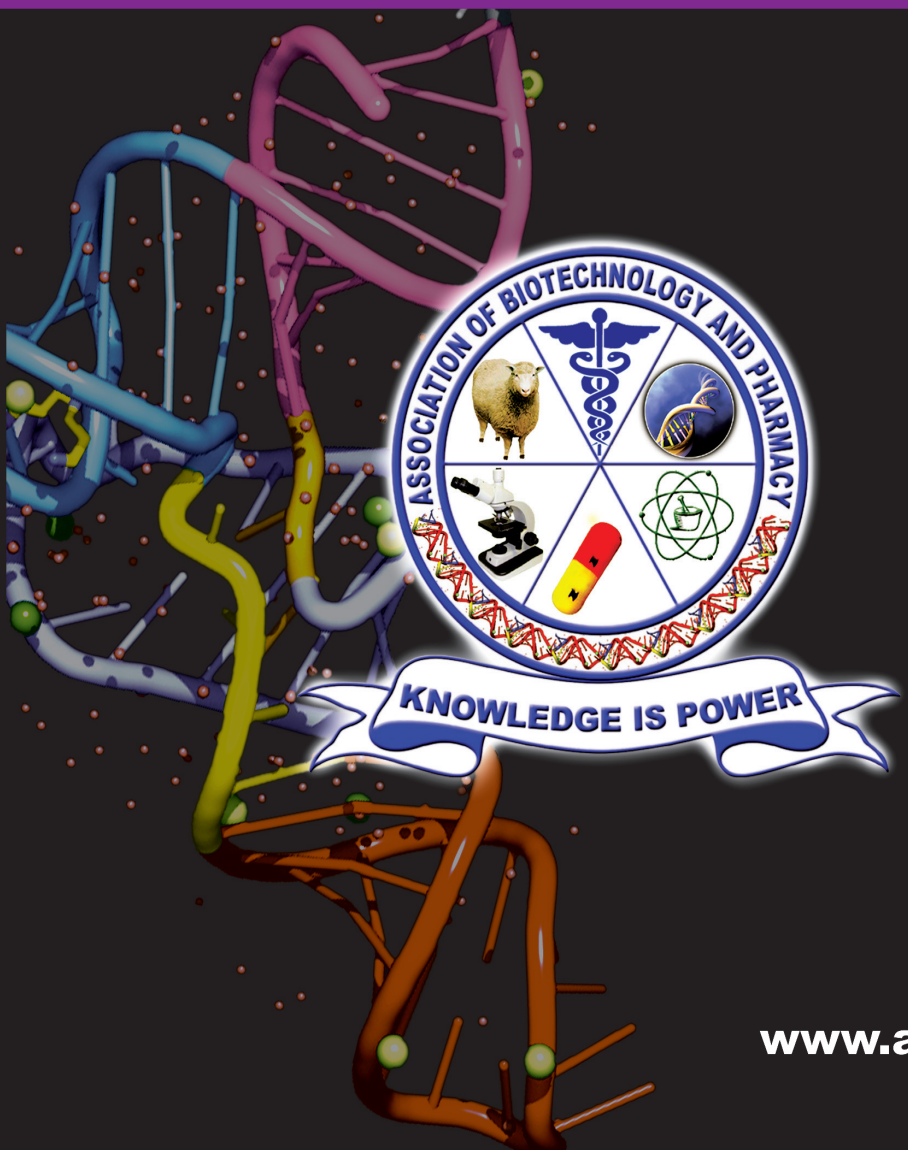
ISSN 0973-8916

Current Trends in Biotechnology and Pharmacy

Volume 5 Issue 1 January 2011



www.abap.co.in

Current Trends in Biotechnology and Pharmacy
ISSN 0973-8916 (Print), 2230-7303 (Online)

Editors

Prof. K.R.S. Sambasiva Rao, India
krssrao@abap.co.in

Prof. Karnam S. Murthy, USA
skarnam@vcu.edu

Editorial Board

Assistant Editors

(Electronic Version)
www.abap.co.in

ISSN 0973-8916

Current Trends in Biotechnology and Pharmacy

(An International Scientific Journal)

Volume 5 Issue 1 Januray 2011

www.abap.co.in

Indexed in Chemical Abstracts, EMBASE, ProQuest, Academic Search™, DOAJ, CAB Abstracts, Index Copernicus, Ulrich's Periodicals Directory, Open J-Gate Pharmoinfonet.in Indianjournals.com and Indian Science Abstracts.

Current Trends in Biotechnology and Pharmacy
Vol. 5 (1) January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

Association of Biotechnology and Pharmacy

(Regn. No. 28 OF 2007)

The *Association of Biotechnology and Pharmacy (ABAP)* was established for promoting the science of Biotechnology and Pharmacy. The objective of the Association is to advance and disseminate the knowledge and information in the areas of Biotechnology and Pharmacy by organising annual scientific meetings, seminars and symposia.

Members

The persons involved in research, teaching and work can become members of Association by paying membership fees to Association.

The members of the Association are allowed to write the title ***MABAP*** (Member of the Association of Biotechnology and Pharmacy) with their names.

Fellows

Every year, the Association will award Fellowships to the limited number of members of the Association with a distinguished academic and scientific career to be as Fellows of the Association during annual convention. The fellows can write the title ***FABAP*** (Fellow of the Association of Biotechnology and Pharmacy) with their names.

Membership details

(Membership and Journal)	India	SAARC	Others
Individuals - 1 year	Rs. 600	Rs. 1000	$100
LifeMember	Rs. 4000	Rs. 6000	$500
Institutions - 1 year	Rs. 1500	Rs. 2000	$200
(Journal only) Life member	Rs. 10000	Rs.12000	$1200

Individuals can pay in two instalments, however the membership certificate will be issued on payment of full amount. All the members and Fellows will receive a copy of the journal free

Association of Biotechnology and Pharmacy
(Regn. No. 28 OF 2007)
#5-69-64; 6/19, Brodipet
Guntur - 522 002, Andhra Pradesh, India

Current Trends in Biotechnology and Pharmacy
Vol. 5 (1) January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

Current Trends in Biotechnology and Pharmacy

ISSN 0973-8916

Volume 5 (1) **January - 2011**

CONTENTS

Current Trends in Biotechnology and Pharmacy
Vol. 5 (1) January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

Information to Authors

The *Current Trends in Biotechnology and Pharmacy* is an official international journal of *Association of Biotechnology and Pharmacy*. It is a peer reviewed quarterly journal dedicated to publish high quality original research articles in biotechnology and pharmacy. The journal will accept contributions from all areas of biotechnology and pharmacy including plant, animal, industrial, microbial, medical, pharmaceutical and analytical biotechnologies, immunology, proteomics, genomics, metabolomics, bioinformatics and different areas in pharmacy such as, pharmaceutics, pharmacology, pharmaceutical chemistry, pharma analysis and pharmacognosy. In addition to the original research papers, review articles in the above mentioned fields will also be considered.

Call for papers

The Association is inviting original research or review papers in any of the above mentioned research areas for publication in Current Trends in Biotechnology and Pharmacy. The manuscripts should be concise, typed in double space in a general format containing a title page with a short running title and the names and addresses of the authors for correspondence followed by Abstract (350 words), 3 to 5 key words, Introduction, Materials and Methods, Results and Discussion, Conclusion, References, followed by the tables, figures and graphs on separate sheets. For quoting references in the text one has to follow the numbering of references in parentheses and full references with appropriate numbers at the end of the text in the same order. References have to be cited in the format below.

Mahavadi, S., Rao, R.S.S.K. and Murthy, K.S. (2007). Cross-regulation of VAPC2 receptor internalization by m2 receptors via c-Src-mediated phosphorylation of GRK2. Regulatory Peptides, 139: 109-114.

Lehninger, A.L., Nelson, D.L. and Cox, M.M. (2004). Lehninger Principles of Biochemistry, (4^{th} edition), W.H. Freeman & Co., New York, USA, pp. 73-111.

Authors have to submit the figures, graphs and tables of the related research paper/article in Adobe Photoshop of the latest version for good illumination and allignment.

Authors can submit their papers and articles either to the editor or any of the editorial board members for onward transmission to the editorial office. Members of the editorial board are authorized to accept papers and can recommend for publication after the peer reviewing process. The email address of editorial board members are available in website www.abap.in. For submission of the articles directly, the authors are advised to submit by email to krssrao@abap.co.in or editor@abap.co.in

Authors are solely responsible for the data, presentation and conclusions made in their articles/ research papers. It is the responsibility of the advertisers for the statements made in the advertisements.

Current Trends in Biotechnology and Pharmacy
Vol. 5 (1) 972-981 January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

Ethosomes: A Tool for Transdermal Drug Delivery

Ambekar Abdul Wahid*[1], Nagaraju Ravouru[2] and Sawant Ramesh Lakshman[1]
[1]Department of Pharmaceutics, P.D.V.V.P.F's College of Pharmacy, Vilad Ghat M.I.D.C, Ahmednagar – 414111 (M.S), India
[2]Department of Pharmaceutics, Institute of Pharm. Technology, Sri Padmavati Mahila Visvavidyalayam (Women's University) Tirupati, A.P., India
* For Correspondence – wahidambekar@gmail.com

Abstract

Since skin offers an excellent barrier to molecular transport, as stratum corneum is the most formidable barrier to the passage of most of the drugs, except for highly lipophilic, low molecular weight drugs. Various attempts have been made for enhanced drug delivery into the body through the intact skin including using lipid vesicles like liposome, niosomes etc. Classic liposomes are of little or no value as carriers for transdermal drug delivery because they do not deeply penetrate the skin. One of the major advances in vesicle research is the finding that some specially designed vesicles possessed properties that allowed them to successfully deliver drugs in deeper layer of skin. Ethosome is one of the specially designed lipid carrier recently developed by Touitou *et al*, showing enhanced skin delivery. Ethosome are characterized by prolong physical stability with respect to liposomes.

Key Words: Stratum corneum (SC), Liposome, Niosome, Classic Liposome, Ethosome.

Introduction

Transdermal delivery of drugs through the skin to the systemic circulation offers many advantages as compared to traditional drug delivery systems such as increased patient acceptability, avoidance of gastrointestinal disturbances and first pass metabolism of the drug (1, 2). Although the skin as a route for drug delivery can offers many advantages, the barrier nature of the skin makes it difficult for most drugs to penetrate into and permeate through it (3). During the past decades there has been wide interest in exploring new techniques to overcome the stratum corneum barrier (4, 5). Various physical and chemical methods has been investigated to overcome stratum corneum barriers such as iontophoresis, sonophoresis, radio frequency, electroporation, lipid vesicles like liposomes, niosomes, transferosomes etc (6,7,8).

Drug encapsulated in lipid vesicles prepared from phospholipids and nonionic surfactant serves as nontoxic penetration enhancer for drugs due to its amphiphilic nature. Lipid rich vesicles like liposome and niosomes can be used for encapsulation hydrophilic and lipophilic as well as low and high molecular weight drugs (9,10) However these lipid rich vesicles has a problem of poor skin permeability. Introduction of ethosomes, another novel lipid carrier developed by Touitou *et al* has shown enhanced skin permeation as compared to conventional liposomes (2, 11)

Ethosomes

Ethosomes were developed by Touitou *et al* as noninvasive vesicular carriers very similar

to liposomes produced in the presence of ethanol comprise of hydroalcoholic or hydro/alcoholic/ glycolic phospholipids with high concentration of alcohol may be upto 50 % (2, 6, 12, 13, 14).

The ethosomal system consists of phospholipids, ethanol and water (15) The phospholipids with various chemical structure includes phosphatidylcoholine (PC), hydrogenated PC, phosphatidylethanolamine (PE), phosphatidy lglycerol (PPG), phosphatidylinositol (PI), hydrogenated PC etc (16). The nonaqueous phase range between 22 % to 70 %. The alcohol may be ethanol or isopropyl alcohol. Dyes or amphiphilic fluorescent probe such as D – 289, Rhodamine – 123 , fluorescence isothiocynate (FITC), 6 – carboxy fluorescence are often added to ethosomes for characterization study (3, 17,18).

Effect of high alcohol concentration: Ethanol is an established permeation enhancer and is proposed that it fluidizes the ethosomal lipids and stratum corneum bilayer thus allowing the soft, malleable vesicles to penetrate the disorganized lipid bilayer (8). The relatively high concentration of ethanol (20 – 50 %) is the main reason for better skin permeation ability and is packed less tightly than conventional vesicles but has equivalent stability and better solubility of many drugs (3,19). Moreover the vesicular nature of ethosomal formulation could be modified by varying the components ratio and phospholipids (20). Ethanol confers a surface negative net charge to the ethosome which causes the size of vesicles to decrease. The size of ethosomal vesicles increase with decreasing ethanol concentration (3).

Advantages of ethosomes

1. Enhanced permeation of drug molecules to and through the skin to the systemic circulation (3, 14).
2. Contrary to deformation liposomes, ethosomes improve skin delivery of drugs both under occlusive and non-occlusive conditions (22).
3. Since composition and components of ethosomes are safe, they have various applications in pharmaceutical, veterinary and cosmetic field.
4. Better patient compliance.
5. Better stability and solubility of many drugs as compared to conventional vesicles.
7. Relatively smaller size as compared to conventional vesicles.

Limitations of ethosomes

1. Poor yield (23).
2. In case if shell locking is ineffective then the ethosomes may coalescence and fall apart on transfer into water.
3. Loss of product during transfer form organic to water media (24)

Methods of preparation ethosomes: Ethosomes can be prepared by two very simple and convenient methods that is hot method (Fig 1 and Fig 2) and cold method (Fig 3) (16, 17, 18, 25)

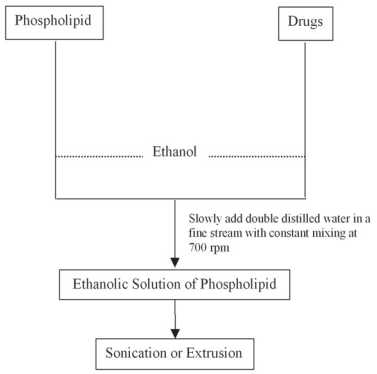


Fig.1. General method for the preparation of ethosomes

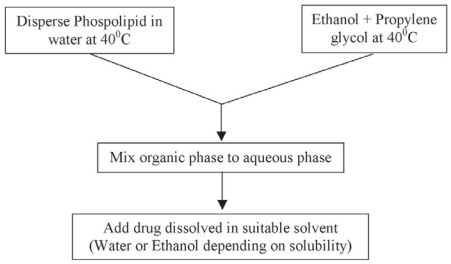


Fig.2. Hot method for the preparation of ethosomes

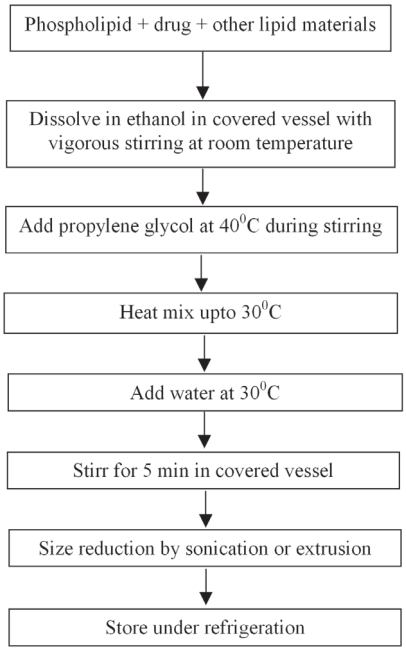


Fig.3. Cold method for the preparation of ethosomes

Various methods of characterization of ethosomes

1. Surface morphology: Transmission electron microscope (TEM) and Scanning electron microscope (SEM) can be used for vesicular shape and surface morphology studies. TEM can be performed using phosphotungstic acid as negative stain (26)

2. Vesicle size and size distribution: Ethosome size and size distribution can be done by dynamic light scattering method (DLS) using computerized inspection system (27, 28).

3. Entrapment efficiency: The ability of ethosomes to efficiently entrap lipophilic and hydrophilic drugs can be measured by ultracentrifugation technique, mini column centrifugation method and fluorescence spectrophotometry (20, 29, 30).

4. Glass transition temperature: The glass transition temperature of the vesicular lipid systems can be determined by using differential scanning calorimetry (DSC) (31).

5. Interfacial tension activity measurement: Interfacial Surface tension activity of ethosomes can be measured in aqueous solution by Du Nouy ring tensiometer (32).

6. Turbidity: It can be measured by nephalometer (14).

7. Vesicle skin interaction study: Vesicle skin interaction study can be done by examining under transmission electron microscopy or confocal laser scanning microscope (CSLM) or fluorescence microscope or eosin – hematoxylin staining. For fluorescence microscopy ethosomes should be loaded with fluorescence marker like rhodamine 123 (20, 27).

7. Degree of deformability or Elasticity measurement: The elasticity of ethosome vesicle membrane can be determined by extrusion method. The ethsomal formulation are extruded through filter membrane (pore diameter 50 nm) using stainless steel filter holder of diameter 25 nm, by applying a pressure of 2.5 bar (32).

8. Zeta potential: zeta potential can be measured by zeto meter or dynamic light scattering method (DLS) (20).

9. Phospholipid – ethanol interaction: Phospholipid – ethanol interaction can be assessed by ^{31}P NMR or by differential scanning calorimeter (DSC) (20).

10. Drug content: Drug content of ethosomal formulation can be quantified by a modified high performance liquid chromatographic technique (HPLC) (27).

11. Stability study: Dynamic light scattering method or transmission electron microscope can be used for assessing the stability of ethosomes (33).

12. Penetration and permeation studies: Depth of skin penetration from ethosome can be determined by confocal laser scanning microscope (CLSM) (34).

13. *In vitro* skin permeation/ deposition study: The in vitro permeation characteristic of ethosomal formulation can be done by using franz diffusion cell with artificial or dialysis bag diffusion or biological membranes such as heat separated human epidermis, human epidermis, male nude mouse abdominal skin, rat abdominal skin, rabbit pinna skin, dermatomed cadaver human skin and rat skin (14,29,35,36,37).

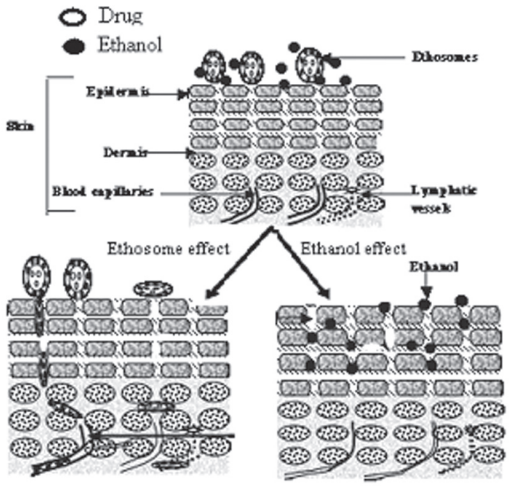


Fig.4. Mode of action of ethosomes

Mode of action of ethosomes

The lipid multilayer, at physiological temperature is densely packed and highly conformational order and hence main barrier for permeation of drugs (38). The enhanced delivery of drugs using ethosomes can be due to interaction between ethosomes and skin lipids. The possible mechanism of interaction is due to "ethanol effect" and "ethosome effect" (39). Ethanol increases the fluidity and decreases the density of lipid molecules by interacting with lipid molecules in the polar head group region which in turn result in increased permeability. Ethosomes effect include penetration of flexible ethosome vesicle through disturbed stratum bilayers and opening of new pathways due to the malleability and fusion of ethosomes with skin lipids, resulting in the release of the drug in deep layers of the skin (3,16,20,22,40).

