (7) *Miscellaneous agents*-Cloricromen, Ditazole.
(II) Non specific platelet inhibitors	Ca^{2+} antagonists, Dipyridamole, Ketanserin, Nafazatron, α-receptor antagonists, β-blockers

(B) Fibrinolytic or thrombolytic agents: These agents activate plasminogen to form plasmin, which degrades fibrin and breaks up thrombi by converting insoluble clot to soluble. They are effectively useful in the treatment of myocardial complications, thrombosis and angina pectoris (11).

Classification of fibrinolytics

(I)	Tissue plasminogen activators	Streptokinase, Urokinase
(II)	Recombinant tissue plasminogen activators	Alteplase, Duteplase, Anistreplase, Reteplase, Saruplase, Tenecteplase, Monteplase
(III)	Miscellaneous agents	ASVIN, Ancrod, Fibrinolysis, Brinase

(C) Anti-coagulant agents: These agents use to inhibit coagulation process of the blood. The coagulation of the blood comprises the formation of fibrin with the involvement of thirteen clotting factors like calcium; several enzymes and various molecules associated with platelets and damaged tissue. The anti-coagulants prevent coagulation by interfering with the clotting factors. They are frequently used to suppress blood clotting in-vitro as well as in-vivo.

Classification of anticoagulants

(I) *In-vitro* or non-medicinal calcium complexing agents	**(II) *In-vivo* or oral anticoagulants or vitamin-K antagonists**	**(III) *In-vivo* and *in-vitro* agents or parenteral anticoagulants**
Citrate sodium, EDTA sodium, Fluorides sodium, Oxalates sodium	(i) Coumarin derivatives-Cyclocumarol, Dicumarol, Ethyl bicoumacetate, Phenpro-couman, Coumatetralyl (ii) Indandione derivatives-Acenocoumarol, Anisindione, Chlorphenindione, Clorindione, Diphenadione, Phenindione (iii) Rodenticides-Anisindione, Brodifacoum, Bromadiolone, Chlorphenindione, Pindone (iv) Warfarin (v) Others agents-Ximelagatran, Tioclomarol	(i) Natural Heparin-Heparin (ii) Low molecular weight heparin-Ardeparin, Bemiparin, Certoparin, Dalteparin, Enoxaparin, Nadroparin, Parnaparin, Reviparin, Tinzaparin (iii) Heparinoids-Ancrod, Sulodexide, Danaparoid, Dermatan sulfate, Dextran sulfate, Heparin sulfate (iv) Synthetic heparins derivatives-Fondaparinux, Idrabiotaparinux (v) Direct thrombin-II inhibitors-*(a) Univalent*-Argatroban, Dabigatran etexilate, Melagatran, Ximelagatran *(b) Bivalent*-Bivalirudin, Hirudin, Lepirudin, Desirudin(vi) Direct Xa inhibitors or Xabans-Apixaban, Betrixaban, Edoxaban, Otamixaban, Rivaroxaban(vii) Miscellaneous agents-Antithrombin-III, Drotrecogin-á or protein-C

Pharmacology of Individual Drugs

(A) Antithrombotic or antiplatelet agents : Ticlopidine is a thienopyridine derivative antiplatelet drug, acts slowly, and takes 3-7 days for peak effect. *Mechanism of action*: ticlopidine act on GPIIb/IIIa fibrinogen receptors which are located on the platelet membrane and inhibits platelet aggregation. *ADME*: onset of action is slow, 3-7 days required to reach maximum effect, converted into an active metabolite. *Dose*: 250mg/BD/with meals. *Adverse effects*: it may cause nausea, vomiting, diarrhoea, abdominal pain, aplastic anaemia, bleeding, haemorrhage, headache, hypercholesterolemia, jaundice, leukopenia, neutropenia, skin rashes, tinnitus and vertigo. *Uses*: it is preferred in the following cases-to prevent restenosis and thrombosis following coronary angioplasty and in prophylaxis of myocardial infarction and stroke, coronary artery diseases, coronary bypass implants, cerebrovascular diseases, venous thrombosis, peripheral vascular diseases, prosthetic heart valves and arteriovenous shunts, acute thrombotic stroke, atrial fibrillation, pulmonary embolism and peripheral arterial occlusion and stable angina pectoris (12).

Epoprostenol (13, 14) is a short acting prostacyclin or PGI_2, useful in treatment of pulmonary hypertension. *Pharmacological actions*: strongly dilate all vascular beds, inhibits platelet aggregation and prevent thrombogenesis and platelet clumping in lungs. *ADME*: administered intravenously as infusion, $t_{1/2}$ is 3

minutes. *Dose*: 0.5mg/i.v. infusion. *Adverse effects*: it may cause nausea, vomiting, chest pain, drowsiness, hyperglycaemia, hypotension and tachycardia. *Uses*: it is preferred in the following cases-to inhibit platelet aggregation during haemodialysis, during cardiac bypass surgery and pulmonary hypertension (15).

Dipyridamole (16) is a powerful coronary vasodilator, anti-platelet agent, suppresses platelet aggregation by potentiating PGI_2 or inhibiting phosphodiesterase that leads to increase cAMP. It also inhibits thrombus formation in the arterial circulation. *Pharmacological actions*: produces anticoagulant effect, reduces contractility of myocardium, it also has membrane stabilizing activity. *Mechanism of action*: suppresses the coronary vascular resistance, improving blood flow and sinus oxygen saturation by increasing the plasma concentration of adenosine by inhibiting the uptake of adenosine into erythrocytes and other body tissues. *ADME*: administered orally, absorption is highly variable, mainly metabolized in liver. *Interactions*: potentiate the action of aspirin and warfarin. *Contra-indicated in*: aortic stenosis, heart failure, exacerbates migraine, hypotension and myasthenia gravis. *Dose*: 100mg/TDS or QID/orally. *Adverse effects*: it may cause angioedema, blurred vision, diarrhoea, dizziness, dry mouth, GIT effects, headache, hot flushes, hypotension, more bleeding during surgery, myalgia, nausea, precipitates cardiac failure, precipitation of glaucoma, rashes, severe bronchospasm, tachycardia, throbbing headache, thrombocytopenia, urine retention and urticaria. *Uses*: it is preferred in the following cases-angina pectoris (17), thromboembolism, post myocardial infarction, post stroke condition, thrombus formation after cardiac surgery, persistent coronary artery aneurysms, supraventricular and ventricular tachycardia, Wolff-Parkinson white syndrome, ischemic attacks and coronary bypass surgery.