Application (Table 1)

1. Pilosebaceous targeting: Pilosebaceous units have been use for localized therapy, particularly for the treatment of follicle related disorders such as acne or alopecia. Ethosomal formulation of minoxidil a lipid soluble drug used for baldness accumulate into nude mice skin two to seven fold higher and thus can be use for pilosebaceous targeting for better clinical efficacy (2,20).

2. Transdermal delivery: Since ethosomes enhance permeability of drug through stratum corneum barrier, it can be use for administration of drugs having poor skin permeation, low oral bioavability, first pass metabolism and dose

Table 1 : Applications of ethosomes

Drugs	**Result**
Anti- viral agents (Zidovudine) (41) (Lamivudine)(27) (Stavudine) (42)	Prolonged drug action, reduced drug toxicity. Control release for prolonged period of time. Improved biological anti-inflammatory activity, sustained effect.
NSAIDS (17,18) (Diclofenac) (Aceclofenac)(43)	Selective and prolong delivery of drug to desired site. Superior to the marketed gel for the topical administration.
Acyclovir (4)	Increased skin permeation and biological activity two to three times.
Topical (44) Photodynamic Therapy (PDT) (5- aminolevulinic acid)	Greater penetration ability than that of liposomes, More entrapment efficiency
Insulin (14,45)	Significant decrease in blood glucose level.
Trihexyphenidyl Hydrochloride(14)	Higher entrapment capacity, improved tansdermal flux, improved patient compliance.
Antibiotic (46) (Erythromycin) (Cannabidol)	Complete inhibition of infection, prolonged drug action. Improved skin deposition and biological activity.
Pilosebaceous (2) Targeting (Minoxidil)	High penetration into deep layers of the skin.
Ammonium (29) glycrrhizinate	Improved biological anti-inflammatory activity, sustained effect.
Salbutamol sulfate (47)	Controlled release rate, enhanced skin permeation.
Propranolol (35)	Better skin permeation.
Testosterone (48)	Significantly higher permeation into the skin increased systemically delivery.
Finasteride (49)	Enhanced percutaneous absorption.
Bacitracin (50)	Reduced drug toxicity.
Methotrexate (51) (MTX)	Enhanced transdermal flux, lower lag time, higher entrapment efficiency and better stability profile
Gold Nanopartical (52)	Gold nanopartical in ethosomes shows enhancement of pharmacological efficacy in transdermal and dermal delivery systems.

dependent side effect. Touitou *et al* reported that the skin permeation of testosterone from ethosomal formulation is nearly 30 times higher than the marketed transdermal patch of testosteronen (Testosterone Patch, Alza). They also concluded that the ethosomal testosterone formulation area of application required to produce the effective plasma concentration was 10 times less than required by commercially gel formulation (15, 20).

3. Topical delivery of DNA: Another important application of ethosomes is their use for topical delivery of DNA molecules. Touitou *et al* demonstrated that better intracellular uptake of DNA, better delivery and expression of genes in skin cells can be achieved by ethosomal formulation (51) Hence was concluded that ethosomes can be used carrier for gene therapy application that require transient expression of genes.

4. Delivery of antiarthritis drug: Arthritis treatment is associated with problems like low bioavailability; first pass metabolism, GIT degradation etc. To overcome above problems ethosomal formulation of antiarthritis drugs can be an alternative as it significantly increase skin permeation, accumulation and biological activity (21)

5. Delivery of antibiotics: Conventional oral therapy of antibiotics is usually associated with several allergic reactions along with side effects and low therapeutic efficacy. Topical delivery of antibiotics is a better choice to increase therapeutic efficacy, but conventional topical preparation possess low permeability to deep skin layers and subdermal tissues. Ethosomes formulation of antibiotics could be highly efficient and over come the problems associated with conventional therapy since they penetrate rapidly into deeper layer of skin and suppress infection at their root (20, 45, 54).

6. Delivery of HIV drugs: An effective antiretroviral therapy is required on a long term basis and is associated with strong side effects (38). Adequate zero order delivery of zidovudine, Lamivudine a potent antiviral agent is required to maintain expected anti – AIDS effect. Subheet Jain *et al* reported that ethosomal formulation of the above drugs prolong the release with increased transdermal flux (28, 29). Conventional topical preparation acyclovir an topically used antiviral drug for treatment of herpes labials show low therapeutic efficiency due to poor permeation through skin as replication of virus take places at the basal dermis. Ethosomal formulation of acyclovir show high therapeutic efficiency with shorter healing time and higher percentage of abortive lesions.

7. Delivery of problematic drug molecules: Oral delivery of large biogenic molecules such as peptides or proteins and insulin is difficult because they are completely degraded in the GIT tract hence transdermal delivery is a better alternative. But conventional transdermal formulation of biogenic molecules such as peptides or protein and insulin has poor permeation. Formulating these above molecules into ethosomes significantly increase permeation and therapeutic efficacy (55)

Patented and marketed formulation of ethosome: Ethosome was invented and patented by Prof. Elka Touitou along with her students of department of pharmaceutics at the Hebrew University School of Pharmacy (17, 18). Novel Therapeutic Technologies Inc (NTT) of Hebrew University has been succeeded in bringing a number of products to the market based on ethosome delivery system. Noicellex ™ an anti – cellulite formulation of ethosome is currently marketed in Japan. Lipoduction ™ another

formulation is currently used in treatment of cellulite containing pure grape seed extracts (antioxidant) is marketed in USA (56). Similarly Physonics is marketing anti – cellulite gel Skin Genuity in London (57). Nanominox© containing monoxidil is used as hair tonic to promote hair growth is marketed by Sinere (58).

Future perspective: For transdermal delivery of drugs, stratum corneum is the main barrier layer for penetration of drug. Various methods have been discovered to enhanced skin penetration of drugs lipid vehicle based enhancement approach has drawn considerable interest in recent past. Studies will continue further to improve skin delivery of drug using lipid vesicles.

Introduction of ethosomes has initiated a new area in vesicular research. Ethosomes has shown promising result and potential for delivery of various agents more effectively. Better control over drug release, non – invasive delivery of small, medium and large size drug molecules can be achieved by ethosomes. Ethosomes can be the promising tool for dermal/transdermal delivery of various agents and can be a alternate formulation for problematic drugs.

Reference:

1. Benson, H.A.E. (2005). Transdermal drug delivery: penetration enhancement techniques. Current Drug Delivery, 2: 23 – 33.
2. Toutitou, E., Dayan, N., Bergelson, L., Godin, B. and Eliaz, M. (2000). Ethosomes novel vesicular carriers for enhanced delivery: characterization and skin penetration properties. Journal of Control Release, 65:403 – 418.
3. Mustafa, M.A.E., Ossama, Y.A., Viviane, F.N. and Nawal, M.K. (2007). Lipid vesicles for skin delivery of drugs. Reviewing three decades of research. International Journal of Pharmaceutics, 332: 1 – 16.
4. Barry, B.W. (2001). Novel mechanism and devices to enable successful Transdermal drug delivery. European Journal of Pharmaceutical Sciences, 14 (2): 101 – 114.
5. Honeywell – Nguyen, P.L. and Bouwatra, J.A. (2005). Vesicles as a tool for transdermal and dermal delivery. Drug Discovery Today: Technologies, 2: 67 – 74.
6. Touitou, E. (2002). Drug delivery across skin. Expert Opinion on Biological Therapy, 2 (7):723 – 733.
7. Ranade, V. (1991). Drug delivery system: Transdermal drug delivery. The Journal of Clinical Pharmacology, 31(6): 401 – 418.
8. Dinesh, D., Amit, A.R., Maria, S., Awaroop, R.L. and Moh. Hassan G.D. (2009). Drug vehicle based approaches of penetration enhancement. International Journal of Pharmacy and Pharmaceutical Sciences, 1 (1): 24 – 45.
9. Schreier, H. and Bovwstra, J. (1994). Liposomes and niosomes as topical drug carriers: dermal and transdermal drug delivery. Journal of Control Release, 30(1): 1 – 15.
10. Touitou, E., Junginger, H.E., Weiner, N.D., Nagai, T. and Mezei, M. (1994). Liposomes as carriers for topical and transdermal delivery. Journal of. Pharmaceutical Sciences, 83: 1189 -1203.
11. Touitou, E., Godin, B. and Weiss, C. (2000). Enhanced delivery of drugs into and across the skin by ethosomal carriers. Drug Development Research, 50: 406 – 415.
12. Touitou, E. and Godin, G. (2008). Skin nonpenetrating sunscreens for cosmetic and

pharmaceutical formulations. Clinics in Dermatology, 26: 375 -379.

13. Touitou, E., Godin, B., Dyan, N. and Weiss, C., Piliponsky, A. and Levi – Scha, F. (2001). Intracellular delivery mediated by an ethosomal carrier. Biomaterials, 22: 3053 – 3059.

14. Dayan, N. and Touitou, E. (2000). Carriers for skin delivery of triexphenidyl HCl: Ethosome Vs Lipsomes. Biomaterials, 21: 1879 – 1885.

15. Touitou, E. (2002). Drug delivery across skin. Expert Opinion on Biological Therapy, 2: 723 – 733.

16. Vyas, S.P. (2009). Theory and practice in novel drug delivery system, (1st edition), CBS Publication and Distributors. New Delhi, pp. 259 – 261.

17. Touitou, E. (1996). Composition of applying active substance to or through the skin. US Patent: 5716638.

18. Touitou, E. (1998). Composition of applying active substance to or through the skin. US Patent: 5540934.

19. Barry, B.W. (2001). Is Transdermal drug delivery research still important today. Drug Discovery Today, 6(19):967 – 971.

20. Biju, S.S., Sushama, T., Mishra, P.R. and Khar, R. K. (2006). Vesicular systems: An overview. Indian Journal of Pharmaceutical Sciences, 68(2): 141-153.

21. Pavan, K.K., Radhika, P.R. and Sivakumar, T. (2010). Ethosomes- A priority in transdermal drug delivery. International Journal of Advances in Pharmaceutical Sciences. 1: 111 – 121.

22. Elsayed, M.M., Abdallah, O.Y, Naggar, V.F. and Khalafallah, N.M. (2007). Deformable liposomes and ethosomes as carriers for skin delivery of ketotifen. Pharmazie, 62: 133 – 137.

23. Laib, S., and Routh, A.F. (2008). Fabrication of colloidosomes at low temperature for the encapsulation of thermally sensitive compounds. Journal of Colloid & Interface Science, 317: 121-129.

24. Swarnlata, S., Rahul, R., Chanchal, D.K., Shailendra, S. (2011). Colloidosomes an advanced vesicular system in drug delivery. Asian Journal of Scientific Research, 4(1): 1 – 15.

25. Verma, D.D. and Fahr, A. (2004). Synergistic penetration effect of ethanol and phospholipids on the topical delivery of cyclosporine A. Journal of Control Release, 97(1): 55 – 66.

26. Guo, J., Ping, Q., Sun, G. and Jiao, C. (2000). Lecithin vesicular carriers for Transdermal delivery of cyclosporine A. International Journal of Pharmaceutics, 194(2): 201 – 207.

27. Subjeet, J., Ashok, K.T., Bharti, S. and Narendra, K.J. (2007). Formulation and evaluation of ethosomes for transdermal delivery of lamivudine. APPS PharmSciTech, 8 (4): E1 – E9.

28. Maghraby, G.M.M., Williams, A.C. and Barry, B.W. (2000). Oestradiol skin delivery from ultradeformable liposomes: refinement of surfactant concentration. International Journal of Pharmaceutics, 196(1): 63 – 74.

29. Donatella, P., Giuseppe, L., Domenico, M., Franco, A. and Massimo, F. (2005). Ethosomes for skin delivery of ammonium glycyrrhizinate permeation through human skin and in vivo anti inflammatory activity on human volunteers. Journal of Controlled

Release, 106: 99 – 110.

30. Fry, D.W., White, J.C. and Goldman, I.D. (1978). Rapid seperation of low molecular weight solutes from liposomes without dilution. Analytical Biochemistry, 90: 809 – 815.

31. Torchilin, V.P. and Weissig, V. (2003). Liposomes a practical approach, (2nd edition), Oxford University Press, Oxford, pp. 36 – 39.

32. Cevc, G., Schatzlein, A. and Blume, G. (1995). Transdermal drug carriers: Basic properties, optimization and transfer efficiency in case of epicutaneously applied peptides. Journal of Controlled Release, 36:3 – 16.

33. Vanden Berge, B.A.I., Swartzendruber, V.A.B. and Geest, J. (1997). Development of an optimal protocol for the ultrastructural examination of skin by transmission electron microscope. Journal Microscopy, 187 (2): 125 – 133.

34. Toll, R., Jacobi, U., Richter, H., Lademann, J., Schaefer, H. and Blume, U. (2004). Penetration profile of microspheres in follicular targeting of terminal hair follicles. Journal of Investigative Dermatology, 123: 168 – 176.

35. Kirjavainen, M., Urtti, A., Valjakka – Koskela, R., Kiesvaara, J. and Monkkoonen J. (1999). Liposome – skin interactions and their effects on the skin permeation of drugs. European Journal of Pharmaceutical Sciences, 7(4): 279 - 286.

36. Lopez – Pinto, J.M., Gonzalez – Rodriguez, ML. and Rabasco, A.M. (2004). Effect of cholesterol and ethanol on dermal delivery from DPPC liposomes. International Journal of Pharmaceutics, 298(1): 1 – 12.

37. Anibinder, D. and Touitou, E. (2005). Testosterone ethosomes for enhanced transdermal delivery. Drug Delivery, 12(5): 297 – 303.

38. Jarivis, B. and Faulds, D. (1999). Lamivudine: a review of its therapeutic potential in chronic hepatits B. Drugs, 58(1): 101 – 141.

39. Barry, B.W. (1987). Mode of action of penetration enhancers on the kinetics of percutaneous absorption. Journal of Control Release, 6: 85 – 97.

40. Sanjay, P. (2007). Ethosomes: A promising tool for transdermal delivery of drug. Pharma Info Net, 5 (3): 1 – 5.

41. Jain, S., Umamaheshwari, R.B., Bhadra, D. and Jain, N.K. (2004). Ethosomes – A novel vesicular carrier for enhanced Transdermal delivery of an anti – HIV agent. Indian Journal Pharmaceutical Sciences, 66 (1): 72 – 81.

42. Sheo, D.M., Sunil, K.P., Anish, K.G., Gyanendra, K.S. and Ram, C.D. (2010) Formulation development and evaluation of ethosome of stavudine. Indian Journal of Pharmaceutical Education and Research, 44(1): 102 – 108.

43. Vivek, D., Dhirendra, K., Shaila, L., Sarvesh P. (2010) Ethosome for enhanced transdermal drug delivery of aceclofenac. International Journal of Drug Delivery, 2 : 81 - 92

44. Yi-Ping, F., Yi-Hung, T., Pao-chu, W. and Yaw – Bin, H. (2008). Comparison of 5-aminolevulinic acid – encapsulated liposome versus ethosome for skin delivery for photodynamic therapy. International Journal of Pharmaceutics, 356: 144 – 152.

45. Banga, A.K. and Chein, Y.W. (1993).

Hydrogel – based iontotheraputic delivery devices for transdermal delivery of peptide/ protein drugs. Pharmaceutical Research, 10: 697 – 702.

46. Godin, B., Touitou, E., Rubinstein, F., Athamna, A., Athamna, M. (2005). A new approach for treatment of deep skin infections by an ethosomal antibiotic preparation: an in vivo study. Journal of Antimicrobial Chemotherapy, 55(6): 989 – 994.

47. Ehab, R.B. and Mina, I.T. (2007). Enhanced transdermal delivery of salbutamol sulfate via ethosomes. AAPS PharmSciTech, 8 (4): E1 – E8.

48. Kaplun – Friscкhoff, Y. and Touitou, E. (1997). Testosterone skin permeation enhancement by menthol through formation of eutectic with drug and interaction with skin lipids. Journal of Pharmaceutical Sciences, 86: 1394 – 1399.

49. Yuefeng, R., Feiyue, Z., Xingguo, Z., Jianqing, G., Wenquan, L. (2008). *In vitro* percutaneous permeation and skin accumulation of finasteride using vesicular ethosomal carriers. AAPS PharmSciTech, 9(3): 860 – 865.

50. Godin, B. and Touitou, E. (2004). Mechanism of bacitracin permeation enhancement through the skin and cellular membrane from an ethosomal carrier. Journal of Controlled Release, 94: 365 – 379.

51. Vaibhav, D., Dinesh, M., Tathagata, D., Manoj, N., Saraf, D.K. and Jain, N.K. (2007). Dermal and transdermal delivery of an anti – psoriatic agent via ethanolic liposomes. Journal of Controlled Release, 123: 148 – 154.

52. Patricia de la, P., *et al*. (2009). Gold nanoparticals generated in ethosomes bilayers, as revealed by cryo –electron – tomography. Journal of Physical Chemistry. B, 113(10): 3051 – 3057

53. Touitou, E. (2002). Composition and methods for intracellular delivery. PCT/IL02/00516.

54. Jia-You, F., Chi-Tzong, H., Wen-Ta, C., Ying-Yue, W. (2001). Effect of liposomes and niosomes on skin permeation of enoxacin. International Journal of Pharmaceutics, 219 (1): 61 – 72.

55. Jain, S., Jain, P. and Jain, N.K. (2003). Transfersomes: a novel vesicular carrier for enhanced transdermal delivery: development, characterization and performance evaluation. Drug Development and Industrial Pharmacy, 29: 1013 – 1026.

56. www.ntt-inc.com/Opportunities/Products/ tabid/76/Default.aspx

57. www.ecademy.com/module.php/ mod=list&lid=203723

58. www.sinere.com/nanominox_en.html

Current Trends in Biotechnology and Pharmacy
Vol. 5 (1) 982-992 January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

Fermentation of Enzymatically Saccharified Groundnut shell for Fuel Ethanol Production by *Pichia stipitis* NCIM 3498

Chandra Sekhar Gajula[1], Radhika Konakalla[1], Chandel Anuj Kumar[1], Ravinder Rudravaram[2] and Lakshmi Narasu Mangamoori[1]*

[1]Centre for Biotechnology, JNT University, Hyderabad – 500 085, AP, India.
[2]Celestial Labs Limited, Banjara Hills, Hyderabad – 500 034, AP, India.
*For Correspondence - mangamoori@gmail.com

Abstract

Ethanol production was evaluated from Groundnut shell (GS), a readily available potential feedstock, for production of fermentable sugars. Sodium hydroxide pretreated GS was enzymatically saccharified (50 °C, pH 5.0, 50 h) using cellulolytic enzyme cocktail (cellulase, xylanase, β–glucosidase etc.) from a novel isolate, *Aspergillus sp*. RCAL-5. It showed cellulolytic enzyme production (Filter paperase (FPase) 0.86 U ml^{-1}, Carboxy methyl cellulase (CMCase) 1.25 U ml^{-1}, β-glucosidase 1.68 U ml^{-1} and xylanase 4.57 U ml^{-1}) after 4 days of incubation at 28 °C using delignified wheat bran as a carbon source. The maximum yield of monomeric sugars from delignified substrate was 0.64 g g^{-1} with a saccharification efficiency of 63.12 %. The fermentation performance of *Pichia stipitis* NCIM3498 was assessed using GS enzymatic hydrolysate (49 g l^{-1} sugars) for ethanol production in 10 l fermenter. A maximum of ethanol production (19.4 g l^{-1}) was obtained with a yield (0.46 g g^{-1} sugar utilized) and productivity (0.23 g l^{-1} h^{-1}).