Aspirin (16, 18) is an acetylsalicylic acid, possesses antiplatelet activity and useful in the treatment of thrombosis. *Pharmacological actions*: prevents atheromatous plaques formation in arteries, inhibits PGs synthesis in vessel wall and the release of ADP from platelets. *Mechanism of action*: inhibition of platelet activation, stickiness or aggregation by irreversible inhibition of platelet cyclo-oxygenase and thromboxane synthetase which leads to depletion of prostacyclin (PGI_2) and thromboxane (TXA_2) which is key platelets activator (19). *Precautions*: asthma, hepatic impairment, peptic ulceration, pregnancy, renal impairment, uncontrolled hypertension. *Contra-indicated in*: peptic ulceration, haemophilia, bleeding disorders, breast feeding and children less than 15 years. *Dose*: 75-300mg/day. *Adverse effects*: it may cause bronchospasm, GIT complications, and haemorrhage. *Uses*: it is preferred in the following cases-thrombus formation after cardiac surgery, persistent coronary artery aneurysms, prophylaxis of stroke, stable angina, myocardial infarction, atrial fibrillation, claudication, eclampsia in pregnancy and diabetic retinopathy.

Clopidogrel (20) is a glycoprotein receptor antagonist, and inhibits platelet aggregation. *Mechanism of action*: suppressed platelet aggregation by inhibition of GPIIb/IIIa receptors. *ADME*: well absorbed after oral administration, platelet aggregation inhibition achieved within 90 minutes, metabolized quickly. *Interactions*: action enhanced by aspirin. *Contra-indicated in*: breast feeding and bleeding. *Dose*: 300mg flowed by 75mg/day/orally. *Adverse effects*: it may cause hemorrhage; GIT upsets CNS disorders, biliary disorders, and hepatic complications. *Uses*: it is preferred in following thromboembolic complications-stroke, myocardial infarction, peripheral arterial disease and unstable angina (21).

Ketanserin (22, 23) is a $5HT_2$, á-adrenergic and dopaminergic receptor blocker. *Pharmacological actions*: it reduces blood pressure and Inhibits vasoconstriction, bronchoconstriction and platelet aggregation by

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blocking the action of serotonin. *ADME*: administered orally, metabolized in liver to an inactive metabolite ketanserinol, $t_{1/2}$ is 10-18 hours, excreted unchanged in urine. *Dose*: 40mg/ orally/TDS. *Adverse effects*: it may cause nausea, postural hypotension, palpitation, fatigue, dryness of mouth, headache, sedation and dizziness. *Uses*: it is preferred in following cases-aggression, anxiety, carcinoid syndrome, depression, diabetic gastroparesis, dyspepsia, emesis, gastroeso phageal reflex disease, hypertension, inflammation, irritable bowel syndrome, migraine, nociception, Parkinsonism, peripheral vascular diseases, schizophrenia and urticaria.

Abciximab (24) is a humanized, nonantigenic, potent platelet aggregation inhibitor, Fab fragment of a chimeric monoclonal antibody against GP IIb/IIIa. *Mechanism of action*: it prevents platelet aggregation by blocking glycoprotein IIb/IIIa receptors involved in platelet aggregation. *ADME*: administered as bolus injection followed by slow i.v. infusion, $t_{1/2}$ is 10-30 min, but effect remains for 24 hours. *Dose*: 0.25mg/kg, i.v., 10-60 min before PCI, followed by 10µg/min for 12 hours. *Adverse effects*: it may cause haemorrhage, thrombocytopenia, constipation, ileus and arrhythmias. *Uses*: it is preferred in the cases of unstable angina and as adjuvant to coronary thrombosis (25).

Eptifibatide (26) is an antiplatelet heptacyclic peptide GP IIb/IIIa receptor antagonists. It is derived from a protein found in the venom of the south eastern pygmy rattlesnake. *ADME*: it is administered as bolus injection initially followed by slow i.v. infusion. It has short half life and excreted in urine. *Contra-indicated in*: thrombocytopenia, renal insufficiency and allergy. *Adverse effects*: it may cause bleeding, thrombocytopenia, hypotension, cardiovascular failure, arrhythmias and severe allergic reactions. *Uses*: it is preferred in cases like myocardial infarction (25).

Terutroban (27, 28) is a potent, long acting antiplatelet antithrombotic agent, selective antagonist of thromboxane receptors. *Mechanism of action*: it blocks thromboxane induced platelet aggregation and vasoconstriction. *ADME*: it is active orally and $t_{1/2}$ is 6-10 hours. *Uses*: it is effective in animal models of thrombosis, atherosclerosis and diabetic neuropathy and recently undergoing phase-III development for the secondary prevention of acute thrombotic complications of atherosclerosis (29).

Beraprost (30) is a prostacyclin (PGI_2) analogue has vasodilatory, antiplatelet and cytoprotective properties. *Pharmacological actions*: it enhances liver enzymes, triglycerides and bilirubin. *Mechanism of action*: it binds to prostacyclin receptors and reduces the release of intracellular calcium and thus cause relaxation of the smooth muscle cells and vasodilation. *ADME*: administered orally and should be taken with food. *Precautions*: may enhancing bleeding tendency in patients on anticoagulants, antiplatelet or fibrinolytic agents, and menstruating women. *Dose*: 120-180 µg/day. *Contra-indicated in:* patients with haemorrhage and pregnancy. *Interactions:* any drug intensifies bleeding. *Adverse effects*: it may cause headache, hot flushes, nausea, GIT upsets, bleeding tendency and dizziness. *Uses*: it is preferred in cases like thrombosis and atherosclerosis.

(B) Fibrinolytic or thrombolytic agents : *Tissue plasminogen activator* (31) is fibrin-selective but does not activate systemic plasminogen. It is synthesized by recombinant technology and is also obtained from cultured human melanoma cells. *Mechanism of action*: it induces fibrinolysis of the formed thrombus by preferentially activating plasminogen bound to fibrin. *Dose*: 100 mg/ *i.v.*/3 hours. *Uses*: it is preferred in the treatment of deep vein thrombosis (32, 33).

Alteplase (34) is an enzyme that catalyzes tissue plasminogen to plasmin and is commonly used to prevent clot-related myocardial disorders.

Mechanism of action: it dissolves blood clots by converting plasminogen into plasmin that digests fibrin, fibrinogen, and other proteins. *Interactions*: Its action accelerates in combination with heparin. *Contraindicated in:* bleeding, defective homeostasis, trauma, surgical procedures, stroke, acute pericarditis, hypoglycaemia and hyperglycaemia. *Dose*: 100 mg/*i.v.* infusion. *Adverse effects*: it may cause adverse effects like nausea, vomiting, fever, arrhythmias, allergy, hypotension, intracranial haemorrhage, and GIT bleeding. *Uses*: it is preferred in cases of angina pectoris, as an anticoagulant, intravascular thrombosis, ischemic diseases, myocardial infarction, pulmonary embolism, and to dissolve thrombi.

Streptase or streptokinase (35) is obtained from â-haemolytic Streptococci group-C. It combines with circulating plasminogen to form an activation complex, which then causes limited proteolysis of other plasminogen molecules to plasmin. Antistreptococcal antibodies present due to the initial dose of streptokinase make a loading dose necessary in the beginning. *Mechanism of action*: activates the conversion of plasminogen (profibrinolysin) into plasmin (fibrinolysin), which stimulates the conversion of fibrin (insoluble) into fibrin fragments (soluble). *Dose*: 250,000 units followed by 100,000 units/ hour for 1-3 days/*i.v*. *Contraindicated in:* active internal bleeding, bleeding diathesis, cerebral tumour. If hemostasis is important, pregnancy, previous cerebrovascular accident, recent cranial trauma, surgery within ten days, and uncontrolled hypertension. *Adverse effects*: it may cause anaphylaxis, bronchospasm, hypersensitivity, fever, hypotension, and arrhythmias. *Uses*: it is preferred in cases of acute arterial thromboembolism, acute myocardial infarction, acute thrombotic stroke, deep venous thrombosis, local thrombolysis in the anterior chamber of the eye, myocardial infarction, and unstable angina.

Urokinase (36) is a proteolytic but not antigenic, fibrin-selective, thrombolytic, or fibrinolytic agent. It is developed by recombinant technology as pro-urokinase and is also derived from the human kidney (is present in urine). It is converted to urokinase from pro-urokinase upon its binding to fibrin. *Mechanism of action*: it directly converts plasminogen into plasmin. *Contraindicated in:* bleeding risks, vascular aneurysm, endocardial thrombi, and allergy to streptokinase. *Dose*: 3,00,000 Units/h for 12 hours/*i.v*. *Adverse effects*: it may cause fever, bleeding, GIT bleeding, and haemolytic stroke. *Uses*: it is preferred in cases like central deep vein thrombosis such as superior vena cava syndrome and ascending thrombophlebitis. It is also effective in acute coronary thrombosis, myocardial infarction, and multiple pulmonary emboli.

Anistreplase (37) or anisoylated plasminogen-streptokinase activator complex is a human plasminogen and streptokinase. The anisoyl group is removed in blood by a hydrolytic deacylation process. *Dose*: 30 units/*i.v*. infusion over 2-5 minutes. *Adverse effects*: it may cause hypotension and allergy.

Tenecteplase (38) is developed by the recombinant technique and is a mutant of alteplase containing 527 amino acids. It is more fibrin-selective and more resistant to plasminogen activator inhibitor-1. It is given as a single bolus injection of 30-50 mg. It has a longer $t_{1/2}$ and greater efficacy.

Saruplase (39) is a full-length, human, unglycosylated, single-chain polypeptide containing 411 amino acids, urokinase type plasminogen activator and also known as prourokinase. It is obtained by recombinant technology from *E. coli*. It is a fibrin-specific fibrinolytic agent and is effectively used for the treatment of thrombotic disorders such as acute myocardial infarction. *Dose*: 20 mg/*i.v*. bolus followed by a 60 mg infusion for 60 minutes. *Uses*: it is preferred in thrombotic disorders and myocardial infarction.

(C) Anti-coagulant agents : Warfarin (40-42) is a coumarin derivative, structurally similar to

vitamin-K, used as oral anticoagulating agent because it prevents clot formation. *Mechanism of action*: antagonizes the production and action of vitamin-K, and also inhibiting the reduction of it into active metabolite. *ADME*: well absorbed after oral administration, bound to albumin, metabolized by conjugation and glucuronic acid in liver, $t_{1/2}$ is 36-44 hours, and excreted in urine and stool. *Interactions*: action potentiated by antibiotics, cephalosporins, aspirin, salicylates, phenylbutazone, cotrimoxazole, sulphonamide, phenytoin, probenecid, moxalactum, carbenicillin, tolbutamide, chloramphenicol, metronidazole, antiplatelet agents, alcohol, cimetidine, anabolic hormones, antimalarials, ciprofloxacin and action inhibited by barbiturates, rifampin, griseofulvin and oral contraceptives. *Contra-indicated in*: colitis, peptic ulcer, pregnancy, severe hypertension, bacterial endocarditis, renal failure and hepatic disorders. *Dose*: 10-15mg/day/orally. *Adverse effects*: it may cause alopecia, anorexia, dermatitis, diarrhoea, fever, gangrene, haemorrhage, hepatic dysfunctions, nausea, vomiting, pancreatitis, hypersensitivity, rashes, jaundice, necrosis, pyrexia and teratogenicity. *Uses*: it is preferred in cases like cerebrovascular diseases, defibrination syndrome, myocardial infarction, pulmonary embolism, rheumatic heart diseases, vascular surgery, venous thrombosis and also used as anticoagulant.

Danaparoid (43) is a mixture of heparin glycosamineglycans obtained from porcine intestinal mucosa. *Mechanism of action*: stimulates inhibition of factor Xa by antithrombin. *ADME*: administered through subcutaneously or intravenously, $t_{1/2}$ is 24 hours. *Contra-indicated in*: renal failure. *Uses*: it is preferred in cases like deep venous thrombosis and heparin induced thrombocytopenia.