Key words: Groundnut shell: Ethanol: *Aspergillus* sp. RCAL-5: *Pichia stipitis*: Cellulase: Enzymatic saccharification

Introduction

Ethanol made from biomass can be an attractive, safer and clean energy option (1, 2). Among various forms of biomass, agro crop residues are particularly well suited for energy applications because of its large – scale availability, low cost and environmentally benign production (3, 4). An important issue regarding the bioethanol production is whether the process is economical or not. The cost of raw material for ethanol production is an important parameter in establishing a cost effective technology.

After recovery of groundnut pods, groundnut shell (GS) is a readily available material worldwide for ethanol production. The alkaline method of pretreatment disrupts the cell wall and thus improves the amenability of enzymes to polysaccharides in the pant cell wall. Enzymatic hydrolysis is an attractive approach to render the monosaccharides from lignocellulosics accessible for fermentation (5, 6). However, the cost of cellulolytic enzymes accounts for about 60 % of the total cost of a bioconversion process (7). In-house enzyme production is always preferable choice to bring down the overall cost of cellulosic ethanol. *Aspergillus sp*. is widely known for its capability to produce cellulolytic enzymes utilizing cheap carbon sources. Among the various

pentose sugars fermenting yeast, *Pichia stipitis* has shown promise for industrial application for ethanol production due to its ability to ferment wider range of sugars rapidly with a high ethanol yield and apparently produces no xylitol (8).

In this communication, enzymatic hydrolysis of NaOH delignified GS was carried out using cellulases derived from *Aspergillus sp*. RCAL-5 for the recovery of fermentable carbohydrates. The fermentation of GS enzymatic hydrolysate was undertaken for ethanol production from *P. stipitis* NCIM3498 under batch conditions using 10 l capacity fermenter.

Materials and Methods

Raw Material

GS were collected from Chagalamarri, Kurnool Dt. Andhra Pradesh. The material was further processed in a laboratory disintegrator to attain a particular size between 4-10 mm followed by washing with tap water until the washings were clear and colorless and then gently dried at 40 °C for overnight. This material was used throughout all the experiments.

Delignification

750 g of dry GS pulverized material was suspended in 1 N NaOH solution (1:10 ratio) and autoclaved at 121 °C for 30 min. The contents were filtered with two layers of muslin cloth and the solid residue was repeatedly washed with water until the pH of the filtrate became neutral. The residue was dried at 40 °C for overnight and subsequently used for enzymatic hydrolysis experiments.

Microorganisms and media

P. stipitis NCIM3498 was procured from National Collection of Industrial Microorganisms (NCIM), NCL, Pune, India. It was revived and maintained on MGYP (Malt extract 0.5%, glucose1%, Yeast extract 0.5% and Peptone 0.5%, Agar 2%) slants at 30 °C. The culture cultivation conditions of *P. stipitis* NCIM3498 were followed as described by Nigam (9). *Aspergillus sp*. RCAL-5 showing cellulase activity on 2% CMC agar (Figure 1a, 1b) was isolated from the discarded lemon feedstock from a local vegetable market and maintained on Potato Dextrose Agar medium, subcultured at monthly and kept at 4 °C.

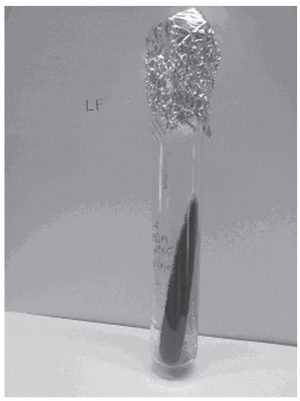


Fig. 1 (a). *Aspergillus sp.* RCAL-5, a novel isolate from natural resources grown on Potato Dextrose Agar medium

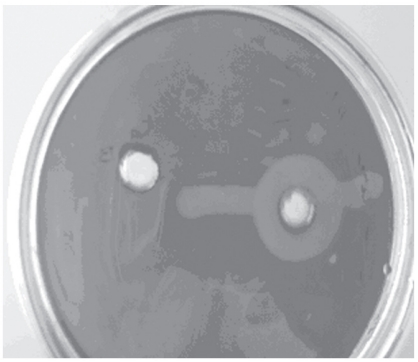


Fig. 1(b). Culture supernatant showing Cellulase activity on 2%CMC agar and control without any cellulase activity.

Inoculum preparation and cellulase production from *Aspergillus sp.* RCAL-5

The inoculum for cellulase production from *Aspergillus sp*. RCAL-5 was prepared according to the method of Chandel et al. (10) after some modifications. The medium ingredients were kept same except carbon source. NaOH pretreated wheat bran (1 N, 110 °C, 15 min) was used as a sole carbon source (10 g l^{-1}). Cellulase was produced by *Aspergillus sp*. RCAL-5 using the growth medium consisted of (g l^{-1}): alkali pretreated wheat bran 10, peptone 3, yeast extract 3, KH_2PO_4 2, $MgSO_4$ 1, $CaCl_2$ 1 and olive oil 2. Erlenmeyer flasks (1 l) containing of 250 ml growth medium was inoculated with a spores suspension (10^4 spores ml^{-1}) and incubated at 28 °C for 4 days at 150 rpm. Parametric optimization studies were done using one parameter at-a-time approach to know the most influential growth parameters for the maximum cellulase production from isolate, *Aspergillus sp*. RCAL-5 under shake flask conditions. The parameters involved were - effect of incubation time (1-7 days), carbon source (dried distillery grains, wheat bran, bengal gram, groundnut shell), nitrogen source (yeast extract, peptone, ammonium nitrate, sodium nitrate, casein, potassium nitrate, ammonium chloride), pH (3.5–6.5), temperature (24-36 °C) and agitation (50-250 rpm). The obtained culture filtrate was recovered by centrifugation (10,000 rpm for 10 min at 4 °C) and assayed for FPase (Filter paperase), CMCase (Carboxy methyal cellulase), β-glucosidase and xylanase. The recovered culture filtrates were subsequently lyophilized (Scanvac, Coolsafe, Denmark) and the enzyme powder was used for saccharification experiments.

Enzymatic hydrolysis

Enzymatic hydrolysis of 532.5 g (dry wt.) of NaOH pretreated GS was carried out in 10 L round bottom glass vessel containing 8 l of citrate buffer (pH 5.0, 50 mM, 50 °C) at 100 rpm. The cellulosic substrate was soaked in the citrate buffer for 2 h before adding the enzymatic solution. Sodium azide was added at a concentration of 0.005 % to restrict any microbial growth during the course of enzymatic hydrolysis. The substrate soaked in citrate buffer was supplemented with enzyme cocktail from *Aspergillus sp*. RCAL-5: cellulase 30 FPU g^{-1}, β-glucosidase 55 U g^{-1} and xylanase 150 U g^{-1} of the dry substrate. Samples were withdrawn at various intervals, centrifuged and supernatant analyzed for total reducing sugars released.

The saccharification efficiency was calculated as:

$$\text{Hydrolysis (\%)} = \frac{\text{Reducing sugar concentration obtained x 0.98x100}}{\text{Amount of NaOH pretreated substrate taken}}$$

Inoculum preparation and ethanol fermentation

Inoculum was prepared by harvesting the yeast cells grown for 24 h at 30 °C in the culture medium containing 10.0 g l^{-1} each of xylose and glucose with the medium defined by Nigam (9) at 150 rpm.

The fermentation medium consisted of 7 l groundnut shell enzymatic hydrolysate supplemented with the defined media ingredients described by Nigam (9). Fermentation of GS enzymatic hydrolysate (49 g l^{-1} total reducing sugar) was carried out in 10 l capacity glass fermenter (Sartorius B Plus, Bangalore, India) with a working volume of 7 l. The GS enzymatic hydrolysate (7 l volume) was taken and supplemented with the other medium ingredients as described by Nigam (9) to form a complete medium prior to sterilization at 120 °C for 20 min.

After sterilization and cooling the medium, 3% (v/v) of inoculum was aseptically transferred to fermentation medium through peristaltic pump. The fermentation was run at agitation 300 rpm, aeration 2 l min^{-1}, temperature 30 °C, and pH 5.5 automatically adjusted with 2 N HCl and 2 N NaOH. Antifoam (Silicone oil), 3.0 ml was also added to the fermentation medium to avoid the excessive foaming. Sterile syringes were used to take samples at regular time intervals during fermentation for estimation of residual sugars, biomass and ethanol.

Analytical methods

CMCase, FPase, xylanase and β-glucosidase activities were assayed as the method mentioned in Saha *et al.* (11). One unit (U) of each enzyme activity is defined as the amount of enzyme, which produces 1 µmol reducing sugar as glucose or xylose in the reaction mixture per minute under the above-specified conditions. β-glucosidase activity was determined by the release of p-nitrophenol from p-nitrophenyl-β-D-glucoside. Total reducing sugars (TRS) were estimated by dinitrosalicylic acid method of Miller (12). Ethanol was estimated by gas chromatography (GC) (Agilent 4890D, USA) as described by Chandel *et al.* (8). For yeast cell mass determination, biomass was harvested by centrifugation and washed with 0.9 % (w/v) NaCl in duplicates using 5 ml samples, dried at 105 °C for 2 h.

Results and Discussion

Chemical composition

GS used in this investigation contained 35.7% cellulose and 18.7% hemicellulose, which constitutes total carbohydrate amount of 54.4% on dry solid (DS) basis (Table 1) (13).

Table 1. Composition of Groundnut shell cell wall on % dry weight basis. [16]

Component	% dry weight
Volatile solids	68.7
Ash content	5.9
Organic carbon	48.3
Total nitrogen	0.8
Cellulose	35.7
Hemicellulose	18.7
Lignin	30.2

Parametric optimizations for FPase production from a novel isolate, *Aspergillus sp.* RCAL-5 under shake flask conditions

Parametric studies were conducted to know the best parameter ranges influencing the maximum enzyme production from *Aspergillus sp*. RCAL-5 under one parameter at-a-time strategy.

Optimization of incubation time

Incubation time was optimized by measuring the maximum FPase production from the day 1 to 7 at pH 4.5, 150 rpm, NaOH pretreated wheat bran as carbon source (10 g l^{-1}), yeast extract as nitrogen source (2.5 g l^{-1}) at 28 °C. Figure 2 presents the results of incubation period study on cellulase production from *Aspergillus sp*. RCAL-5. The maximum FPase production (0.75 FPU ml^{-1}) was observed after 4 days of incubation subsequently showing a regular down fall. In a study by Kocher *et al.* (14) maximum cellulase production (FPase 0.09 and CMCase 0.12 IU ml^{-1}) was observed after 8 days by *T. harzianum* 8230 using rice straw as carbon source.

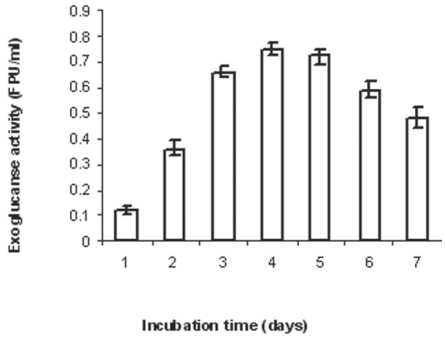


Fig. 2. Effect of incubation time on exoglucanase production by *Aspergillus* sp. RCAL-5. (pH 4.5, 150 rpm, wheat bran (10 g l^{-1}), yeast extract (2.5 g l^{-1}), temp. 28 °C.)

Effect of carbon sources

Effect of various NaOH pretreated carbon sources (dried distillery grains(DDG), wheat bran, bengal gram, groundnut shell) was studied for maximal FPase production under the following conditions (pH 4.5, temp. 28 °C, agitation 150 rpm, yeast extract as nitrogen source (2.5 g l^{-1}) and incubation time (4 days). Each substrate was pretreated with dilute sodium hydroxide solution (1 N NaOH, 110 °C, 15 min). A maximum of 0.86 FPU ml^{-1} activity was found using pretreated wheat bran (10 g l^{-1}) as a carbon source (Figure 3).

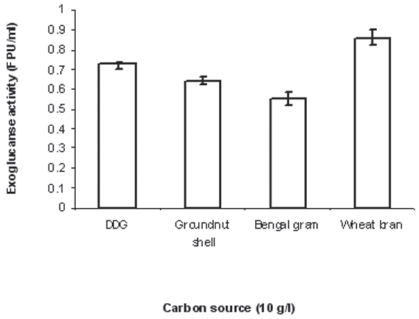


Fig. 3. Effect of carbon source on exoglucanase production by *Aspergillus* sp. RCAL-5 under following conditions. (pH 4.5, temp. 28 °C, Agitation 150 rpm, yeast extract (2.5 g l^{-1}) and incubation time (4 days)).

Effect of nitrogen sources

Effect of various nitrogen sources (yeast extract, peptone, ammonium nitrate, sodium nitrate, casein, potassium nitrate, ammonium chloride) was studied for maximal FPase production at conditions (pH 4.5, temp. 28 °C, agitation 150 rpm, wheat bran 10 g l^{-1}, Incubation time (4 days). Among all the nitrogen sources tested, yeast extract (2.5 g l^{-1}) showed the maximum FPase production (0.85 FPU ml^{-1}). Figure 4 shows the effect of different nitrogen sources on cellulase production from *Aspergillus sp*. RCAL-5. Umikalsom *et al.* (15) showed the maximum FPase production (1.4 U ml^{-1}) with peptone (6 g l^{-1}) as nitrogen source from the *Chaetomium globosum* strain 414.

Effect of pH

The effect of pH for cellulase production by *Aspergillus sp.* RCAL-5 was investigated within the range of 3.5–6.5. The pH was adjusted either with 1N NaOH or 1N HCl. It was grown for 7 days under submerged culture conditions (temp. 28 °C, agitation 150 rpm, wheat bran 10 g l^{-1}, yeast extract 2.5 g l^{-1}, incubation time (4 days). Figure 5 shows that maximum FPase (0.79 FPU ml^{-1}) production was obtained at pH 4.5 using NaOH pretreated wheat bran as sole carbon source. In general, lower pH is favored for higher cellulase titer production from fungi when grown on different lignocellulosic substrates.

Effect of temperature

The effect of temperature on the cellulase production was examined at the range of 24-36 °C. The results were given in Figure 6. The optimum temperature for maximum FPase (0.84 FPU ml^{-1}) production was found at 28 °C (agitation 150 rpm, wheat bran 10 g l^{-1}, yeast extract 2.5 g l^{-}, pH 4.5, incubation time (4 days). These results are in well accordance with the study of Narasimha et al. (16) who reported

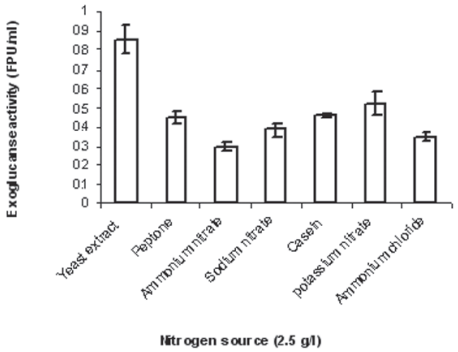


Fig. 4. Effect of nitrogen on exoglucanase production by *Aspergillus* sp. RCAL-5 under following conditions (pH 4.5, temp. 28 °C, agitation150 rpm , wheat bran 10 gl^{-1}, incubation time (4 days)).

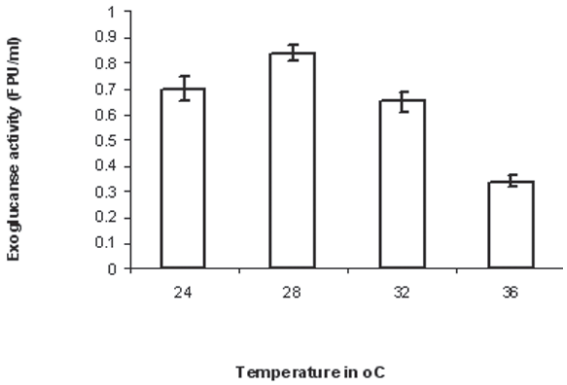


Fig. 6. Effect of temperature on exoglucanase production by *Aspergillus* sp. RCAL-5 under following conditions. (agitation 150 rpm, wheat bran 10 g l^{-1}, yeast extract 2.5 g l^{-1}, pH 4.5, incubation time (4 days)).

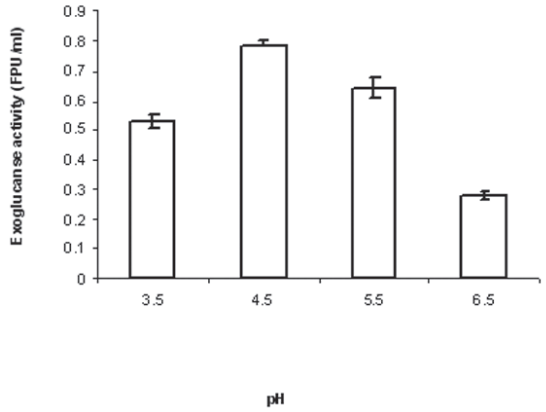


Fig. 5. Effect of pH on exoglucanase production by *Aspergillus* sp. RCAL-5 under following conditions. (temp. 28 °C, agitation 150 rpm, wheat bran 10 g l^{-1}, yeast extract 2.5 g l^{-1}, incubation time (4 days)).

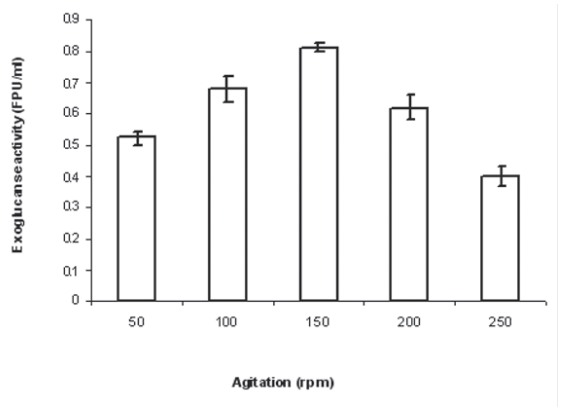


Fig. 7. Effect of agitation on exoglucanase production by *Aspergillus* sp. RCAL-5 under following conditions. (temp. 28 °C, wheat bran 10 g l^{-1}, yeast extract 2.5 g l^{-1}, pH 4.5, incubation time (4 days)).

maximum cellulase production (0.775 IU ml^{-1}) using saw dust as carbon source from *A.niger* grown at 28°C.

Recently, Deshpande et al. (17) observed maximum cellulase activity (0.22±0.04 IU ml^{-1}) at the incubation temperature of 30 °C.

Effect of Agitation

Effect of different agitation rates ranging from 50-250 rpm were studied for maximum FPase (0.81 FPU ml^{-1}) production under the conditions (temp. 28 °C, wheat bran 10 g l^{-1}, yeast extract 2.5 g l^{-1}, pH 4.5). From the Figure 7, it is

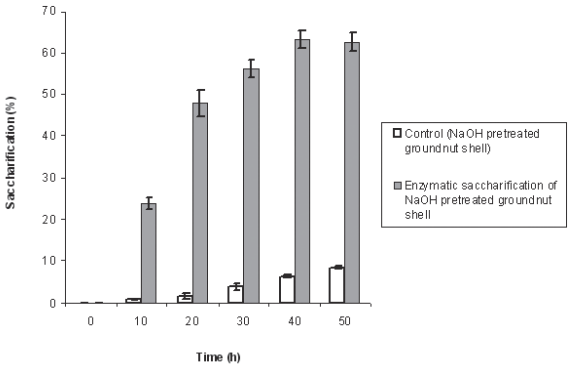


Fig. 8. Enzymatic hydrolysis of control and NaOH pretreated Groundnut shell using the cellulases derived from *Aspergillus sp*. RCAL-5. The hydrolytic conditions were: substrate and enzyme concentration (1: 15), 50 mM citrate buffer, pH 5.0, 50 °C at 100 rpm. The enzyme cocktail (cellulase 30 FPU, β-glucosidase 55 U and xylanase 150 U) was loaded per gram substrate.