Dalteparin (44) is a low molecular weight heparin and having antiplatelet, antithrombotic activities. *Mechanism of action*: inhibition of thrombus and clot formation by blocking clotting factor Xa and IIa. *ADME*: administered subcutaneously, crosses placental barrier and secreted in breast milk, $t_{1/2}$ is about 4.5 hours, excreted out in urine. *Contra-indicated in*: hypersensitivity, benzyl alcohol, severe thrombocytopenia, uncontrolled bleeding and pregnancy. *Adverse effects*: it may cause local irritation and erythema at the site of injection, chills, fever, pain, hematoma, urticaria, thrombocytopenia, hyperkalemia, haemorrhage and asthma. *Uses*: it is preferred in cases like deep vein thrombosis and pulmonary embolism and also used as adjunct to antineoplastic chemotherapy.

Heparin (45) is a rapid but short acting anticoagulant, obtained from porcine intestine mucosa and bovine lung tissue. It is a mixture of acidic substances, naturally synthesized in body and stored in mast cells and basophils. It is useful in thromboembolic diseases or to prevent further clotting. *Pharmacological actions*: acts as anticoagulant in vitro and in vivo, inhibits platelet aggregation, clear lipemia of plasma by releasing lipoprotein lipase from blood vessels and tissues. *Mechanism of action*: it bound to antithrombin-III and inactivates factor Xa and thrombin. *ADME*: administered i.v. or s.c, metabolized by heparinase in liver and excreted in urine. *Contra-indicated in*: haemophilia, haemorrhage, hypersensitivity, liver disease, neurosurgery, ocular surgeries, peptic ulcer, piles, recent cerebral haemorrhage, sever hypertension, tuberculosis. *Dose*: 5000-15000 units/12 hours. *Adverse effects*: it may cause allergy, alopecia, haemorrhage, hematuria, hyperkalemia, osteoporosis and spontaneous fractures, renal and hepatic dysfunctions, skin necrosis, suppress cell mediated immunity, suppress wound healing and thrombocytopenia. *Uses*: it is preferred in cases like acute peripheral arterial occlusion, myocardial infarction, thrombosis on prosthetic heart valves, to keep blood outside the body in fluid state, to prevent clotting during haemodialysis or bypass surgery, to prevent clotting in open heart surgery, to prevent post operative deep venous thrombosis and pulmonary embolism, unstable angina and venous thrombosis.

Dabigatran etexilate (46, 47) is a prodrug, substrate of P-glycoprotein transporter and use as oral anticoagulant. *Mechanism of action*: it reversibly inhibits the active site of thrombin. *ADME*: administered orally rapidly and completely converted to dabigatran by esterases in plasma and liver. 6% bioavailability reach within 2 hours, $t_{1/2}$ is 14-17 hours and about 80% of drug as such excreted out through kidneys. *Contra-indicated in*: elderly patients over the age of 75 years due to GIT bleeding. *Interactions*: quinidine reducing the clearance of dabigatran but amiodarone increases dabigatran level upto 50%. *Dose*: 150 mg/BD/orally. *Adverse effects*: it may cause gastrointestinal bleeding. *Uses*: it is preferred in cases like venous thromboembolism, stroke or systemic embolism in patients with atrial fibrillation.

Apixaban (46, 47) is a potent, oral, reversible, direct, highly selective, active drug and use as oral anticoagulant. *Mechanism of action*: it prevents coagulation by blocking thrombin or generation of factor Xa and thrombin activity. It inhibits free and clot-bound factor-Xa, and prothrombinase activity. Activation of factor-X to factor-Xa via the intrinsic and extrinsic pathway plays a central role in the cascade of blood coagulation. It indirectly inhibits platelet aggregation induced by thrombin. *ADME*: administered orally rapidly absorbed, maximum plasma concentration achieved within 3 hours, $t_{1/2}$ is 8-14 hours and excreted out through kidneys. *Contra-indicated in*: patients receiving azole-antimycotics and HIV protease inhibitors. *Dose*: 2.5 mg/BD/orally. *Adverse effects*: it may cause any type of bleeding. *Uses*: it is preferred in cases like venous thromboembolism following elective hip or knee replacement surgery and stroke in patients with atrial fibrillation.

Conclusion

Thrombosis is one of the leading cause of thromboembolic disorders affecting million peoples worldwide. Thromboembolic disorders such as pulmonary emboli, deep vein thrombosis, strokes and heart attacks are the main causes of morbidity and mortality in developed countries. Hence, antithrombotics play a pivotal role as agents for the prevention and treatment of thromboembolic disorders. The various products having antithrombotic activity include antithrombotics, fibrinolytics and anticoagulants have enlisted in the article. A brief description, pharmacological actions, mechanism of action, ADME, dose, precautions, interactions, contra-indications, adverse effects and therapeutical potential have been described in the present review. This article includes general as well as recent pharmacological information on different aspects of antithrombotic, fibrinolytic and anticoagulant drugs that may be useful for their better understanding by users and health care professionals.

References

1. Dickneite, G., Seiffe, D., Diehl, K.H., Rogers, M. and Czech, J. (1995). Pharmacological characterization on a new 4-amidinophenyl-alanine thrombin-inhibitor. Thromb Res, 77: 357-368.
2. Hirsh, J., O'Donnell, M. and Weitz, J.I. (2005). New anticoagulants. Blood, 105: 453-463.
3. Hirsh, J., O'Donnell, M. and Eikelboom, J.W. (2007). Beyond unfractionated heparin and warfarin: current and future advances. Circulation, 116: 552-560.
4. Tripathi, K.D. (2008). Essentials of medical pharmacology: drugs affecting coagulation, bleeding and thrombosis (6th edition), Jaypee Brothers medical publishers (P) Ltd., New Delhi, pp. 593-611.
5. Schattauer, G. and Stuttgart. (2001). Fibrinolysis and cellular hemostasis; accelerated thrombosis in a rabbit model of carotid artery thrombosis with liposome-encapsulated and microencapsulated streptokinase. Blood Coagulation, 4: 64.
6. Chart, I.S. and Sanderson, J.H. (1979). General aspect of blood coagulation system. Pharmacy Therapeutics, 5: 229-233.

7. Tortora, G.J. and Grabowski, S. (2000). Principles of anatomy and physiology (7th edition), Harper Collins College Publishers, New York, pp. 583.

8. Richard, C., Becker, M.D. and Durbam, N.C. (2003). Understanding the dynamics of thrombin in cardiovascular disease: pathophysiology and biochemistry for the clinician. Am. Heart Journal, 149: 52-58.

9. Chong, B.H. (2003). Thrombin an ideal target for pharmacological inhibition: a review of direct thrombin inhibitors. J. Thrombosis and Hemostasis, 121: 1471-1478.

10. Rickles, F.R. and Falanga, A. (2001). Molecular basis for the relationship between thrombosis and cancer. Thromb Res, 102: V215-V224.

11. Maheshwari, K.K. (2012). Drugs pharmacology: anti-platelet agents (Ist edition), Vallabh Prakashan, New Delhi, pp. 190-195.

12. Berglund, U., von-Schenck, H. and Wallentin, L. (1985). Effects of ticlopidine of platelet function in men with stable angina pectoris. Thromb Haemost, 54: 808-812.

13. Payton, C.D., Belch, J.J.F. and Jones, J.M.B. (1985). Successful treatment of thrombotic thrombocytopenic purpura by epoprostenol infusion. Lancet, 1(8434): 927-928.

14. Bobbio-Pallavicini, E., Porta, C., Tacconi, F., Gugliotta, L., Centurioni, R. and Vianelli, N. (1994). Intravenous prostacyclin (as epoprostenol) infusion in thrombotic thrombocytopenic purpura. Haematologica, 79: 429-437.

15. McLaughlin, V.V., Genthner, D.E., Panella, M.M. and Rich, S. (1998). Reduction in pulmonary vascular resistance with long-term epoprostenol (prostacyclin) therapy in primary pulmonary hypertension. N Engl J Med, 338: 273-277.

16. Muller, T.H. (2001). Inhibition of thrombus formation by low-dose acetylsalicylic acid, dipyridamole, and their combination in a model of platelet-vessel wall interaction. Neurol, 57(2): S8-S11.

17. Sbar, S. and Schlant, R.C. (1967). Dipyridamole in the treatment of angina pectoris. A double-blind evaluation. JAMA, 201: 865-867.

18. Miner, J. and Hoffhines, A. (2007). The discovery of aspirin's antithrombotic effects. Tex Heart Inst Journal, 3: 1791-1796.

19. Mehta, S.C. and Kar, A. (2011). Pharmaceutical pharmacology: antiplatelet drugs (Ist edition), New age international (P) Ltd Publishers, New Delhi, pp. 498-499.

20. Savi, P., Heilmann, E. and Nurden, P. (1996). Clopidogrel: an antithrombotic drug acting on the ADP-dependent activation pathway of human platelets. Clin Appl Thromb Hemost, 2: 35-42.

21. Sabatine, M.S., Cannon, C.P., Gibson, C.M., Lpez-Sendn, J.L., Montalescot, G. and Theroux, P. (2005). Addition of clopidogrel to aspirin and fibrinolytic therapy for myocardial infarction with ST-segment elevation. N Engl J Med, 352: 1179-1189.

22. Awouters, F. (1985). The pharmacology of ketanserin, the first selective serotonin S2-antagonist. Drug Development Res, 6: 263-300.

23. Mysliwiec, M., Malyszko, J., Pawtak, K., Borawski, J. and Malyszko, J.S. (1995). Effect of ketanserin on platelet function and bleeding time in uremic patients treated with erythropoietin. Thrombosis Res, 78(6): 541-546.

24. Hayes, R., Chesebro, J.H., Fuster, V., Dangas, G., Fallon, J.T. and … Sharma,

S.K. (2000). Antithrombotic effects of abciximab. The Am J Cardiol, 85(10): 1167-1172.

25. Sharma, H.L. and Sharma, K.K. (2011). Principles of pharmacology (Ist edition), Paras Medical Publishing House, Hyderabad, pp. 915-919.

26. Gribble, G.W. (2010). Heterocyclic scaffolds-II: indoles: synthesis, properties and applications. Springer, pp. 11.

27. Sorbera, L.A., Serradell, N., Bolos, J. and Bayes, M. (2006). Terutroban sodium. Drugs of the Future, 31(10): 867-873.

28. Spreitzer, H. (2007). Neue wirkstoffe-terutroban (in German). Osterreichische Apothekerzeitung, 3: 116.

29. Hennerici, M.G., Bots, M.L., Ford, I., Laurent, S. and Touboul, P.J. (2010). Rationale, design and population baseline characteristics of the perform vascular project: an ancillary study of the prevention of cerebrovascular and cardiovascular events of ischemic origin with terutroban in patients with a history of ischemic stroke or transient ischemic attack trial. Cardiovascular Drugs and Therapy, 24(2): 175-180.

30. Melian, F.E. and Goa, K.L. (2002). Beraprost: a review of its pharmacology and therapeutic efficacy in the treatment of peripheral arterial disease and pulmonary arterial hypertension. Drugs, 62(1): 107-133.

31. Astrup, T. (1966). Tissue activators of plasminogen. Federation Proceedings, 25(1): 42-51.

32. Pilger, E., Obernosterer, A., Decrinis, M., Aschauer, M., Doder, S. and Lafer, M. (1992). Pilot trial of recombinant tissue type plasminogen activator in deep venous thrombosis. Fibrinolysis, 6: 148.

34. Doggrell, S.A. (2001). Alteplase: descendancy in myocardial infarction, ascendancy in stroke. Expert Opin Investig Drugs, 10: 2013-2029.

33. Kishore, K. and Krishan, P. (2009). Pharmacology of recombinant or genetically engineered drugs. J Young Pharmacist, 1(2): 141-150.

35. Bhattacharya, S.K., Sen, P., Ray, A. and Das, P. (2005). Pharmacology (2nd edition), Reed Elsevier India Private Limited, New Delhi, pp. 207-209.

36. Iqbal, O., Messmore, H., Hoppensteadt, D., Fareed, J. and Wehrmacher, W. (2000). Thrombolytic drugs in acute myocardial infarction. Clin Appl Thrombosis/ Hemostasis, 6: 1-13.

37. Anderson, J.L. (1988). Anistreplase (APSAC) and future trends in thrombolytic therapy. J Interv Cardiology, 1: 285-290.