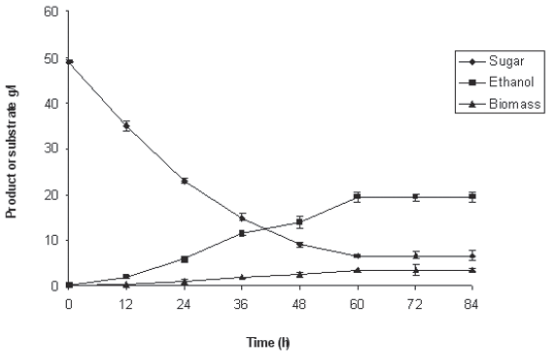


Fig. 9. The time course of growth, sugar utilization and ethanol production by *P. stipitis* NCIM 3498 at 30 °C using Groundnut shell enzymatic hydrolysate (pH 5.5). Initial sugar concentration was 49 g l^{-1} and the final ethanol was 19.4 g l^{-1} after 84 h.

evident that maximum FPase production was achieved at 150 rpm followed by a gradual downfall upon increasing the agitation rates. Agitation has a very important role for the production of various metabolites under shake flask cultivation conditions. Optimum agitation is required for the appropriate air supply as well as adequate nutrient availability to the microorganism (18). We observed a maximum of cellulase production (0.81 U ml^{-1}) at 150 rpm. Gomes et al. (18) found that shifting of agitation speed from 120 rpm to 180 rpm showed 2-fold (2.3 U ml^{-1} of CMCase) increment in enzyme production.

Our observation for cellulase production from this novel isolate could be fairly compared with the previous study of Vyas *et al.* (19) and Deshpande *et al.* (17).

Cellulase production under optimized set of conditions

The cellulolytic enzyme production profile by *Aspergillus sp*. RCAL-5 under optimized conditions is given in Table 2. The organism showed all the main enzymes required for the depolymerization of structural polysaccharides from the delignified substrate. *Aspergillus sp*. RCAL-5 showed maximum cellulase (0.88 FPU ml^{-1}) after 4 days of incubation, exhibiting higher level of CMCase than FPase activity, which is a normal pattern among cellulolytic organisms (20,

Table 2. Hydrolytic enzymes production profile by *Aspergillus sp*. RCAL-5 under optimized conditions (agitation 150 rpm, temp. 28 °C, wheat bran 10 g l^{-1}, yeast extract 2.5 g l^{-1}, pH 4.5 and after 4 days of incubation).

Enzyme	Activity (U ml^{-1})
FPase	0.86
CMCase	1.25
β – glucosidase	1.68
Xylanase	4.57

The data presented are averages of three independent analysis.

21). The higher β-glucosidase activity could have resulted in releasing higher amount of total sugars in the GS enzymatic hydrolysate. Recently, Dien et al. (5) also observed a key role of β-glucosidase in monosaccharide yields after enzymatic hydrolysis of hot water treated corn fiber.

Enzymatic hydrolysis

Enzymatic hydrolysis of NaOH pretreated GS was carried out using concentrated enzymatic solution of (cellulase, 30 FPU g^{-1}; β-glucosidase, 55 U g^{-1}; xylanase, 150 U g^{-1} of the dry substrate) *Aspergillus sp.* RCAL-5. Time course of enzymatic saccharification of GS is shown in Figure 8. Saccharification of NaOH pretreated GS shell yielded maximum sugars (0.64 g g^{-1}) with the hydrolysis efficiency of 63.12%. These findings can be fairly compared with the reports of Sharma *et al.* (22) who observed 59.8 % saccharification efficiency from sunflower hulls after pretreatment with sodium hydroxide 0.5% (w/v). Saha and his coworkers (11) also showed an enzymatic hydrolytic efficiency of 64 % total carbohydrates (485 mg g^{-1}) from wheat straw using the commercial cellulases. The hydrolytic efficiency of lignocellulosic substrates also depends upon the substrate used. Recently, Chandel *et al.* (10) reported 77.85±0.45% hydrolytic efficiency from a weed material, *Saccharum spontaneum* using the cellulases derived from *A. oryzae* MTCC 1846.

Ethanol fermentation

A typical fermentation profile of GS enzymatic hydrolysate using *P. stipitis* NCIM 3498 utilizing 49 g l^{-1} total sugars is shown in Figure 9. A regular increase in ethanol production was observed till 84 h of fermentation and declined thereafter. About 86.5 % of the available sugars were utilized within 84 h giving an ethanol yield of 0.46 g g^{-1} sugar utilized. The fermentation parameters viz. ethanol yield, biomass yield, ethanol productivity etc. are summarized in Table 3.

Table 3. Fermentation parameters of the *P. stipitis* NCIM3498 when grown in the Groundnut shell enzymatic hydrolysate

Parameters studied	Values
Initial sugar concentration ($g_s\ l^{-1}$)	49
Sugar utilization (%)	86.50
Ethanol ($g_p\ l^{-1}$)	19.4
Ethanol yield ($g_p\ g_s^{-1}$)	0.46
Biomass ($g_x\ l^{-1}$)	3.4
Biomass yield ($g_x\ g_s^{-1}$)	0.08
Volumetric ethanol productivity ($g_p\ l^{-1}\ h^{-1}$)	0.23
Biomass productivity ($g_x\ l^{-1}\ h^{-1}$)	0.04
Sugar uptake rate ($g_s\ l^{-1}\ h^{-1}$)	0.50
Specific ethanol productivity ($g_p\ g_x^{-1}\ h^{-1}$)	0.06

Total incubation time- 84 h. The data presented are averages of three independent analysis.

Among the xylose fermenting yeasts, *P. stiptis* has been promising in utilizing maximum fermentable sugars present in lignocellulose hydrolysates which in turn reflects in the higher ethanol production with a greater yield. In general, the fermentation performance of *P. stipitis* is lower than the conventionally used ethanologen, *Saccharomyces cerevisiae* or *Zymomonas mobilis*. But looking the ability of *P. stipitis* for utilization of almost all sugars present in the lignocellulose hydrolysates, this microorganism is considered ideal in biorefineries. *S. cerevisiae* or *Z. mobilis* utilize C_6 sugars or sucrose highly efficiently but their inability to utilize C_5 sugars make them inappropriate candidates for biorefineries (3, 4). Our results were similar according to the earlier reports summarized in Table 4.

Table 4. Bioethanol Production from various agro residues by different strains of *P. stipitis*

Agro residues	Sugar concentration (g/L)	Strain	Ethanol production (g/L)	Reference
Hazelnut shell	50	*Pichia stipitis*	16.79	23
Wheat straw	46	*Pichia stipitis*. NRRL Y-7124.	12.9	24
Saccharum spontaneum	53.9	*Pichia stipitis* NCIM3498.	21.82	25
Prosopis juliflora	18.24	*Pichia stipitis* NCIM3498	7.13	26
Deoiled rice bran	33.55	*Pichia stipitis* NCIM3499	12.47	8
Lantana camara (red sage)	19.4	*Pichia stipitis* NCIM3498	5.16	27
Yellow poplar	89.2	*Pichia stipitis* KCCM 12009	28.7	28
Sugar maple	59.9	*Pichia stipitis*. NRRL Y-7124	14.3	29

Conclusions

GS after pretreatment with alkali yielded 63.12 % hydrolytic efficiency using the enzymes from a novel isolate, *Aspergillus sp*. RCAL-5. It was optimized for cellulase production under shake flask conditions.

The obtained GS enzymatic hydrolysate was fermented with *P. stipitis* NCIM3498 for ethanol production at 10 l fermenter level. A maximum of 19.4 g l^{-1} ethanol was produced with the yield (0.46 g g^{-1} sugar utilized) and productivity (0.23 g l^{-1} h^{-1}). This study proved GS as a potential, renewable and low cost biomass for ethanol production on a commercial scale.

Acknowledgements

Authors are highly thankful of Mr. G. Balanarsimha Reddy for providing the Groundnut shell and technical help for this study.

References

1. Chandel, A.K., Singh, O.V., Chandrasekhar, G., Rao, L.V. and Narasu, M.L. (2010). Key-drivers influencing the commercialization of ethanol based biorefineries. Journal of Commercial Biotechnology, 16:239-257.
2. Herrera, S. (2004). Industrial biotechnology- a chance at redemption. Nature Biotechnology, 22:671-675.
3. Wyman, C.E. (2007). What is (and is not) vital to advancing cellulosic ethanol. Trends in Biotechnology, 25: 153-157.
4. Lynd, L.R., Laser, M.S., Bransby, D., Dale, B.E., Davison, B., Hamilton, R., Himmel, M., Keller, M., McMillan, J.D., Sheehan, J. and Wyman, C.E. (2008). How biotech can transform biofuels. Nature Biotechnology, 26:169-172.

5. Dien, B.S., Jung, H.J.G., Vogel, K.P., Casler, M.D., Lamb, J.F.S., Iten, L., Mitchell R.B. and Sarath, G. (2006). Chemical composition and response to dilute-acid pretreatment and enzymatic saccharification of alfalfa, reed canary grass, and switch grass. Biomass and Bioenergy, 30: 880-891.

6. Suvarna Laxmi, G., Sathish, T., Subba Rao, Ch., Brahmaiah, P., Hymavathi, M. and Prakasham, R.S. (2008). Palm fiber as novel substrate for enhanced xylanase production by isolated *Aspergillus sp.* RSP-6. Current Trends in Biotechnology and Pharmacy, 2 (3): 447-455.

7. Tangnu, S.K., Blanch, H.W. and Wilke, C.R. (1981). Enhanced production of cellulases, hemicellulase and â-glucosidase by *Trichoderma reesei* (Rut C-30). Biotechnology and Bioengineering, 23:1837-1849.

8. Chandel, A.K., Mangamoori, L.N., Ravinder, R., Ravindra, P. and Rao, L.V. (2009a). Bioconversion of De-Oiled Rice Bran (DORB) Hemicellulosic Hydrolysate into Ethanol by *Pichia stipitis* NCM3499 under Optimized Conditions. International Journal of Food Engineering, 5:1-15.

9. Nigam, J.N. (2002). Bioconversion of water-hyacinth (*Eichhornia crassipes*) hemicellulose acid hydrolysate to motor fuel ethanol by xylose–fermenting yeast. Journal of Biotechnology, 97:107-116.

10. Chandel, A.K, Lakshmi Narasu, M., Chandrasekhar, G., Manikyam, A. and Venkateswar Rao, L. (2009b). Use of *Saccharum spontaneum* (wild sugarcane) as biomaterial for cell immobilization and modulated ethanol production by thermotolerant *Saccharomyces cerevisiae* VS3. Bioresource Technology, 100:2404-2410.

11. Saha, B.C., Iten, L.B., Cotta, M.A. and Wu, Y.V. (2005). Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. Process Biochemistry, 40:3693-3700.

12. Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry, 31:426-428.

13. Raveendran, K., Anuradda, G. and Kartic, C. (1995). Khilart Influence of mineral matter on biomass pyrolysis characteristics. Fuel, 74:1812-1822.

14. Kocher, G., Kalra, K. and Banta, G. (2008). Optimization of cellulase production by submerged fermentation of rice straw by *Trichoderma harzianum* Rut-C 8230. The Internet Journal of Microbiology, 5: 2.

15. Umikalsom, M.S, Ariff, A.B., Shamsuddin, Z.H., Tong, C.C., Hassan, M.A. and Karim, M.I.A. (1997). Production of cellulase by a wild strain of *Chaetomium globosum* using delignified oil palm empty-fruit-bunch fibre as substrate. Applied Microbiology and Biotechnology, 47:590-595.

16. Narasimha, G., Sridevi, A., Buddolla, V., Subhosh, C.M. and Rajsekhar, R.B. (2006). Nutrient effect on production of cellulolytic enzymes by *Aspergillus niger.* African Journal Biotechnology, 5:472-476.

17. Deshpande, P., Nair, S. and Khedkar, S. (2009). Water Hyacinth as Carbon Source for the Production of Cellulase by *Trichoderma reesei*. Applied Biochemstry and Biotechnology, 158:552-560.

18. Gomes, I., Sarkar, P.K., Rahman, S.R., Rahim, M.A. and Gomes, D.J. (2007). Production of Cellulase from *Talaromyces emersonii* and Evaluation of Its Application in Eco-Friendly Functional Finishing of

Jute-Based Fabrics Isidore Gomes1. Bangladesh Journal of Microbiology, 24:109-114.

19. Vyas, A., Vyas, D. and Vyas, K.M. (2005). Production and optimization of cellulases on pretreated groundnut shell by *Aspergillus terreus* AV49. Journal of Scientific & Industrial Research, 64:281-286.

20. Singh, A. and Kumar, P.K. (1991). *Fusarium oxysporum*: status in bioethanol production. Critical Review in Biotechnology, 11(2):129-47.

21. Wen, Z., Liao, W. and Chen, S. (2005). Production of cellulase by *Trichoderma reesei* from dairy manure. Bioresource Technology, 96:491-499.

22. Sharma, S.K., Kalra, K.L. and Kocher, G.S. (2004). Fermentation of enzymatic hydrolysate of sunflower hulls for ethanol production and its scale-up. Biomass Bioenergy, 27:392-402.

23. Arslan, Y. and Eken-Saraçoðlu, N. (2010). Effects of pretreatment methods for hazelnut shell hydrolysate fermentation with *Pichia Stipitis* to ethanol. Bioresource Technology, 101(22):8664-8670.

24. Nigam, J.N. (2001). Ethanol production from wheat straw hemicellulose hydrolysate by *Pichia stipitis*. Journal of Biotechnology. 87(1):17-27.

25. Chandel, A.K., Singh, O.V., Rao, L.V., Chandrasekhar, G. and Narasu, M.L. (2011). Bioconversion of novel substrate *Saccharum spontaneum*, a weedy material, into ethanol by *Pichia stipitis* NCIM3498. Bioresource Technology, 102:1709-1714.

26. Gupta, R., Sharma, K.K. and Kuhad, R.C. (2009). Separate hydrolysis and fermentation (SHF) of *Prosopis juliflora*, a woody substrate, for the production of cellulosic ethanol by *Saccharomyces cerevisiae* and *Pichia stipitis*-NCIM 3498. Bioresource Technology, 100(3):1214-1220.

27. Kuhad, R.C., Gupta, R., Khasa, Y.P. and Singh, A. (2010). Bioethanol production from *Lantana camara* (red sage): Pretreatment, saccharification and fermentation. Bioresource Technology, 101(1): 8348-8354.

28. Cho, D.H., Shin, S., Bae, Y., Park, C., Kim, Y.H. (2010). Enhanced ethanol production from deacetylated yellow poplar acid hydrolysate by *Pichia stipitis*. Bioresource Technology, 101:4947-4951.

29. Stoutenburg R.M., Perrotta, J.A., Amidon, T.E., and Nakas, J.P. (2008). Ethanol production from â membrane purified hemicellulosic hydrolysate derived from sugar maple by *Pichia stipitis* NRRL Y-7123. Bioresources, 3(4):1349-1358.

Current Trends in Biotechnology and Pharmacy
Vol. 5 (1) 993-997 January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

In vitro Antibacterial Activity in Seed extracts of *Phoenix sylvestris* Roxb (Palmae), and *Tricosanthes dioica* L (Cucurbitaceae)

Vijay Kothari*
Institute of Science, Nirma University, Ahmedabad-382481, India.
*For Correspondence - vijay.kothari@nirmauni.ac.in

Abstract

Seeds of *Phoenix sylvestris* Roxb (Palmae), and *Tricosanthes dioica* L (Cucurbitaceae) were investigated for their *in vitro* antibacterial activity against few gram-negative and gram-positive bacteria. Extracts of the plant seeds were prepared in methanol, ethanol, and chloroform by microwave assisted extraction method. Their antibacterial activity was investigated by disc diffusion and broth dilution methods. Ethanol extract of *P. sylvestris* was found active (bacteriostatic) against both gram-positive and gram-negative organisms with MIC (minimum inhibitory concentration) values of 481 and 410 µg/mL against *Salmonella paratyphi A* and *Staphylococcus epidermidis*, respectively. Phytochemical screening revealed presence of phenols, alkaloids, and flavones in it. This study indicated various extracts of *P. sylvestris,* and *T. dioica* seeds to possess antibacterial activity.

Key words: Antibacterial, *Phoenix sylvestris, Tricosanthes dioica,* MAE (microwave assisted extraction)

Introduction

Plants are rich source of drugs, either as direct remedies or template for production of synthetic drugs. Numerous studies made throughout the world have demonstrated wide occurrence of antimicrobial compounds in higher plants (1). Due to emergence of drug resistant strains of pathogenic bacteria, it has become important to investigate plants as sources of novel antimicrobials, as they may inhibit bacteria by a mechanism different than that of currently used antibiotics (2).

Present study was aimed at screening various extracts of seeds from two plants namely- *Phoenix sylvestris* Roxb (Palmae; common name- Khajur), and *Tricosanthes dioica* L (Cucurbitaceae; common name- Parvar) for their antibacterial activity. Seeds of the latter has been reported to possess haemagglutinating and antifungal activity, and are rich in fatty acids (3).

Material and Methods

Seeds of both the plants *P. sylvestris,* and *T. dioica* were procured from local market of Ahmedabad city. They were authenticated for their unambiguous identity by Prof. Y. T. Jasrai, Head of Botany Dept., Gujarat University, Ahmedabad.

Extraction: Seeds were extracted in three different solvents (Merck, Mumbai, India) – methanol, ethanol (50%), and chloroform using the microwave assisted extraction (MAE) method (4). Dry seed powder was soaked into the solvent in a ratio of 1:50, and subjected to microwave

heating (Electrolux EM30EC90SS) at 720 W. Total heating time was kept 90, 70, and 180 second for methanol, ethanol, and chloroform respectively, with intermittent cooling. This was followed by centrifugation (at 10,000 rpm for 15 min.), and filtration with Whatman paper # 1 (Whatman International Ltd., Maidstone, England). Solvent was evaporated from the filtered extract and then the dried extracts were reconstituted in: (i) their respective solvents for disc diffusion assay, and (ii) dimethyl sulfoxide (DMSO) for broth dilution assay. Reconstituted extracts were stored under refrigeration for further use. Extraction efficiency was calculated as percentage weight of the starting dried plant material. Extraction efficiency ranged from 2.8-13.6 %, with highest (13.6%) being in case of methanol extract of *T. dioica* seeds.

Bacterial strains: *Staphylococcus aureus* MTCC 737, *Streptococcus pyogenes* MTCC 442, *Staphylococcus epidermidis* MTCC 435, *Escherichia coli* MTCC 723, *Aeromonas hydrophila* MTCC 1739, *Salmonella paratyphi A*, *Shigella flexneri* MTCC 1457, *Vibrio cholerae* MTCC 3906, and *Pseudomonas oleovorans* MTCC 617. *S. paratyphi A* was procured from Department of Microbiology, Gujarat University, Ahmedabad, while the rest were obtained from Microbial Type Culture Collection (MTCC), Chandigarh.

Disc diffusion assay (DDA) : This was performed by Kirby-Bauer method as per NCCLS guidelines (5). 500 µL of inoculum (adjusted to 0.5 McFarland standard) was spread on surface of Muller-Hinton agar medium (HiMedia, Mumbai, India). Sterile discs (6 mm diameter) made of Whatman paper # 1 were dipped into the test extract and were put onto the agar surface after complete drying. Discs dipped into pure solvents (separate disc for each solvent) after drying were applied as negative control. Commercially available discs of either streptomycin or Ofloxacin (HiMedia) served as positive control. Plates were then incubated at 35ºC for 24 h. After incubation plates were observed for zones of inhibition, and their diameter were measured. Studies were performed in triplicates.

MIC determination : MIC (minimum inhibitory concentration) determination was carried out using microbroth dilution method as per NCCLS guidelines (5). Assay was performed in a 96-well microtitre plate. Total volume of the assay system in each well was kept 200 µL. Cation-adjusted Muller-Hinton broth (HiMedia) was used as growth medium. Inoculum density of the test organisms was adjusted to that of 0.5 McFarland standard. Broth was dispensed into wells of microtitre plate followed by addition of test extract and inoculum. Extracts (reconstituted in DMSO) were serially diluted into each of the wells. A DMSO control was included in all assays (6). Gentamicin (HiMedia) served as positive control. Plates were incubated at 35ºC for 16-20 h, before being read at 655 nm in a plate reader (BIORAD 680). MIC was recorded as the lowest concentration at which no growth was observed. All MICs were determined on three independent occasions. Concentration at which growth was inhibited by 50% was recorded as IC_{50} value.

After reading the plates for MIC, subculturing was made on nutrient agar plate from the wells showing no growth, so as to determine whether the extract is bactericidal or bacteriostatic. Growth on the plate indicated bacteriostatic action, absence of growth indicates bactericidal action.

Total activity: Total activity (mL/g) was calculated as (7): Amount extracted from 1 g (mg) / MIC (mg/mL).

Activity index: Activity index was calculated as (8)- zone of inhibition by extract / zone of inhibition by antimicrobial agent used as positive control

Phytochemical screening: Active extracts were tested for presence of alkaloids, flavones, phenolics, and flavonoids as described below (8,9).

Alkaloids: Dragendorff reagent was used to test the presence of alkaloids. The presence of alkaloids was indicated by the appearance of yellow precipitate when few drops of reagent were added to the solution.

Flavones: Test solution (500 µl) was mixed with 100 µl of absolute alcohol, 0.02 g of p-dimethyl amine benzaldehyde and two drops of conc. Hcl. Development of red or pink colour indicated the presence of flavones.

Flavonoids: An aqueous solution of the extract was treated with 10% ammonium hydroxide solution. Yellow florescence indicates the presence of flavonoids.

Phenols: Ferric chloride was used to test the presence of phenols. The presence of phenols was indicated by appearance of green colouration when the reagent was added to extract.

Results and Discussion

Results of disc diffusion assay (table 1) indicated *S. paratyphi A* to be susceptible to all the extracts tested against it. *S. pyogenes*, and *S. auerus* exhibited no notable susceptibility to any of the test extracts. Ethanol extract of *P. sylvestris* (50 mg/mL) produced same size of inhibition zone as that produced by ethanol extract of *T. dioica* (100 mg/mL) against *S. paratyphi A*. As the former did it at exactly half the concentration of the latter, it can be said to be twice as potent as the latter. Methanolic extract of *P. sylvestris* produced smallest zone of inhibition against *S. paratyphi A*, despite its concentration being higher than all other test extracts. Activity index (AI) was calculated for the extracts which exhibited activity in disc diffusion assay, as written in parentheses in table 1. Methanolic extract of *T. dioica* registered highest values of AI against *S. paratyphi A*, and *P. oleovorans*. As ethanolic extract of *P. sylvestris* was found to be effective against both gram-positive as well as gram-negative bacteria, it can be said to have a broad spectrum of activity (10). It was further investigated for its MIC values against susceptible bacteria. It was capable of inhibiting the growth of *S. paratyphi A*, and *S. epidermidis* at concentrations of 481 and 410 µg/mL, respectively (Table 2). It was found to be bacteriostatic in action, as organisms were able to revive growth when transferred on nutrient agar free from extract. Total activity of this extract against both the organisms was above 100 mL/g. Total activity is a measure of the amount of material extracted from a plant in relation to the MIC of the extract, fraction or isolated compound. It indicates the degree to which the active fractions or compounds present in 1 g can be diluted and still inhibit growth of the test organism (7). Phytochemical tests indicated the presence of phenols, alkaloids, and flavones in ethanolic extract of *P. sylvestris* seeds.

Conclusion

This study indicated various extracts of *P. sylvestris,* and *T. dioica* seeds to possess antibacterial activity. These extracts should be further investigated for identification of active constituent(s). Active constituent(s) once separated can be subjected to compatible techniques such as mass spectrometry, IR and NMR spectroscopy for structural studies.

Acknowledgements

Author thank Nirma Education and Research Foundation (NERF) for financial assistance, Prof. Y. T. Jasrai (Botany Dept., Gujarat University) for seed authentication, and Prof. S. R. Dave (Microbiology Dept., Gujarat University) for providing one of the test organisms.

Table 1. Disc diffusion assay of *P. sylvestris* and *T. dioica* seed extracts

Organism	*P. sylvestris*		*T. dioica*		Positive control		
	Diameter of zone of inhibition (mm)						
	M[1]	**Et**[2]	**M**[3]	**Et**[4]	**Ch**	**O**	**S**
A. hydrophila	0	0	0	0	11±2.8 (0.61)	-	18±2.0
E. coli	0	0	0	0	-	28±1.8	-
S. aureus	FI	0	0	0	-	17±2.0	28±2.5
S. epidermidis	0	9±1.2 (0.47)	FI	0	-	-	19±1.8
S. paratyphi A	11±1.8 (0.5)	17±2.0 (0.77)	18±1.6 (0.81)	17±2.0 (0.77)	- -	- -	22±1.0
S. flexineri	0	9±1.8 (0.29)	FI	20±2.0 (0.64)	0	31±2.4	-
V. cholerae	9±1.0 (0.45)	FI	FI	FI	-	27±1.4	20±2.2
P. oleoverans	0	FI	21±2.8 (0.80)	FI	FI	26±1.4	-
S. pyogenes	0	0	0	0	0	15	20±2.2

All values are means±SD of three experiments.
FI: Faint inhibition without clear zone; O: Ofloxacin (5 µg /disc), S: Streptomycin (10 µg /disc)
M: Methanol; Et: Ethanol; Ch: Chloroform
Conc. of extract into which disc was dipped (mg/mL): [1]260, [2]50, [3]120, [4]100
Figures in parentheses indicate activity index. Negative controls did not cause any inhibition of growth.

Table 2. Results of broth dilution assay

Extract	Organism	IC_{50} (µg/mL)	MIC (µg/mL)	Total activity (mL/g)
Ethanol extract of	*S. paratyphi* A	<400	481	103.95
P. sylvestris seeds	*S. epidermidis*	<380	410	121.95

References

1. Joseph, I., and Singh, R. (2008). Antimicrobial activity of selected medicinal plants, *Craetva magna* (Linn.), *Pongamia glabra* (Linn.), and *Areca catechu* (Linn.). Ethnobotany Leaflets, 12: 995-1002.

2. Tomoko, N., Takashi, A., Hiromu, T., Yuka, I., Hiroko, M. Munekazu, I., Totshiyuki, T., Tetsuro, I., Fujio, A., Iriya, I., Tsutomu, N., and Kazuhito, W. (2002). Antibacterial activity of extracts prepared from tropical and subtropical plants on methicillin resistant *Staphylococcus aureus*. Journal of Health Sciences, 48: 273-276.

3. Khare, C.P. (2004). Encyclopedia of Indian medicinal plants. Springer, Germany, pp. 208-357.

4. Kothari, V.O., Punjabi, A.B., and Gupta, S.R. (2009). Optimization of microwave assisted extraction of *Anona squamosa* seeds. The Icfai University Journal of Life Sciences, 3(1): 55-60.

5. Jorgensen, J.H., and Turnidge, J.D. (2003). Susceptibility test methods: Dilution and disk diffusion methods. In: *Manual of clinical microbiology* (8th edition),. Ed, PR Murray, ASM International, New York, USA, pp. 1108-1127.

6. Wadhwani, T., Desai, K., Patel, D., Lawani, D., Bahaley, P., Joshi, P., and Kothari, V. (2009). Effect of various solvents on bacterial growth in context of determining MIC of various antimicrobials. The Internet Journal of Microbiology, 7(1).

7. Eloff, J.N. (2004). Quantifying the bioactivity of plant extracts during screening and bioassay-guided fractionation. Phytomedicine, 11: 370-371.

8. Borgio, J.F., Thorat, P.K., and Lonkar, A.D. (2008). Antimycotic and antibacterial activities of *Gynandropis pentaphylla* DC extracts and its phytochemical studies. The Internet Journal of Microbiology, 5(2).

9. Wagner, H., Bladt, S., and Zgainski, E. (1983). Plant Drug Analysis (2nd edition), Springer, Berlin, Germany.

10. Talaro, K.P. (2008). Foundations in Microbiology: Basic Principles (6th edition), McGraw Hill, New York, USA, pp. 350.

Current Trends in Biotechnology and Pharmacy
Vol. 5 (1) 998-1003 January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

Optimal Production Conditions, Cultural and Physiological Characterization of an Active-inhibitory Compound(s) Producing *Streptomyces* Bb36 Isolate against Multiresistant Bacterial pathogens

Ismail Saadoun[1]* and Mohammad Alawawdeh[2]
[1]Department of Applied Biology, University of Sharjah, Sharjah, UAE, [2]Department of Applied Biological Sciences, Jordan University of Science and Technology, Irbid-22110, Jordan
*For correspondence - isaadoun@sharjah.ac.ae

Abstract

A *Streptomyces* Bb_{36} isolate was found to inhibit multi-drug resistant bacterial pathogens. When the activity of Bb_{36} *Streptomyces* isolate was compared to 23 standard antibiotics, data revealed that this isolate may not produce any of these antibiotics except cefotaxime and cefuroxime sodium. Bb_{36} isolate began to produce inhibitory bioactive compounds against *Escherichia coli* and *Staphylococcus aureus* after 5 days and optimally in yeast-extract malt-extract (YEME) broth at 28 °C with inhibition zone diameter = 8 mm. Ethanol extraction and evaporation of the culture filtrate revealed activity against *E. coli* and *S. aureus* at MIC of 10 and 5 mg ml^{-1} of the crude residues of evaporated ethanol extract with 8 and 11 mm inhibition zone diameter, respectively.

Key words: Bioactive, Compound, Conditions, Extraction, *Streptomyces*

Introduction

Actinomycetes and particularly members of the genus *Streptomyces* are the most important industrial microorganisms because of their capacity to produce numerous secondary metabolites particularly antibiotics (5, 10) that may ultimately find application as anti–infective, anti–cancer agents or other pharmaceutically useful compounds (1). However, in the past 20 years and due to the increase in the resistance of numerous pathogens to common drugs, the frequency of pathogens that cause invasive diseases has increased dramatically (2). For this reason, intensive screening programs for antibiotics are still running in different countries of the world and mainly from genus *Streptomyces* which provide a rich source of antibiotics (3, 12).

The traditional approach to/for isolation has been the use of terrestrial soils, which provide a rich source of these microorganisms (4). However, screening programs are still being initiated in various countries for isolation of such antibiotic-producing *Streptomyces* strains mainly from soil samples with new strategies that combine broad spectrum of activity against test pathogenic bacteria or fungi that are resistant to several known antibiotics.

In the course of our screening program for new antibiotics, *Streptomyces* Bb_{36} strain, was isolated and characterized. The bioactive compound(s) produced by this strain showed broad-spectrum activity against several pathogenic bacteria that are resistant to different antibiotics. Therefore, the purpose of this study was to examine the optimal conditions for bioactive compounds production by Bb_{36} *Streptomyces* isolate in addition to the extraction of these substances.

Vol. 5 (1) 998-1003 January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

Materials and Methods

Bb_{36} Streptomyces isolate and test organisms

In a previous study, Saadoun and Gharaibeh (7) characterized two *Streptomyces* isolates (Bb_{36} and Z_{11}) which inhibited the growth of multiresistant bacterial pathogens (*Escherichia coli, Staphylococcus aureus* and *Klebsiella pneumonia*). These pathogens were sensitive to at least one of these antibiotics (CTX: Cefotaxime , STR: Streptomycin , CXM: Cefuroxime, GN_{10}: Gentamycin). However they were resistant to at least five of these antibiotics (GN_{10}: Gentamycin; AM_{10}: Amoxicillin; STR: Streptomycin; OFF: Offloxacin (Novecin); COT: Cotimoxazole; ERY: Erythromycin; OXC: Oxaxillin; PEN: Penicillin; VAN: Vancomycin; CLT: Cephaloyhin; AUG: Augmantine; CTX: Cefotaxime; CXM: Cefuroxime sodium; TCP: Teicoplanin; TET: Tetracyclin; CMD: Cefamandole; CHL: Chloramphenicol; TOB: Tobramycin; CPR: Ciprofloxacin; SXT: Sulphamethoxazole; IMP: Impecillin; CB_{100}: Carbenicillin).

Optimal conditions for antibiotic production

Culture media **:** Bb_{36} *Streptomyces* isolate was grown in submerged cultures in 250 ml flasks containing 50 ml of the following broth culture media: Tryptone Yeast Extract (TYE), Nutrient Broth (NB), Yeast Extract Malt Extract (YEME) and Inorganic Salt Starch (ISS) (8). The flasks were inoculated with 1 ml of Bb_{36} active *Streptomyces* spore suspension and incubated at 28°C ± 1 for 21 days with shaking at 100 rpm.

Agar well diffusion method : The inhibitory activity was tested on Muller Hinton agar plates (8). Three cores of 8 mm diameter were removed from the agar plates that have pre-seeded with the test organisms using a sterile swab. Wells were filled up with a 100 µl supernatant broth of 3, 5, 7, 14 and 21 days culture. The plates were incubated at 37°C ± 1 for 48 h, and then inhibition zones were visualized and recorded.

Fermentation **:** Bb_{36} *Streptomyces* isolate that showed the highest activity against the pathogens in the above media was selected for seed culturing by growing the producer strain (Bb_{36}) on oatmeal agar at 28ºC ± 1 for 14 days. After growth, the whole aerial mycelium was scrapped by a loop and suspended in 10 ml of sterile distilled water. Aliquots of 0.5 ml of the spore suspension was inoculated in 250 ml Erlenmeyer flasks containing 50 ml of the production medium, pH=7.2. Twenty flasks of the broth medium were used for large-scale fermentation. All seed cultures were grown at 28ºC ± 1 with shaking in a rotary shaker incubator (TEQ, Portugal) at 100 rpm for 21 days. After fermentation, 5 ml of culture broth were transferred to a sterile tube and centrifuged at 5000 rpm for 15 min at 4 °C.

Extraction : The remaining broth was extracted with 4 volumes of absolute ethanol for overnight at 4ºC. After extraction, the pellet was discarded and the supernatant was evaporated using rotary evaporator (Heidolph, Germany) then the dry weight of the ethanol evaporate extract was determined.

Determination of MIC for the crude ethanol evaporates**:** Dried ethanol evaporate of Bb_{36} extract was suspended in 10 ml of distilled water to have a concentration of 0.1 g of the ethanol evaporate/ml. Three cores of 8 mm diameter were removed from the Mueller Hinton agar plates that have pre-seeded with the test organisms using a sterile swab. Holes were filled up with the dissolved ethanol evaporate extract (100 µl) and plates were incubated at 37°C ± 1 for 48 h then inhibition zones were visualized and recorded (6).

MIC of the ethanol evaporate was determined and the activity of the ethanol evaporate against the pathogens was compared to the activity of the broth production medium.

Cultural and physiological characterizations of Bb_{36} Streptomyces isolate **:** *Streptomyces* isolate (Bb_{36} strain) was characterized morphologically and physiologically according to the International *Streptomyces* project (ISP) (11) and as described by Saadoun et al. (9).

Results

Determination of optimum culture media for inhibitory compound(s) production **:** When the activity of Bb_{36} *Streptomyces* isolate was compared to 23 standard antibiotics, data revealed that this isolate may not produce any of the tested antibiotics except cefotaxime and cefuroxime sodium. Table 1 shows several broth media that have been examined to test the ability of the active *Streptomyces* strain Bb_{36} to produce inhibitory bioactive compounds against the tested pathogens. Data showed that strain Bb_{36} optimally produce inhibitory bioactive compound(s) against *E. coli* and *S. aureus* after 5 days when cultured in YEME broth with an inhibition zone diameter of 8 and 13 mm, respectively. Also, growth as measured by dry weight biomass production in the YEME broth medium was followed for 14 days (Table 1). Results from Table 1 demonstrated that Bb_{36} *Streptomyces* isolate prefer to produce these inhibitory substance(s) in YEME broth medium at the expense of growth.

Partial isolation and purification of the active compound(s) **:** It is necessary to extract and purify the substance responsible for the antibiotic activity, so further tests for evaluation were carried out with a simple ethanol extraction of the antibiotic from cells grown in liquid cultures. Table 2 shows the MIC of the evaporated water-soluble ethanol extract against *S. aureus* and *E. coli*. Ethanol extraction and evaporation of the

Table 1. Activity of Bb_{36} isolate at different incubation intervals and culture media.

Isolate	Broth Medium[a]	Final pH			Dry Weight (mg/ml)			Activity (mm)[b]					
					Days			*E. coli*			*S. aureus*		
		5	7	14	5	7	14	5	7	14	5	7	14
Bb36	TYE	8.53	8.75	8.65	1.2	0.0	0.0	13	-	-	10	-	-
	NB	8.36	8.66	8.76	0.2	0.6	1.0	-	-	-	-	-	-
	YEME	6.36	6.72	8.15	2.4	2.6	3.5	8	8	8	13	11	11
	ISS	6.42	6.70	8.08	2.4	2.9	3.3	-	-	-	-	-	-

[a] TYE: Tryptone Yeast Extract; NB: Nutrient Broth; YEME: Yeast Extract Malt Extract; ISS: Inorganic Salt Starch

[b]No activity was recorded at 3 and 21 days

Table 2. Activity of ethanol evaporate extract of *Streptomyces* B36 isolate.

	Extract Concentration (mg/ml)									
Bacteria	10	9	8	7	6	5	4	3	2	1
S. aureus	13[a]	13	13	13	13	11	-	-	-	-
E. coli	8	-	-	-	-	-	-	-	-	-

[a]Diameter of inhibition zone (mm, mean value, *N*= 3).

culture filtrate revealed activity against *E. coli* and *S. aureus* at MIC of 10 and 5 mg ml^{-1} with 8 and 11 mm diameter of inhibition zone, respectively (Table 2).