38. Dunn, C.J. and Goa, K.L. (2001). Tenecteplase: a review of its pharmacology and therapeutic efficacy in patients with acute myocardial infarction. Am J Cardiovasc Drugs, 1: 51-66.

39. Vermeer, F., Bosl, I., Mexer, J., Bar, F., Charbonnier, B. and Windeler, J. (1999). Saruplase is a safe and effective thrombolytic agent; observation in 1998 patients: results of the pass study, practical applications of saruplase study. Thromb Thrombilysis, 8: 143-150.

40. Carter, B.L. (1991). Therapy of acute thromboembolism with heparin and warfarin. Clin Pharmacy, 10: 503-518.

41. Horton, J.D., Pharm, D., Bruce, M. and Bushwick, M.D. (1999). Warfarin therapy: evolving strategies in anticoagulation. Am Fam Physician, 59(3): 635-646.

42. Ansell, J., Hirsh, J., Hylek, E., Jacobson, A., Crowther, M. and Palareti, G. (2008).

Pharmacology and management of the vitamin K antagonists. Chest, 133: 160S-198S.

43. Van-Barlingen, H.H.J.J., Van-Beek, A., Erkelens, D.W. and De-Bruin, T.W.A. (1997). Danaparoid: an antithrombotic agent without major impact on triglyceride hydrolysis capacity in human. J Internal Medicine, 242: 125-129.

44. Dunn, C. and Sorkin, E.M. (1996). Dalteparin sodium: a review of its pharmacology and clinical use in the prevention and treatment of thromboembolic disorders. Drugs, 52(2): 276-306.

45. Ofusu, F.A., Buchanan, M.R., Anavari, N., Smith, L.M. and Blajchman, M.A. (1989). Plasma anticoagulant mechanisms of heparin, heparan sulphate and dermatan sulphate, Ann N Y Acad Sci, 556: 123-131.

46. Eikelboom, J.W. and Weitz, J.I. (2010). New anticoagulants. Circulation, 121(13): 1523-1532.

47. Weitz, J.I. and Gross, P.L. (2012). New oral anticoagulants: which one should my patient use?. Hematology, 1: 536-540.

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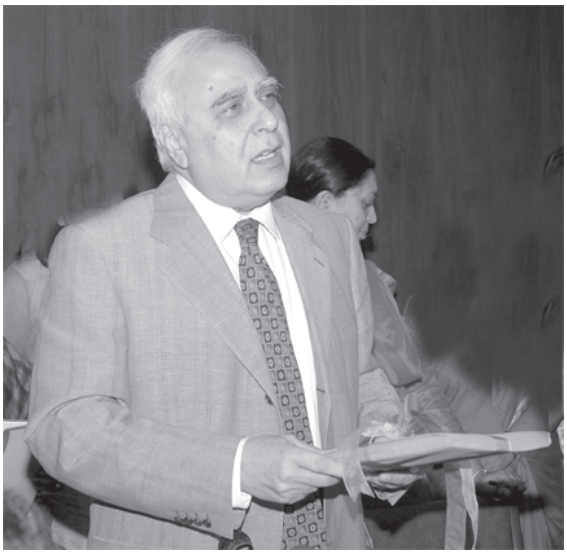
NEWS ITEM

DBT given dedicated efforts in promoting biotechnology in India : Shri - Kapil Sibal

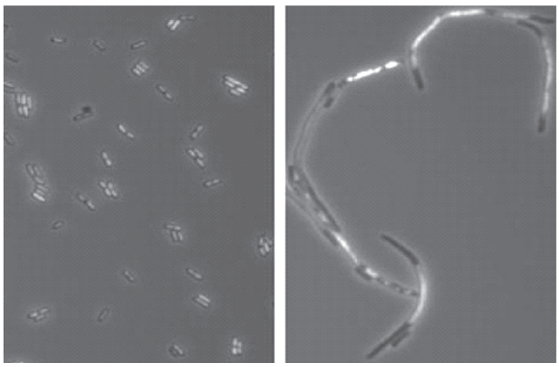


The 21st Foundation day and award distribution ceremony of the Department of Biotechnology (DBT) was celebrated on 12th March, 2007 at the National Centre for Plant Genome Research (NCPGR), JNU, New Delhi. Presided over by Shri Kapil Sibal, Hon'ble Minister for Science & Technology and Earth Sciences, the function was attended by representatives of foreign missions in India, former Secretaries of DBT and other senior officers of various scientific departments.Dr. M. K. Bhan, Secretary, DBT, took the opportunity to address the gathering and assure them that the Department would continue to make efforts for the country to be recognized as a global leader in the field of biotechnology.

The Hon'ble Minister presented the Biology Scholarships for the year 2006 to 56 students who topped in the CBSE and other Board Examinations for year 2005-06 and pursuing Biology as a main subject at graduate level. Thereafter, National Bio-scientist Award for Career Development, Young and Senior Women Bioscience Awards, Innovative Young Biotechnologists Award, and Distinguished Biotechnologist Awards were also presented to the awardees.The occasion was also used for the commercial launch of the Filarial Antibody Spot/ Immunoblot Assay System for Rapid Detection of Filariasis developed by the Centre for Biotechnology, Anna University (Tamil Nadu). Shri Kapil Sibal in his address appreciated efforts of DBT and its staff members for their untiring and dedicated efforts in promoting biotechnology in India through various schemes and programs. He also stressed the need for expanding the physical and technical resources of DBT in the near future for facing new challenges.