Characters of Bb_{36} Streptomyces isolate **:** Growth characteristics on different agar media are summarized in Table 3. Data were recorded after 14 days of incubation at 28 °C. *Streptomyces* Bb_{36} isolate form a yellow aerial mycelium and distinctive reverse side pigment and develop a spiral hyphae arrangements when grown on ISP medium No. 3. Culture of *Streptomyces* Bb_{36} isolate can grow at the expense of glucose, sucrose, xylose, arabinose, ramnose, manitol and

Table 3. Cultural and physiological characteristics of Bb_{36} *Strptomyces* isolate

Medium	Growth	Aerial Mycelium	Reverse Side	Soluble Pigment
Nutrient Agar	+3 Good	No Sporulation	White/Cream	No Pigment
Glucose-Asparagine Agar	+4 V. Good	Yellow	Yellow	No Pigment
Glycerol Asparagine Agar (ISP Medium No. 5)	+4 V. Good	Cream	L. Brown	No Pigment
Inorganic Salt Starch Agar (ISP Medium No. 4)	+3 Good	Cream	V.L. Brown	No Pigment
Glucose YE Malt Extract (ISP Medium No. 2)	+4 V. Good	Cream	V.L. Brown	No Pigment
Oatmeal Agar (ISP Medium No. 3)	+3 Good	Cream	Light Yellow	No Pigment
Tyrosine Agar (ISP Medium No. 7)	+4 V. Good	Cream	Brown	L. Yellow
Tryptone YE Broth (ISP Medium No. 1)	+1 Poor	-	-	V.l.Yellow

Physiological characters of Bb_{36} *Streptomyces* isolate

Melanin Production on ISP medium No.6	-
Growth at	
10°C	Slight or moderate growth
22°C	Good
28°C	Very Good
37°C and 45°C	No Growth
Growth in Glucose-YE-Malt Extract +	
0% NaCl	Very Good
7.5% NaCl	No Growth
10% and13% NaCl	No Growth
Carbon Utilization	
Glucose, Xylose, Sucrose, Arabinose, Rhamnose, and Meso-Inositol	Positive
Cellulose, Rafinose, Meso-Inositol	Negative

but not on cellulose, rafinose and meso-inositol. Melanin production on ISP-7 medium (Peptone-Iron Agar) for Bb36 isolate is negative. NaCl tolerance is low (less than 7.5%) and showed no growth at 37°C and 45°C; however, slight or moderate, good and very good growth was observed at 10°C, 22°C and 28°C, respectively (Table 3).

Discussion

Experiments on optimization for antibacterial compound(s) production showed that Yeast-Extract Malt-Extract (YEME) broth medium was the most suitable for production of the antibacterial compound(s) as indicated by the maximum inhibition zone diameter.

The minimum inhibitory concentration (MIC) for the evaporated water-soluble ethanol extract was 5 and 10 mg/ml against *S. aureus* and *E. coli*, respectively. This shows that the antibacterial compound(s) from *Streptomyces* strain (Bb_{36}) was less active, when it compared to the MIC value of the tested drugs. There are various factors affecting the activity, the solvent used for extraction may not be suitable for it or the solvent may not properly extracted the compound. The MIC is not constant for a given agent, because it is affected by the nature of the test organism used, the inoculums size, and the composition of the culture medium, the incubation time, and aeration. In addition, these studies were based on a crude form of the compounds and not on completely purified one.

Acknowledgments

This research was funded by Islamic Organization for Education, Culture and Science (ISESCO) (Grant No. 42/02).

References

1. Bibb, M. J. (2005). Regulation of secondary metabolism in streptomycetes. Microbiology 8, 208-215.
2. Georgopapadakou, N. H. (1998). Antifungal mechanism of action and resistance established and novel drugs. Microbiology 1, 547-557
3. Goodfellow, M. and O'Donnell, A.G. (1989). Search and discovery of industrially significant actinomycetes. *In* Microbial Products: New approaches. Ed., S. Baumberg, I. Hunter and M. Rhods, Cambridge University Press, pp. 343-383.
4. Labeda, D.P. and Shearer, M.C. (1990). Isolation of actinomycetes for biotechnological applications. *In* Isolation of biotechnological organisms from nature. Ed., D.P. Labeda, McGrraw-Hill Publishing Company, pp. 1-19.
5. Lee, J. Y. and Hwang, B. K. (2002). Diversity of antifungal actinomycetes in various vegetative soils of Korea. Microbiology 48, 4067-417
6. Malik, M. A. (1997). Isolates of soil actinomycetes with potential for phytotoxon production. Journal of Chemical Ecology 23, 2683-2693
7. Saadoun, I. and Gharaibeh, R. (2002). The *Streptomyces* flora of Badia region of Jordan and its' potential as a source of antibiotics active against antibiotic-resistant bacteria. Journal of Arid Environments 53, 365-371.
8. Saadoun, I. and Muhana, A. (2008). Optimal production conditions, extraction, partial purification and characterization of inhibitory compound(s) produced by *Streptomyces* Ds-104 isolate against multi-drug resistant *Candida albicans*. Current Trends in Biotechnology and Pharmacy 2, 402-420.

9. Saadoun, I., Wahiby, L., Ababneh, Q., Jaradat, Z., Massadeh, M. and AL-Momani, F. (2008). Recovery of soil streptomycetes from arid habitats in Jordan and their potential to inhibit multi-drug resistant *Pseudomonas aeruginosa* pathogens. World Journal of Microbiology and Biotechnology 24, 157-162.

10. Sahin, N. (2003). Investigation of the antimicrobial activity of some *Streptomyces* isolates. Turkish Journal of Biology 27, 79-84.

11. Shirling, E.B., and Gottlieb, D. (1966). Methods for characterization of *Strptomyces* species. International Journal of Systematic Bacteriology 16, 313-340.

12. Takizawa, M., Colwell, R.R. and Hill, R.T. (1993). Isolation and diversity of actinomycetes in the Chesapeake Bay. Applied and Environmental Microbiology 59, 997-1002.

Current Trends in Biotechnology and Pharmacy
Vol. 5 (1) 1004-1010 January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

Effect of Growth regulators, Sucrose and Glutamine on Shoot Regeneration from Node Derived Callus Cultures of Rubber yielding species - *Parthenium argentatum*

A. Maruthi Rao[1], I. Sampath Kumar[1], P. Sri Laxmi[2], P. Maheshwari Rao[2], P. Hima Kumari[2], S. Anil Kumar[2] D. Madhavi[2], E. Venu Madhav[3] and P. B. Kavi Kishor[2*]

[1]Plant Biotechnology Research Lab, Department of Botany, Telangana University, Nizamabad 503 322, India
[2]Department of Genetics, Osmania University, Hyderabad 500 007, India
[3]Department of Biotechnology, Achayra Nagarjuna University, Nagarjuna Nagar, - 522 510, India
*For correspondence - pbkavi@yahoo.com

Abstract

The nodal explants of guayule (*Parthenium argentatum* Gray) when cultured on Murashige and Skoog's (MS) medium fortified with different concentrations of kinetin (KN), 6-benzylaminopurine (BAP), 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4-D + BAP, napthaleneacetic acid (NAA) and NAA + BAP produced callus tissues and few shoots. The percent frequency of callus was around 90% with 0.5 mg/l BAP and NAA. The high frequency shoot regeneration from the node derived callus of guayule was observed when MS medium was supplemented with BAP, NAA + BAP with varying concentrations of sucrose. Sucrose at 3-4% levels increased the number of shoots formed (12-15) per 250 mg of callus. Glutamine at 200 mg/l level further enhanced the number of shoots formed (22-25) per callus mass. The percent frequency of shoot regeneration from callus tissues was high (88%) when the medium was fortified with 0.1 mg/l NAA + 2.5 mg/l BAP, 200 mg/l glutamine and 2% sucrose. The ability of the callus tissues of guayule remained more or less same for shoot regeneration over a period of 200 days; but decreased thereafter. Shoots rooted with 15 to 75% frequency and the whole plants when transferred to pots survived with 60% frequency.

Key words : Callus cultures, plant regeneration, guayule (*Parthenium argentatum* Gray).

Introduction

Parthenium argentatum Gray belongs to the family Asteraceae and is a highly branched perennial shrub, which at maturity ranges from 0.3 to 1.0 m in height and 0.6 to 1.2 m in width (1, 2, 3, 4). It is a native of the Chihuahuan desert with a natural history of exposure to water stress (5). Plants in the field may be established from nursery grown seedlings and cuttings (6). Rubber is a macromolecular polyisoprenoid found in over 2000 species (7, 8, 9). In recent years, guayule which accumulates rubber within the ordinary stem bark parenchyma cells and contains high molecular mass rubbers comparable to *H. brasiliensis* has attracted research interest as an additional source of rubber (10, 11, 12). The major limitations for the commercial production of guayule rubber are its low rubber yields and inconsistent field establishment by direct seedlings. This is because of susceptibility of seedlings to water and salinity stresses (13, 14, 15). The decrease in rubber yield in water – stressed plants is attributed to reduced biomass production (13, 16). Therefore, there is a need either to create variation in guayule or generate transgenics which can resist water and salt

stresses. Efficient methods of plant regeneration from tissue cultures are necessary for the selection or the creation of useful variants and also for taking up genetic engineering studies. Subramanian and Zavala *et al* (17, 18) reported *Parthenium* callus cultures that developed roots and shoots. Mass propagation of guayule by *in vitro* organ and callus cultures was developed by Dhar *et al* (19). Present study deals with the effect of different growth regulators, glutamine and sucrose concentration on the regeneration of shoots from node derived callus cultures of guayule.

Materials and Methods

Nodal explants measuring 0.5 to 0.8 cm from 2-year-old shrubs of guayule were surface sterilized with 0.1% mercuric chloride for 6-8 minutes followed by washing with sterile glass distilled water. The explants were inoculated one into each test tube containing 15 ml of MS agar medium (20) supplemented with different combinations and concentrations of growth regulators. The pH of the medium was adjusted to 5.8 before solidifying with 0.8% agar. Callus tissues initiated from the nodal explants were sub-cultured routinely onto MS medium containing 0.2 mg/l NAA + 0.2 mg/l IAA + 1 mg/l BAP and incubated at 26 ± 2 ^{0}C. Callus was sub-cultured for every 25-30 days. For generation of shoots, approximately 250 ± 20 mg of callus was used. For rooting of the *in vitro* derived shoots, half the MS salt concentration but with full strength of iron was used with varying concentrations of auxins either in light (30 μE m^{-2} s^{-1}) or in dark at 26 ± 2 ^{0}C. Before transferring the rooted shoots, they were washed thoroughly to remove the adhered agar if any into earthen pots containing a mixture of soil, sand and farmyard manure in the ratio of 1:1:1 and grown in the net-house for acclimatization. Hoagland solution was added during this period once in every 2-days. Percentage survival of plants was recorded after one month of transfer to the pots.

Results and Discussion

Effect of growth regulators on callus initiation from nodal explants : NAA or 2,4-D alone, 2,4-D and BAP together produced only callus but not organogenesis. With an increase in the concentration of KN and BAP from 1 mg/l to 5 mg/l, there is a gradual decrease in the frequency of callus production (Table 1) but increase in the multiple shoot induction directly from the nodal explants (data not shown). Nodal explants producing only callus with different plant growth regulators is 75% with 0.5 mg/l KN; 90% with 0.5 mg/l BAP; 70% with 1 mg/l 2,4-D; 50% with 0.5 mg/l NAA + 0.5 mg/l BAP but 92% with 0.5 mg/l NAA.

Effect of sucrose and glutamine on number of shoots formed per callus mas : The number of shoots formed per callus mass (250 ± 20 mg) is shown in Table 2. BAP at the concentrations of 0.1 mg/l and 1 mg/l in the medium (devoid of glutamine) exhibited 49% and 58% frequency of shoot regeneration respectively. The frequency of shoot regeneration from the callus of guayule increased considerably when 0.1 mg/l NAA and 2.5 mg/l BAP were used together. Sucrose at 0.5% did not show any organogenetic response. Shoot differentiation was 85% with the incorporation of 0.1 mg/l NAA + 2.5 mg/l BAP (devoid of glutamine) along with 4% sucrose in the medium. However, 6% sucrose drastically reduced the shoot forming ability of the callus. Addition of 200 mg/l of glutamine greatly improved the number of shoots from 12-15 to 22-25 per calli (Table 3). Thus, the concentration of glutamine and sucrose in the medium greatly affected the percentage frequency of shoot regeneration in guayule. The frequency of calli showing shoot regeneration is high (88%) when

Table 1. Morphogenetic response of nodal explants of guayule (*Parthenium argentatum* Gray) to different hormonal treatment.

Hormonal concentration (mg/l)	Number of explants inoculated	Percentage of explants giving callus
No hormone	20	-
0.5 KN	15	75
1.0 KN	18	35
0.5 BAP	24	90
1.0 BAP	25	30
0.5 2,4-D	18	60
1.0 2,4-D	20	70
1.0 2,4-D + 0.5 BAP	22	32
1.0 2,4-D + 1 BAP	28	44
1.0 2,4-D + 2 BAP	31	50
2.0 2,4-D + 3 BAP	32	56
0.5 NAA	20	92
1.0 NAA	26	90
0.5 NAA + 0.5 BAP	20	50

*Data collected from 10 cultures per treatment.

Table 2. Influence of sucrose on organogenetic response of node derived callus of guayule.

MS medium with hormones (mg/l)	Sucrose (g/l)	Number of calli cultured	Number of shoots formed per callus mass (250 ± 20 mg)
0.1 BAP	20	18	3-4
1.0 BAP	20	20	6-8
0.1 NAA + 2.5 BAP	20	18	5-8
0.5 NAA + 3.0 BAP	20	18	8-11
0.1 NAA + 2.5 BAP	5	16	-
0.1 NAA + 2.5 BAP	10	20	3-4
0.1 NAA + 2.5BAP	20	20	5-8
0.1 NAA + 2.5 BAP	30	18	12-15
0.1 NAA + 2.5 BAP	40	19	12-14
0.1 NAA + 2.5 BAP	60	25	3-4

*Data collected from 10 cultures per treatment.

Table 3. Effect of glutamine on organogenetic response of node derived callus of guayule.

MS medium with hormones (mg/l)	Glutamine (mg/l)	Sucrose (g/L)	Number of calli cultured	Number of shoots formed per callus mass (250±20 mg)
0.1NAA + 2.5 BAP	200	20	20	13-15
0.1 NAA + 2.5 BAP	200	20	25	22-25
0.1 NAA + 2.5 BAP	200	20	23	22-25
0.1 NAA + 2.5 BAP	200	20	20	10-15
0.02 NAA + 2.5 BAP	200	20	18	18-20

*Data collected from 10 cultures per treatment.

MS medium was supplemented with 0.1 mg/l NAA + 2.5 mg/l BAP + 200 mg/l glutamine and 2% sucrose. NAA in combination with BAP was found more effective in shoot formation than BAP alone.

Effect of age of callus on the percent frequency of shoot regeneration and rooting of shoots : Shoot regeneration ability decreased from 84 to 30% with an increase in the age of the callus from 100 to 250 days (Table 4). Shoots obtained from the callus tissues were transferred to MS agar medium fortified with either IAA or NAA or 2,4-D. Increasing the concentrations of IAA (0.04 mg/l to 0.5 mg/l) or NAA (0.08 mg/l to 0.5 mg/l) enhanced the percentage of cultures rooting at the cut ends of shoots when incubated in dark. On the other hand, 2,4-D produced roots both in light as well as in dark. The synthetic auxin 2,4-D in the medium (0.04 mg/l and 0.06 mg/l) seemed to be better when maintained in light compared to higher concentrations for rooting of shoots. The number of roots differentiated per shoot for three hormones is shown in Table 5. Plantlets were later transferred to pots containing sand and soil mixture in the ratio of 1: 1. Plants were covered with glass beakers to maintain humidity and watered with Hoagland nutrient solution at 3-4 day intervals. Glass beakers were removed after two weeks of transfer to the pots. The frequency of survival was 60% and the plants did not show any morphological variations.

Table 4. Regenerating ability of node derived callus cultures of guayule (*Parthenium argentatum* Gray) from long-term cultures.

Age of callus (in days)	% frequency of shoot regeneration
50	88
60	85
100	84
200	85
250	30

*Data collected from 10 cultures per treatment.

Arreguin et al (21) established *Parthenium* cultures from stems. Zavala et al (18) obtained axenic cultures from seedling explants or its cotyledons or leaf blades, petioles and roots. MS medium supplemented with 0.5

Table 5. Treatment of excised shoots with IAA, NAA, 2,4-D and the percentage of cultures rooting at cut end.

Treatment (mg/l)	% of cultures rooting at cut ends		Number of roots per culture
	Light	Dark	
0.02 IAA	-	-	-
0.04 IAA	-	50	1.0
0.06 IAA	-	15	1.0
0.08 IAA	-	65	2.5
0.1 IAA	-	70	2.5
0.5 IAA	-	75	3.0
0.02 NAA	-	-	-
0.04 NAA	-	-	-
0.06 NAA	-	-	-
0.08 NAA	-	70	3.5
0.1 NAA	-	72	5.5
0.5 NAA	-	74	6.5
0.02 2,4 – D	35	38	3.5
0.04 2,4 – D	70	45	4.5
0.06 2,4 – D	72	50	8.5
0.08 2,4 – D	60	50	9.0
0.1 2,4 – D	55	42	8.0
0.5 2,4 – D	48	30	4.0

*Data collected from 10 cultures per treatment.

mg/l 2,4-D and 1 mg/l BAP formed optimum callus and growth in *Parthenium hysterophorus* (22). On the other hand, MS medium containing 0.18 mg/l IAA and 0.02 mg/l KN showed good growth of the callus (17). Callus cultures of guayule exhibited superior growth with a combination of 1 mg/l kinetin, 0.2 mg/l NAA and 2 mg/l inositol (18). In the present study, lower concentrations of KN, BAP (0.5 mg/l), 2,4-D (1 mg/l) and NAA (0.5 mg/l) seemed good for callus initiation and also when 2,4-D plus BAP were used together. Subramanian et al (17) reported shoots from stem derived callus tissues of *P.hysterophorus* on MS medium incorporated with IAA (1.75 mg/l) plus either KN (1.01 mg/l) or BAP (1.13 mg/l). Zavala et al (18) reported shoot formation from root or hypocotyl derived callus tissues of *P.argentatum* on Mahlberg's medium fortified with KN (0.1 mg/l), 2,4-D (0.2 mg/l), NAA (0.2 mg/l) and 2 mg/l inositol. MS medium containing 0.1 mg/l KN and 10 mg/l 2(3,4-dichlorophenoxy) triethylamine derivative obtained shoots from callus (23). Shoot differentiation on MS medium supplemented with 0.1 mg/l BAP was reported by Staba and Nygaard (24). In the present study, addition of glutamine (200 mg/l) resulted in the highest frequency (84-88%) of shoot regeneration. There is an increase in the

shoot regeneration ability from the callus tissues when the medium was supplemented with more than 2%. Higher sucrose (4%) in the medium along with 0.1 mg/l NAA + 2.5 mg/l BAP showed 85% frequency of shoot regeneration in the present study. But, 6% sucrose drastically reduced the shoot forming ability. So, in *Parthenium*, the concentration of glutamine and sucrose in the medium greatly affected the shoot regenerating ability. Increase in the age of the callus decreased the percent frequency of shoot regeneration.

Zavala et al (23) reported the formation of roots from callus cultures on a solid medium containing inositol (1-2 mg/l) or inositol and casein hydrolysate (1 mg/l), and different concentrations of 2,4-D, KN or NAA. Differentiation of roots was also shown from callus tissues grown on MS medium containing 0.1 mg/l each of 2,4-D, BAP and kinetin or 2,4-D and 6-dimethylallylamino purine (24). In the present study, MS medium containing 0.5 mg/l IAA or NAA was the best for root initiation of shoots grown in dark and increase in the concentration of 2,4-D from 0.02 mg/l to 0.08 mg/l, increased the percentage of cultures rooting at the cut ends.

References

1. Foster, M.A., Flower, J.L., Kleine, L.G., Grote, M.M. and Puppala, N. (2002). Performance of direct seeded and transplanted guayule. Ind Crops Prod., 15: 23-31.

2. Foster, M.A. and Coffelet, T. (2005). Guayule agronomics: Establishment irrigated production and weed control. Ind Crops Prod., 22: 27-40.

3. Veatch, M.E., Ray, D.T., Mau, C.J.D. and Cornish, K. (2005). Growth, rubber and resin evaluation of two-year-old transgenic guayule. Ind Crops Prod., 22: 65-74.

4. Y Kema, R.E. and Stutz, J.C. (1991). Isolation, identification, and pathogenecity of *Fusarium* Spp. from guayule in Arizona. Plant Disease. 75: 736–738.

5. Maren, E., Veatch –Blohm, Dennis Ray, T. and William Mc Closkey, B. (2006). Water – stress – induced changes in resin and rubber concentration and distribution in green house – grown guayule. Agron J., 98: 766-773.

6. Nishimura, M.S., Emerson, R., Hata, T., Kageyama, A., (1944). The propagation of guayule from cuttings. American Journal of Botany. 31: 412-419.

7. Archer, B.L., Audley , B.G., (1973). In Phytochemistry (Nord, F., and Miller, L., Eds) Vol 2, pp. 310-343, Van Nostrand Rein Hold, New York.

8. Backhaus, R.A. (1985). Rubber formation in plants: a mini review. Isr J Bot, 34: 283-293.

9. Zhiqiang Pan, Francis Durst, Daniele Wreck Reichhart, Harold Gardner, W., Bilal Camara, Katrina Cornish and Ralph Backhaus, A. (1995). The major protein of guayule rubber particles is a cytochrome P450: Characterization based on cDNA cloning and spectroscopic analysis of the solubilized enzyme and its reaction products. The Journal of Biological Chemistry. 270: 8487–8494.

10. Gomez, J.B. (1975). In Proceedings of the International Rubber Conference. Vol. 2. pp. 143-164, Rubber research institute of Malaya, Kualampur.

11. Jeong Kim, Stephen B. Ryu., Yeon Sig Kwak., Hunseung Kang., (2004). A novel cDNA from *Parthenium argentatum* Gray enhances the rubber biosynthetic activity *in*

vitro. Journal of Experimental Botany J Exp Bot., 55: 377-385.

12. Mooir Broek, H. and Cornish, K. (2000). Alternative sources of natural rubber – mini review. Applied Microbiology and Biotechnology. 53: 355-365.

13. Ehrler, W.L., Bucks, D.A. and Nakayama, F.S. (1985). Relations among relative leaf water content, growth, and rubber accumulation in guayule. Crop Sci., 25: 779-782.

14. Nakayama, F.S. and Bucks, D.A. (1984). Crop water stress index, soil water, and rubber yield relations for the guayule plant. Agron. J., 76: 791-794.

15. Tipton, J.L. (1988). Drought and salinity stress effects on guayule seedling emergence. J Amer Soc Hort Sci., 113: 129-133.

16. Nakayama, F.S., Bucks, D.A., Roth, R.L. and Gardner, B.R. (1991). Guayule biomass production under irrigation. Bioresourse Technol., 35: 173-178.

17. Subramanian, V. and Subba Rao, P.V. (1980). *In vitro* culture of the allergenic weed *Parthenium hysterophorus* L. Plant Sci Lett, 17: 269-277.

18. Zavala, M.E., Biesboer, D.D. and Mahlberg, P.G. (1980). Abstract: 3rd International Guayule Conference, Pasadena, California.

19. Dhar, A.C., Kavi Kishor, P.B. and Rao, A.M. (1989). *In vitro* propagation of guayule (*Parthenium argentatum)* – a rubber yielding shrub. Plant Cell Reports. 8: 489-492.

20. Murashige, T., Skoog, F., (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum. 15: 473-497.

21. Arreguin, B. and Bonner J. (1950). The biochemistry of rubber formation in the guayule. II. Rubber formation in aseptic tissue cultures. Arch Biochem, 26: 178-186.

22. Wickham, K., Rodriquez, E. and Arditti, J. (1980). Comparative phytochemistry of Parthenium hysterophorus L. (Compositae) tissue cultures. Bot Gaz. 141: 435-439.

23. Dastoor, M.N., Schubert, W.W. and Petersen, G.R. (1981). Preliminary results of in vitro propagation of guayule. J Agr Food Chem., 29: 686-688.

24. Staba, E.J. and Nygaard, B.G. (1983). *In vitro* culture of guayule. Z pflanzen Physiol, 109: 371-378.

Current Trends in Biotechnology and Pharmacy
Vol. 5 (1) 1011-1020 January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

Protein Expression and Microscopic Evidence of White Spot Syndrome Virus (WSSV) in Tiger prawns *Penaeus monodon*

Bhatt, S.S.[a*], Chavda, D. V.[a] and Sreepada, R. A.[b]
[a]B.R. Doshi School of Biosciences, Zoology Laboratory, Bakrol Vadtal Road, Satellite Campus, Sardar Patel University, Vallabh Vidhyanagar 388120, India
[b]National Institute of Oceanography, Dona Paula, Goa 403 004, India
*For Correspondence - bhatt.sujata@gmail.com

Abstract

Shrimp culture is being practiced in large areas of coastal farms of South Gujarat region in Western India. The disease caused due to white spot syndrome virus (WSSV) has been detected in the cultured *Penaeus monodon* in this region. White spots were clearly visible in the carapace of infected shrimps. The scanning electron micrographs of the upper and lower surfaces of carapace revealed the presence of white spots measuring about 78µm to 135µm. In the infected shrimps, rod shaped viral particles have also been identified from the epidermis associated with carapace by TEM using negative staining. The histo-pathological analysis of gills, hepatopancreas, gut and muscles of *P. monodon* infected with WSSV indicated hypertrophied nuclei, presence of intranuclear inclusion bodies and chromatin margination in gill epithelial cells and necrosis of gill lamellae. In the infected muscles, necrosis, haemocytic infiltration and degeneration of muscle fibers were clearly evident. Damage to the mucosal epithelial cells of gut was accompanied by haemocytic inflammatory response. Inflammatory response and necrotic changes in hepatopancreas of infected shrimps have been observed. Hypertrophied and piknotic nuclei as well as infiltration of viral particles into connective tissues associated with vacuolization were observed in all the analyzed infected tissues. Changes in the expression of proteins have been detected by SDS-PAGE in the infected muscles and hepatopancreas. Four new proteins of 79kDa, 58kDa, 36kDa and 17kDa were found to be differentially expressed in muscles. In hepatopancreas, three proteins viz. 46kDa, 20kDa and 17kDa were differentially expressed in infected shrimps; whereas 62kDa protein, which was expressed in healthy shrimps, was not expressed in infected shrimps. No change in the protein expression pattern was detected in gills. The current study has shown that WSSV, besides causing damage to the tissues, has also drastically altered the protein expression patterns of the infected tissues of *P. monodon.*

Key words: WSSV, protein expression, *Penaeus monodon*, SDS-PAGE

Introduction

Intensive shrimp aquaculture is one of the most important aquaculture practices in the world. Intensive culture practice has also resulted in the development of diseases including viral diseases in shrimp culture units (1). White spot syndrome virus (WSSV) is one of the highly pathogenic shrimp viruses affecting the shrimp culture worldwide with severe economic losses; and its characterization is critical for developing effective control methods (2,3). Though, prawns infected with WSSV or YHV have been reported to be safe for human consumption (4); the

pathogenic effects of WSSV infected shrimps in human cannot be completely ruled out. The virus was first reported in India during 1994 (5) and it has been isolated and characterized from Indian shrimp, *Penaeus indicus* and *Penaeus monodon* (6,7). The WSSV has also been found to be pathogenic to other aquatic hosts like crabs, copepods and also to fresh water prawns (6,7).

White spot syndrome virus is characterized by the development of white spots in exoskeleton (8,9) and is reported to infect mainly tissues of mesodermal and ectodermal in origin (10). Various diagnostic methods like histology, immunological methods, polymerase chain reaction, in-situ hybridization and several other methods are being used for the identification of WSSV infection (1,11-13). The WSSV infection to shrimp is also known to show changes in the expression of proteins in several tissues (14).

Shrimp culture is being extensively practiced in the coastal farms of South Gujarat (India). During the monitoring of hygienic conditions of these aquaculture units, WSSV has been observed in cultured *P. Monodon* in growout ponds by morphological observations. The present study was undertaken to determine the effect of WSSV on the cultured *P. monodon* and identification of pathogen. During the investigations, the morphology and chemical composition of white spots of inside and outside surfaces of carapace were examined by scanning electron microscopy (SEM) along with energy-dispersive X-ray analysis (EDX). The WSSV virus has been identified in the cuticular epithelium by transmission electron microscopy (TEM) with negative staining. Damage caused by viruses to different tissues have been analysed by histopathological techniques. Along with histopathological studies, changes in the expression of proteins in several tissues of WSSV infected shrimps have also been studied.

Materials and Methods

1. Sample collection and tissue processing : *Penaeus monodon* with white spots on carapace and other body parts as well as without white spots (healthy shrimps) were collected from the costal farms of South Gujarat (Olpad, Surat district, 21° 19' 60N, 72° 45' 0E) region of Western India during June-2008 and brought to the laboratory at 4 ºC. Carapace from the shrimps with and without infection were collected and stored at -20ºC for morphological analysis of white spot and for the study of change in the chemical composition of cuticle of white spot region. Hepatopancreas and muscles from infected and healthy shrimps were removed and stored at -20ºC to study the changes in the expression of proteins during infection. Epidermis from the carapace of infected shrimps is removed and stored at -20ºC for identification of pathogen. Hepatopancreas, muscles, gills and gut from both infected and healthy shrimps were fixed to study histopathological changes in these tissues during infection.

2. Scanning electron microscopy and EDX : For the morphological analysis of white spots, the carapaces of diseased shrimps were air dried and the morphology and size of white spots were examined under SEM (Philips ESEM series). The EDX quantification of calcium (Ca), carbon (C), oxygen (O) and phosphorus (P) from upper and lower surface of the carapace for both infected (from white spot region) and healthy shrimps has been performed with SEM.

3. Negative Staining : Epidermis from the carapace region of infected *Penaeus monodon* was removed and homogenized in sterile brackish water at 4ºC (1:9 V/W), centrifuged at 8510×g at -4ºC for 5 min and the supernatant was filtered through a 0.45ì m membrane. The collected filtrate was again centrifuged at 14549×g at -4ºC for 1.5 h and the resulting pellet was resuspended

in few drops of sterilized brackish water prior to negative staining. For negative staining, one drop of suspension was mixed with 4 drops of the mixture of 0.1% bovine serum albumin and 2% phosphotungstic acid (1:2, pH 7.0). The mixture was placed on a grid for 30 to 60 s and excess suspension was removed with filter paper. The preparation was allowed to dry before being examined. The results were observed under TEM (Technai, 20; Philips series) (15).

4. Histopathology : The histopathology of gills, hepatopancreas, muscle and gut were performed from WSSV infected and healthy shrimps. Tissues were blotted free of heymolymph, fixed in Bouin's fluid, sections were cut at 5-8µm and stained with H & E. The photographs were taken with Axioplan image analyzer at 40X and 100X.

5. SDS PAGE Analysis : Muscles and hepatopancreas, 0.5gm each, from infected and healthy shrimps were homogenized in 10ml TN buffer (20 mM Tris-HCl, 400 mM NaCl, pH 7.4), centrifuged at 8510×g for 10min at -4ºC and supernatant was subjected to polyacrylamide gel electrophoresis using 10% polyacrylamide resolving gel and 5% stacking gel (16). The resolving gel (30ml) was polymerized by 300µl of 10% ammonium peroxodisulphate and 70µl of N, N, N, N-Tetramethylenediamine (TEMED). Stacking gel (10ml) was polymerized by adding 50µl APS and 20µl TEMED. The samples were mixed with sample buffer, boiled for 5 min and all gel were run using a dual midi vertical gel electrophoresis system at constant 100V in 1X buffer (25mM Tris, 25mM glycine, pH 8.3, 0.1% SDS). Proteins were visualized by silver staining kit (Genie, Bangalore).

Results

1. Morphological symptoms : The infected shrimps exhibited lethargy, anoxia, opaque musculature and were detected near pond edges. They were detected with white spots on the carapace, on appendages and on other body parts as well as with pink to red body colouration (Fig. 1). The other symptoms of infected *P. monodon* were loose binding of the epidermis with cuticle and enlarged hepatopancreas with yellowish white colouration.

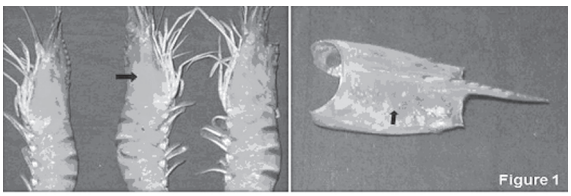


Fig. 1 White spots of *P. monodon*. Arrow indicates the white spots on the cuticle and other body parts.

2. Scanning electron microscopy : The scanning electron micrographs show white spots on both upper and lower surfaces of carapace of infected shrimps. On the inner surface of the carapace, spots were larger in size, usually rounded in shape measuring 25µm to 135µm in diameter. On the outer surface of carapace, white spots were very small in size and measuring between 25 to 32µm in size (Fig. 2a and 2b).

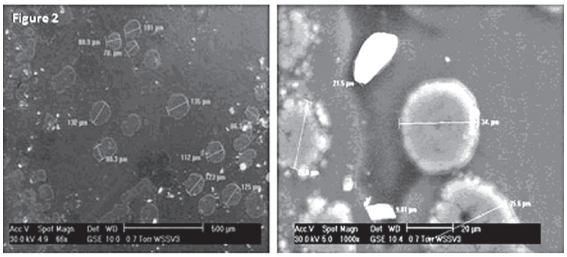


Figure 2a-2b Scanning electron micrograph of white spots of WSSV infected shrimps. Flattened White spot covering the cuticle of carapace (a, b).

EDX quantification of elements like C, O, Ca and P in carapace from both inner and outer surface with SEM in infected and control shrimp indicated that composition of inner and outer surface of white spots have not been changed and were measured as 38%; 32 to 33 %; 0.8 to

0.7% and 29% respectively. At the same time, as compared to control in white spot region, Ca and O were increased; whereas C has decreased (Fig 3a-3d; Table 1).

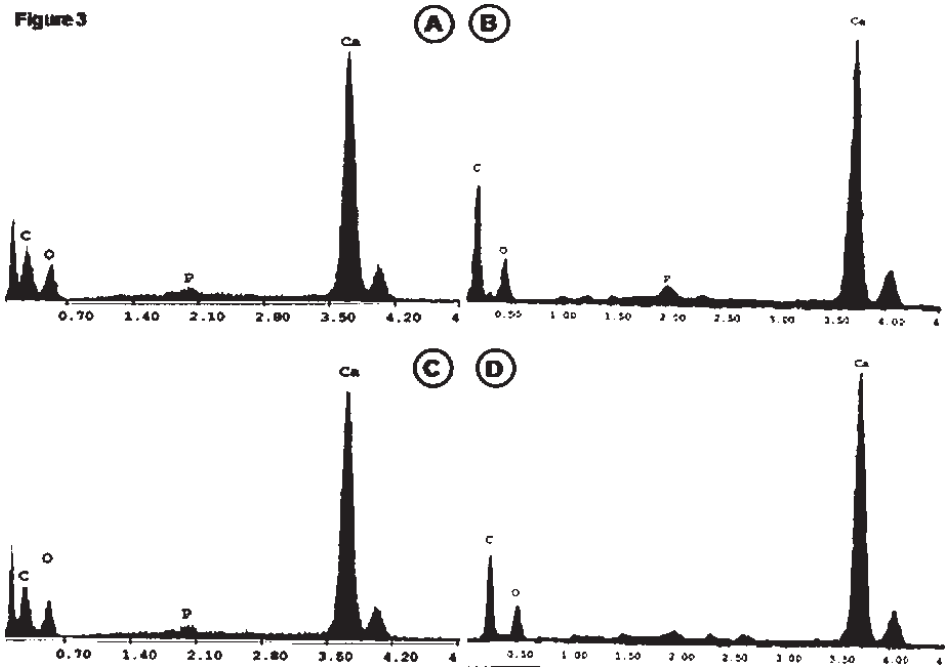


Figure 3a-3d shows the EDX graphs of different levels of carbon (C), oxygen (O), calcium (Ca) and phosphorus (P) of carapace in infected as well as healthy shrimps (Infected lower surface-3a, Control lower surface-3b, Infected upper surface-3c and Control upper surface-3d).

Table 1 shows the values of elements for both upper and lower surfaces of WSSS infected and uninfected shrimps by EDX analysis (Mean, n=4).

Metal	Infected lower surface (%)	Infected upper surface (%)	Control upper surface (%)	Control lower surface (%)
Calcium (Ca)	27.35	27.07	25.53	21.11
Carbon (C)	38.87	38.77	46.03	51.37
Oxygen (O)	32.98	33.39	28.44	26.78
Phosphorus (P)	0.81	0.77	-	0.73

3. Negative Staining : The result of negative staining of the pellet from the filtrate of diseased *Penaeus monodon* epidermis is shown in fig. 4. Viral particles with rod shaped morphology can be seen.

Fig. 4 shows rod shaped viral particles by negative staining in epidermal homogenate.

4. Histopathology : Histo-pathological changes in foregut, gills, hepatopancreas and muscle from tiger shrimp naturally infected with white spot syndrome were observed. The most pronounced changes were observed in gut epithelia. The cells exhibited hypertrophied nuclei with eosinophilic to basophilic inclusion bodies. Large number of nuclei demonstrated central chromatin diminution and margination. Complete condensation of nuclei was also observed (Fig. 5b). Pyknosis and karyorrhexis were evident with the necrosis of tissue leading to changes in the normal architecture of the tissues. The WSSV infection in gills was indicated by hypertrophied nuclei, basophilic inclusion bodies and complete condensation of nuclei. Chromatin margination was detected at lower level (Fig. 5a). Pyknosis and karyorrhexis were more pronounced in connective tissue compartment of hepatopancreas; though damage to the tubular epithelial cells of hepatopancreas was not clearly evident (Fig. 5c). The muscle tissue showed severe necrosis, fragmentation and haemocytic infiltration. Nuclear hypertrophy, chromatin margination and chromatin condensation were also evident at lower level (Fig. 5d).

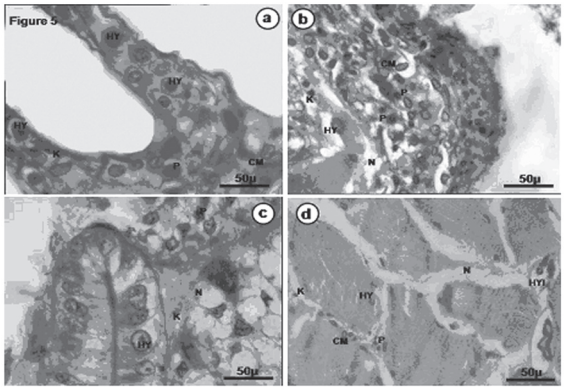


Fig. 5a to 5d tissues of *P. monodon* infected with WSSV. The histopathological changes like hypertrophied nucleus (HY), pyknosis (P), chromatin margination (CM), karyorrhexis (K) and necrosis (N)-in gills (a); gut (b); hepetopancreas (c); and muscle (d) (Fig. 5a to d scale bare=50µm).