SCIENTIFIC NEWS

Breakdown in DNA copying process can cause cancer



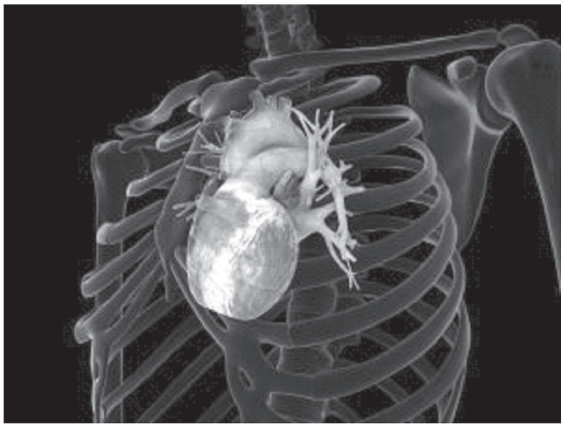
Research led by a scientist at the University of York has thrown new light on the way breakdowns in the DNA copying process inside cells can contribute to cancer and other diseases. Professor McGlynn, who was one of 16 Chairs established at York to mark the University's 50th Anniversary, says: "Our work demonstrates that when organisms try to copy their genetic material, the copying machines stall very frequently which is the first step in formation of mutations that, in man, can cause cancers and genetic disease. They have analysed what causes most of these breakdowns and how, under normal circumstances, cells repair these broken copying machines. Just as importantly, Their work reveals that efficient repair of these breakdowns is very important to avoid corruption of the genetic code.

- P. Suresh Kumar

Meis 1 regulates heart's ability to regenerate after injuries

Researchers at UT Southwestern Medical Center have identified a specific gene that regulates

the heart's ability to regenerate after injuries. Dr. Hesham Sadek, assistant professor of internal medicine in the division of cardiology, and senior author of the study said that they found that the activity of the *Meis1* gene increases significantly in heart cells soon after birth, right around the time heart muscle cells stop dividing. In their observation they found that if the *Meis1* gene is deleted from the heart, heart cells continue to divide through adulthood. The research team demonstrated that deletion of Meis1 extended the proliferation period in the hearts of

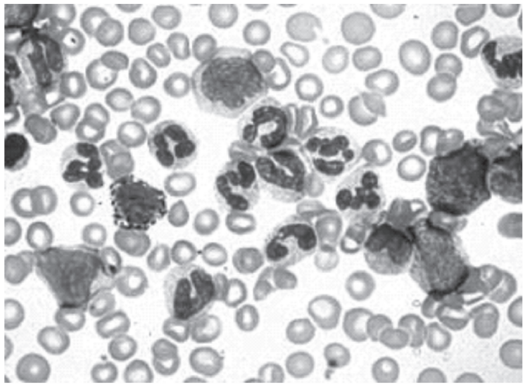


newborn mice, and also re-activated the regenerative process in the adult mouse heart without harmful effect on cardiac functions. This new finding demonstrates that *Meis1* is a key factor in the regeneration process, and the understanding of the gene's function may lead to new therapeutic options for adult heart regeneration. The findings also provide a possible alternative to current adult heart regeneration research, which focuses on the use of stem cells to replace damaged heart cells. *Meis1* is a transcription factor, which acts like a software program that has the ability to control the function of other genes. Dr. Sadek said. As such, *Meis1* could possibly be used as an on/off switch for making adult heart cells divide.

- Anmol Kumar

Enzyme accelerates malignant stem cell cloning in chronic myeloid leukemia

An international team, headed by researchers at the University of California, San Diego School of Medicine, has identified a key enzyme in the reprogramming process that promotes malignant stem cell cloning and the growth of chronic myeloid leukemia (CML), a cancer of the blood and marrow that experts say is increasing in prevalence. Despite the emergence of new therapies, such as tyrosine kinase inhibitors, CML and other leukemias remain problematic because some cancer stem cells avoid destruction and eventually regenerate themselves, a stem cell process known as self-renewal that can result in a return and spread (metastasis) of the disease. It



expressed during embryogenesis to help blood cell development, ADAR1 subsequently turns off and is triggered by viral infections where it protects normal hematopoietic stem cells from attack. In leukemia stem cells, however, overexpression of ADAR1 enhances the missplicing of RNA, which leads to greater self-renewal and therapeutic resistance of malignant stem cells. The findings build upon previous studies by Jamieson and others that elucidate the effects of RNA missplicing and instability. "People normally think about DNA instability in cancer, but in this case, it's how the RNA is edited by enzymes that really matters in terms of cancer stem cell generation and resistance to conventional therapy. The described RNA editing process, which occurs in the context of human and other primate specific sequences, also underscores the importance of addressing inflammation as an essential driver of cancer relapse and therapeutic resistance. It also presents a new target for future therapies. CML is a cancer initiated by a mutant gene called BCR-ABL in blood forming stem cells that leads to an expansion of white blood cells and their precursors. It is typically slow-growing and often not diagnosed until its later stages when there can be a sudden, dramatic increase in malignant cells, known as blast crisis. Median age of diagnosis is 66 years; incidence of the disease increases with age. Despite tremendous advances in BCR-ABL tyrosine kinase inhibitor therapies, the majority of patients relapse if therapy is discontinued, in part as a result of dormant cancer stem cell resistance. This work suggests a novel mechanism for overcoming cancer stem cell

resistance to therapy that may prevent relapse and progression. The estimated prevalence of CML in the United States is 70,000 persons with the disease, projected to steadily increase to approximately 181,000 by 2050. CML is initiated by the mutant BCR-ABL gene, but scientists have not yet identified the cause of the mutation.

The tulip tree reveals mitochondrial genome of ancestral flowering plant

The extraordinary level of conservation of the tulip tree (Liriodendron tulipifera) mitochondrial genome has redefined our interpretation of evolution of the angiosperms (flowering plants). This beautiful 'molecular fossil' has a remarkably slow mutation rate meaning that its mitochondrial genome has remained largely unchanged since the dinosaurs were roaming the earth.

Evolutionary studies make used of mitochondrial (powerhouse) genomes to identify maternal lineages, for example the human mitochondrial eve. Among plants, the lack of genomic data from lineages which split away from the main evolutionary branch early on has prevented researchers from reconstructing patterns of genome evolution. *L. tulipifera* is native to North America. It belongs to a more unusual group of dicotyledons (plants with two seed leaves) known as magnoliids, which are thought to have diverged early in the evolution of flowing plants. By sequencing the mitochondrial genome of *L. tulipifera,* researchers from Indiana University and University of Arkansas discovered that its mitochondrial genome has one of the slowest silent mutation rates (ones which do not affect gene function) of any known genome. Compared to humans the rate is 2000 times slower – the amount of genomic change in a single human generation would take 50,000 years for the tulip tree. The rate is even slower for magnolia trees, taking 130,000 years for the same amount of mitochondrial genomic change. Ancestral gene clusters and tRNA genes have been preserved and *L. tulipifera* still contains many genes lost during the subsequent 200 million years of evolution of flowering plants. In fact one tRNA gene is no longer present in any other sequenced angiosperm. Prof Jeffrey Palmer who led this study explained that by using the tulip tree as a guide we are able to estimate that the ancestral angiosperm mitochondrial genome contained 41 protein genes, 14 tRNA genes, seven tRNA genes sequestered from chloroplasts, and more than 700 sites of protein editing. Based on this, it appears that the genome has been more-or-less frozen in time for millions and millions of years.

- Prudvi

EDUCATION

Ph.D., position in immunology @ University of Oslo : The applicants must hold a master degree in biology or a degree in medicine with a good knowledge of molecular biology, cell biology and immunology is mandatory with Previous experience with relevant research. Applicants with a medical background will have an advantage. Aquaintance with Norwegian language is not required, but successful applicants with good Norwegian skills are eligible for a 4-year program period requiring teaching of medical students (25%). Excellent command of written and oral English is required with Good collaboration skills. Salary ranging from pay grade: 50 – 55 from NOK 416 600 – 451 900 Stimulating and attractive work environment. Membership in the Norwegian Public Service Pension Fund with Attractive welfare benefits**.** The application should attached with Application letter (including a summary of the applicant's scientific work and interests and describing how she/he fits the description of the person we seek), CV (education since high school (place, grade), positions held and details of academic work), list of publications, published abstracts, presentations, copies of educational certificates and transcripts of records, names and contact details of 2-3 referees (name, relation to candidate, e-mail and telephone number) Please also refer to the regulations pertaining to the conditions of employment for research fellowship positions (English translation): http://www.uio.no/admhb/reglhb/personal/

tilsettingvitenskapelig/ansettel... The application with attachments is to be delivered in our electronic recruiting system EasyCruit. Foreign applicants are advised to attach an explanation of their University's grading system. Please remember that all documents should be in English or a Scandinavian language.

OPPORTUNITIES

SRF vacancy at IARI, Regional Station, Kullu invited for Walk-in-interview on 08.05.13 for the posts of SRF, JRFand Field worker to be held on following dates and time at the office of IARI, Regional Station, Katrain, Kullu, HP-175 129 (visit: www.iari.res.in) .Project entitled 'Central sector scheme for implementation of PPV & FRA legislation' under Dr. Chander Parkash Essential Qualification : M.Sc. (Agriculture/ Horticulture / Genetics / Plant Breeding/ Botany/Biotechnology) with Age limit 28 Years Work experience in vegetable improvement / production/ seed production are Desirable. The Duration of the project up to 31st March, 2014 or till termination

Max-Planck Society & DST recruiting JRF at IISER Mohal seeking Applications are invited from Indian nationals to work as Junior Research Fellow (JRF) in a research project funded jointly by the Max-Planck Society, Germany and Department of Science and Technology, India. the Title of the project is Study of RNA splicing using fission yeast Schizosachharomyces pombe. The applicant must work on role of ubiquitin-related modifiers in regulation of RNA splicing. Duration of the project is 2 years (extendable to 3 years). all Emoluments given As per DST rules Essential Qualifications are M.Sc. in any branch of Life Sciences. The applicants should have prior experience working with yeast or RNA splicing or ubiquitin-like proteins. The complete application should send to E-mail: skmishra@iisermohali.ac.in on or befor 1st , May 10, 2013

DST-INSPIRE Faculty Scheme at Delhi Institute of Pharmaceutical Sciences & Research invites applications from eligibl candidates for pre-selection under the DST-INSPIRE Faculty Scheme (Institution mode) . This Scheme has been designed by the Department of Science and Technology, Government of India (DST) to provide contractual research positions to young achievers for independen research and to emerge as future S T leaders. The applicant should have below the age of 32 (35 year for SC/ST candidates) Holding a Ph.D. degree may be considered for a contractual appointment in DIPSAR as DST-INSPIRE Faculty Fellow in areas supported by DST unde this scheme. The amount, duration and conditions of fellowship shall be as per the DST guidelines at http:/ Nrww.inspire-dst.gov.in/faculty scheme.html, which should be carefully read by the candidate before applying.A hard copy of the application, together with self-attested photocopies of all marks sheets and certificates etc should reach Director, Delhi institute of Pharmaceutical Sciences and research, New Delhi-110017 till 30/4/2013. The envelope should be marked Application under INSPIRE Faculty Scheme'. Names of the pre-selected candidates will be forwarded to DST for final decision. The appointment will be strictly as per the terms and conditions of the DST-INSPIRE Faculty Programme. The last date of sending application is 30.04.13

SEMINARS/WORKSHOPS /CONFERENCES

First International and Third National Conference on Biotechnology, Bioinformatics and Bioengineering is an essential annual event of Society for Applied Biotechnology for industry leaders, policy makers, scientists, researchers and other professionals working in the area of biotechnology, bioinformatics and bioengineering. This conference informs scientific community of advances, barriers, opportunities, dealing with challenges and action options through direct exchange of information which may influence science and business endeavors on an international and national scale. Researchers are invited to submit abstract(s) for oral and poster presentation. Abstracts are invited for oral and poster presentation in the technical sessions of the conference. Submission of Abstract - 25 May 2013, Notification of Final Acceptance - 28 May 2013, early-bird registration - 31 May 2013

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ALLIANCE INSTITUTE OF ADVANCED PHARMACEUTICAL AND HEALTH SCIENCES

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Alliance, located conveniently in the heart of Hyderabad, trains industry-ready graduates by bridging education with industry needs in pharmaceutical sciences. Alliance's visionary management built state of the art facilities and laboratories to provide quality education meeting national and international standards.

Collaboration with JNTUH, India

Alliance is having collaboration with **Jawaharlal Nehru Technological University, Hyderabad (JNTUH)**, which is a premier institution with academic and research–oriented programs, offered through the constituent and affiliated colleges. Alliance's syllabi, academic regulations and course structure are **approved by the JNTUH. JNTUH awards the degrees after fulfilling the degree requirements.**

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University of the Pacific, ranks in the top 100 among the 3000 national universities in the United States. Alliance has entered into research collaboration with Thomas J Long School of Pharmacy and Health Sciences, University of the Pacific.

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