5. SDS PAGE : A clear change in expression of proteins in muscles and hepatopancreas has been detected in infected shrimps as compared to uninfected ones during SDS-PAGE analysis. In infected muscle tissue, four newly expressed proteins were detected (Fig. 6b). Two newly expressed proteins are of higher molecular

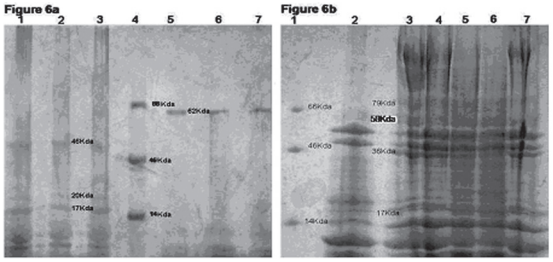


Fig. 6a In infected hepatopancreas, lane 1, 2, 3 shows expression of three proteins 17kDa, 20kDa and 46kDa; lane 5, 6, 7 shows expression of protein from control samples and lane 4 is molecular weight marker band.
Figure 6b In muscle, Lane 3, 4, 5, 6, 7 shows four newly express proteins 79kDa, 58kDa, 36kDa and 17kDa; Lane 1 is molecular weight marker band and lane 2 is for control sample.

weight, 79kDa and 58kDa; whereas two newly expressed proteins are of low molecular weight, 36kDa and 17kDa. Hepatopancreas of infected shrimps exhibited two new protein bands with low molecular weight, 17kDa and 20kDa, as well as a protein band with high molecular weight, 46kDa (Fig. 6a). In both the infected tissues, a newly expressed protein with molecular weight 17kDa has been detected. In hepatopancreas, a 62kDa protein band has not been detected which has been expressed in uninfected shrimps.

Discussion

The outbreak of white spot syndrome in cultured penaeid shrimps have been observed in India since 1983 (5,17). In Gujarat (India), shrimp culture is being practiced in the coastal farms in the southern region; but the outbreak of the diseases in these cultured ponds appears not to have been analyzed systematically. During the monitoring of hygienic condition of these aquaculture units, *P. monodon* with the morphological symptoms of WSSV have been detected. Monitoring of WSSV in the growout ponds has been suggested as one of the health monitoring methods in the farming of shrimps (18). Infected shrimps exhibited lethargy, anoxia, opaque musculature, red body colouration and white spots on carapace as well as appendages. White spot syndrome virus is the extremely virulent, contagious causative agent of white spot syndrome of shrimp; and it causes high mortality and affects commercially cultured marine shrimps (2).

Under the SEM, the white spots were observed as spheres on both inside and outside surfaces of the carapace cuticle. White spots of inside surface were quite larger (25µm to 135µm) as compared to outside surface, where very small white spots (25 to 32µm) have been detected (Fig. 2a-2b). The formation of white spot is a characteristic clinical sign of WSSV infection (1). The crustacean cuticle is secreted by underlying epidermal cells and mainly made up of chitin, calcium carbonate as well as protein (19). Cuticular epithelial cells are one of the most preferred sites of the WSSV and are amongst the first sites to be infected (10). White spots are reported to derive from the abnormalities of cuticular epidermis. Blockage of secretary products from cuticular epithelial cells possibly by WSSV viral infection might have affected cuticle with coloured spots. Presently observed morphological analysis of white spots, analyzed by SEM is found to be similar to the morphological analysis of white spots during WSSV infection reported by Wang et al. (1).

The chemical analysis of white spot by EDX analysis with SEM indicates changes in the levels of certain elements like C, O, P and Ca as compared to healthy shrimps. Though Wang et al. (1) has reported that the chemical composition of the white spot is similar to that of normal cuticle; in the present investigation, a clear increase in Ca and O as well as decrease in C in white spot region has been detected in comparison with healthy shrimps. Damage to the cuticular epithelium by WSSV infection seems to be responsible for the change in the chemical composition of white spots.

Evidence of histopathological manifestation in the target tissues is one of the criteria used in the diagnosis of WSSV infection (9). This paper describes the histopathological changes in foregut, gills, muscles and hepatopancreas of WSSV infected *P. monodon*. The WSSV target tissues are reported to be generally of ectodermal and mesodermal in origin including connective and epithelial tissues, hematopoietic nodules, haemocytes, gills, epidermis, foregut, striated muscles and nerves (2,20). In the present investigation, the cells of infected gut and gills exhibited nuclear hypertrophy, chromatin diminution and margination as well as dense basophilic

intranuclear inclusions (Fig. 5a and 5b). The WSSV infection was reported to have occurred first in the stomach, gill and cuticular epidermis, than subsequently got spread systematically to other tissues of mesodermal and ectodermal origin (21). The presence of dense basophilic intranuclear inclusion and chromatin margination are reported to be characteristic of WSSV infection (19,2). Cytopathological changes in the infected gut and gills clearly suggest WSSV infection in this shrimps. Our findings are in agreement with the cytopathological changes of different tissues of WSSV infected shrimps reported by Chang et al. (10) and Yoganandhan et al. (22). In the infected muscles also, severe necrosis, chromatin margination and dense basophilic intranuclear inclusions (Fig. 5d) have been identified indicating the damage caused by viral infection. Histopathological analysis of hepatopancreas of infected shrimp demonstrated severe pyknosis and karyorrhexis in connective tissue compartment (Fig. 5c). Prominent changes were not seen in the tubular epithelial cells of the hepatopancreas. Our observation is in agreement with Mohan and Shankar (23), who reported that WSSV does not infect tissues of endodermal origin such as hepatopancreatic tubular epithelium. Nuclear Pyknosis and karyorrhexis have been reported to be associated with several viral infections (21). Pantoja and Lightner (24) observed pyknosis and karyorrhexis in animals experimentally infected with WSSV. Presently observed Pyknosis and karyorrhexis in connective tissue cells of hepatopancreas and also in gut indicate the role of viruses in nuclear damage.

The transmission electron microscopy observations of pellet from the filtrate of diseased *P. monodon* cuticular epidermis stained by negative staining indicated the presence of rod shaped viral particles (Fig. 4). These are similar to the viral particles observed in negative staining of spontaneously diseased shrimps reported by Chou et al. (8). Electron microscopic observation conformed that the rod shaped virus was considered to be the main causative agent in the present study. The virus is reported to be highly pathogenic and constitutes a threat to shrimp culture. It is also reported to be transmitted orally or via water (8). Wongteerasupaya et al. (25) first described the TEM morphology of WSSV by negative staining. The presently observed morphology of virus described here from South Gujarat is very similar to the virus previously reported from other parts of India (14).

In the present study, WSSV infected *P. monodon* shows differences in the expression of proteins in muscle and hepatopancreas from uninfected shrimps. In the infected muscle tissue, four new differently expressed proteins with molecular weight 79kDa, 58kDa, 36kDa and 17kDa have been detected. In the infected hepatopancreas, three new proteins with molecular weight 17kDa, 20kDa and 46kDa have been detected. A newly expressed protein with molecular weight 17kDa has been detected in both the tissues (Fig. 6a and 6b). The variation in the pattern of expressions of proteins in tissues of WSSV infected shrimp is possibly related to the intensity of viral infection, degree of pathogenicity and tissue specific disintegration (14). Van Hulten et al. (26) have reported that WSSV virion protein ranges from 18kDa to 28kDa. Presently observed expression of 17kDa protein in infected muscle and hepatopancreas indicates the expression of viral protein and confirms the presence of WSSV infection in both the tissues. Expression of new protein, 17kDa in infected muscle as well as in hepatopancreas is found to be similar to the expression of new protein from WSSV infected shrimps reported by Sathish et al. (27).

In terms of morphological observations, histopathological effects, viral morphology and expression of new proteins in infected tissues,

the virus described here from coastal farms of Gujarat is very similar to white spot syndrome virus. This is believed to be the first report of systematic study of identification of WSSV from the cultured shrimps of South Gujarat region.

Acknowledgement

During the present investigation, the second author DC was supported by a meritorious fellowship from UGC New Delhi, India. We gratefully acknowledge the co-operation from the Office of Assistant Director of Fisheries, Government of Gujarat, Anand, as well as from the fish farmer Shree Girishbhai and Shree Sushil Mahanti from Olapad, Surat District.

References

1. Wang, C.S., Tang, K.F.J., Kou, G.H. and Chen, S.N. (1997). Light and electron microscopic evidence of white spot disease in the giant tiger shrimp, *Penaeus monodon* (Fabricius), and the kuruma shrimp, *Penaeus japonicus* (Bate), cultured in Taiwan. J Fish Dis., 20: 323-331.

2. Rajendran, K.V., Vijayan, K.K., Santiago, T.C. and Rajan, J.J.S. (2005). White spot syndrome virus (WSSV) infection in tiger shrimp *Penaeus monodon*: A non-lethal histopathological rapid diagnostic method using paraffin and frozen sections. Aquacult Int., 13: 341-349.

3. Escobedo-Bonilla, C.M., Alday-Sanz, V., Wille, M., Sorgeloos, P., Pensaert, M.B. and Nauwynck, H.J. (2008). A review on the morphology, molecular characterization, morphogenesis and pathogenesis of white spot syndrome virus. J Fish Dis., 31: 1-18.

4. McColl, K.A., Slater, J., Jeyasekaran, G., Hyatt, A.D. and Crane, M.S.T.J. (2004). Detection of White Spot Syndrome virus and Yellow head virus in prawns imported into Australia. Aust. Vet. J., 82: 69-74.

5. Ramasamy, P. (1995). Diseases of shrimp in aquaculture systems; Diagnosis and therapeutic measures. Vanitha Publications, (Eds.) Madras, Chennai, India, pp. 1-99.

6. Sahul Hameed, A.S., Xavier Charles, M., Anilkumar, M. (2000). Tolerance of *Macrobrachium rosenbergii* to white spot syndrome virus. Aquaculture, 183: 207-213.

7. Sahul Hameed, A.S., Yoganandhan, K., Sathish, S., Murugan, V., Rasheed, M., and Kunthala, J. (2001). Experimental pathogenicity of white spot syndrome virus (WSSV) in two freshwater crabs (*Partelphusa hydrodomous* and *P. pulvinata*). Aquaculture, 201: 179-186.

8. Chou, H.Y., Huang, C.Y., Wang, C.H., Chiang, H.C. and Chu-Fang, Lo (1995). Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaied shrimp in Taiwan. Dis Aquat Org., 23: 165-173.

9. Lightner, D.V. (1996). A Handbook of Shrimp Pathology and Diagnostic Procedures for Diseases of Cultured Penaeid Shrimp. World Aquaculture Society, Baton Rouge, Lousiana, USA, p.304.

10. Chang, P.S., Chu-Fang, Lo, Wang, Y.C. and Kou, G.H. (1996). Identification of white spot syndrome associated baculovirus (WSBV) target organs in shrimp, *Penaeus monodon*, by in situ hybridization. Dis Aquat Org., 27: 131-139.

11. Nadala, Jr. E.C.B., and Loh, P.C. (2000). Dot-blot nitrocellulose enzyme immunoassay for the detection of white-spot virus and yellow head virus of penaeid shrimp. J Virol Methods, 84: 175-179.

12. Tapay, L.M., Cesar, E., Nadala, Jr. E.C.B., and Loh, P.C. (1999). A polymerase chain reaction (PCR) protocol for the detection of various geographical isolates of white spot virus (WSV). J Virol Methods, 82: 39-43.

13. Chang, P.S., Chen, H.C., and Wand, Y.C. (1998). Detection of white spot syndrome associated baculovirus WSBV in experimentally infected wild shrimps, crabs and lobsters by in situ hybridization. Aquaculture, 164: 232-242.

14. Rameshthangam, P. and Ramasamy, P. (2005). Protein expression in white spot syndrome in virus infected *Penaeus monodon* Fabricius. Virus Res., 110: 133-141.

15. Hsin-Yiu-Chou, Chang-Yi Huang, Chuang-Hsiung Wang, Hsien-Choung Chiang, and Chu-Fang Lo (1995). Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaied shrimp in Taiwan. Dis Aquat Org., 23: 165-173.

16. Sambrook, J., and Russell, D. (2001). Molecular Cloning, A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

17. Rajan, P.R., Ramasamy, P., Purushothaman, V., and Brennan, G.P. (2000). White spot baculovirus syndrome virus in the Indian shrimp *Penaeus monodon* and *Penaeus indicus*. Aquaculture, 184: 31-44.

18. Peng, S.E., Lo, C.F., Lin, S.C., Chen, L.L., Chang, Y.S., Liu, K.F., Su, M.S. and Kou, G.H. (2001). Performance of WSSV-infected and WSSV-negative *Penaeus monodon* post larvae in culture ponds. Dis Aquat Org., 46: 165-172.

19. Wang, Y.C., Hassan, M.D., Shariff, M., Zamri, S.M., and Chen, X. (1999). Histopathology and cytopathology of white spot syndrome virus (WSSV) in cultured *Penaeus monodon* from peninsular Malaysia with emphasis on pathogenesis and the mechanism of white spot formation. Dis Aquat Org., 39: 1-11.

20. Lo, C.F., Ho, C.H., Chen, C.H., Liu, K.F., Chiu, Y.L., Yeh, P.Y., Peng, S.E., Hsu, H.C., Liu, H.C., Chang, C.F., Su, M.S., Wang, C.H. and Kou, G.H. (1997a). Detection and tissue distribution of white spot syndrome baculovirus (WSBV) in captured brooders of *Penaeus monodon* with a special emphasis on reproductive organs. Dis Aquat Org., 30: 53-72.

21. Rodrguez, J., Bayot, B., Amano, Y., Panchana, F., Blas Ide, Alday, V. and Calderon, J. (2003). White spot syndrome virus infection in cultured *Penaeus vannamei* (Boone) in Ecuador with emphasis on histopathology and Ultrastructure. J Fish Dis., 26: 439-450.

22. Yoganandhan, K., Sathish, S., Murugan, V., Narayanan, R.B. and Sahul Hameed, A.S. (2003). Screening the organs for early detection of white spot syndrome virus in *Penaeus indicus* by histopathology and PCR techniques. Aquaculture, 215: 21-29.

23. Mohan, C.V. and Curnow, R.N. (1998). Recent findings on white spot syndrome (WSS) of cultured shrimps: Management implications. Fishing Chimes, 18(6): 5-7.

24. Pantoja, C.R. and Lightner, D.V. (2003). Similarity between the histopathology of white spot syndrome virus and yellow head syndrome virus and its relevance to diagnosis of YHV disease in the Americas. Aquaculture, 218: 47-54.

25. Wongteerasupaya, C., Vickers, J.E., Sriurairatana, S., Nash, G.L., Akarajamorn, A., Boonsaeng, V., Panyim, S., Tassanakajon, A., Withyachumnarnkul, B. and Flegel, T. W. (1995). A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*. Dis Aquat Org., 21: 69-77.

26. Van Hulten, M.C.W., Westenberg, M., Goodall, S.D., and Vlak, J.M. (2000b). Identi?cation of two major virion protein genes of white spot syndrome virus of shrimp. Virology, 266: 227-236.

27. Sathish, S., Selvakkumar, C., Sahul Hameed, A.S., and Narayanan, R.B. (2004). 18-kDa protein as a marker to detect WSSV infection in shrimps. Aquaculture, 238:39-50.

Current Trends in Biotechnology and Pharmacy
Vol. 5 (1) 1021-1028 January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

Synthesis and Anti-Inflammatory Activity of some New Chalcones from 3′ - Methyl- 4′ – Hydroxyacetophenone

N. Srinath*, Y. Rajendra Prasasd[1], K. Mukkanti[2], C. Kistayya[1] and B. Bhaskar Rao[1]
Department of Pharmacy, Acharya Nagarjuna University, Guntur – 521250, A.P., India
[1]University College of Pharmaceutical Sciences, Andhra University,
Visakhapatnam-530003, A.P., India
[2]Centre for Pharmaceutical Sciences, J N T University, Hyderabad – 500085
*For Correspondence - srinath_n2k@rediffmail.com

Abstract

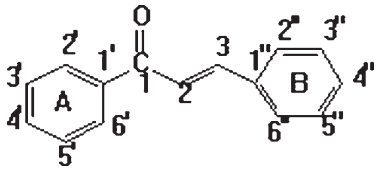
A series of some new chalcones were synthesized by Claisen-Schmidt condensation of 3'-methyl-4'-hydroxyacetophenone with various substituted aromatic / heteroaromatic aldehydes in the presence of alkali. The resulting chalcones were characterized by IR, ^{1}H NMR and Elemental analysis. These chalcones were evaluated for their anti-inflammatory activity and some of them exhibited significant activity when compared with the standard.

Key words: Chalcones, Synthesis, Anti-inflammatory activity

Introduction

Chalcone is a generic term given to compounds bearing the 1, 3-diphenylprop-2-en-1-one frame work, which can be functionalized in the propane chain by the presence of olefinic, keto and / or hydroxyl groups (Fig.1) (1). Chalcones have been reported to exhibit a wide variety of pharmacological activities including antioncogenic (2), antiulcerative (3), analgesic and anti-inflammatory (4), antiviral (5), antimalarial (6) and antibacterial (7) activities. The presence of a reactive á, â – unsaturated keto function is partly responsible for their activities. Chalcones are readily synthesized by the base catalyzed Claisen – Schmidt condensation of an aldehyde and an appropriate ketone in a polar solvent like ethanol and yields may be variable (8,9) ranging from 5 to 80%. In the present communication, we report the reaction of 3'-methyl-4'-hydroxyacetophenone with different aromatic / heteroaromatic aldehydes to form chalcones (B_1–B_{15}). The structures of all the synthesized compounds were assigned on the basis of IR, ^{1}H NMR spectral data and Elemental analysis. These compounds were also screened for their anti-inflammatory activity.

Fig.1



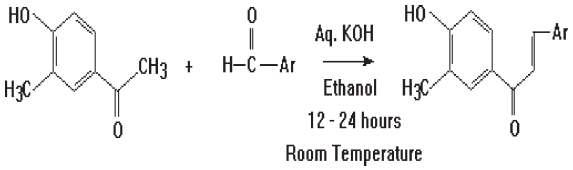
Experimental

Melting points were determined in open capillary tubes and are uncorrected. The IR spectra were recorded in KBr on Perkin Elmer – 337 Infrared Spectrophotometer. The ^{1}H NMR were recorded in $CDCl_3$ on Bruker AMX 400MHz NMR Spectrophotometer using TMS as an internal standard. The Elemental analysis was performed on Perkin Elmer model 2400 series II apparatus. The purity of the compounds was checked by TLC using Silica gel-G (Merck). Column chromatography was performed on Silica gel (Merck, 60-120 mesh).

Vol. 5 (1) 1021-1028 January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

General Procedure for the Preparation of Chalcones : A mixture of 3'-methyl-4'-hydroxyacetophenone (0.005 mol) and various substituted aromatic / heteroaromatic aldehydes (0.005 mol) was stirred in ethanol (30 ml) manually at room temperature for 10 minutes, kept and then an aqueous solution of 50% potassium hydroxide (7.5 ml) was added to it. The mixture was kept overnight at room temperature and then it was poured into crushed ice and acidified with dilute hydrochloric acid. The chalcone derivatives precipitated out as solid. Then it was filtered and crystallized from ethanol (Scheme 1). This procedure remained same with all types of aldehydes.

3'-methyl-4'-hydroxy-acetophenone Aldehyde Chalcone



Scheme 1

Where Ar =

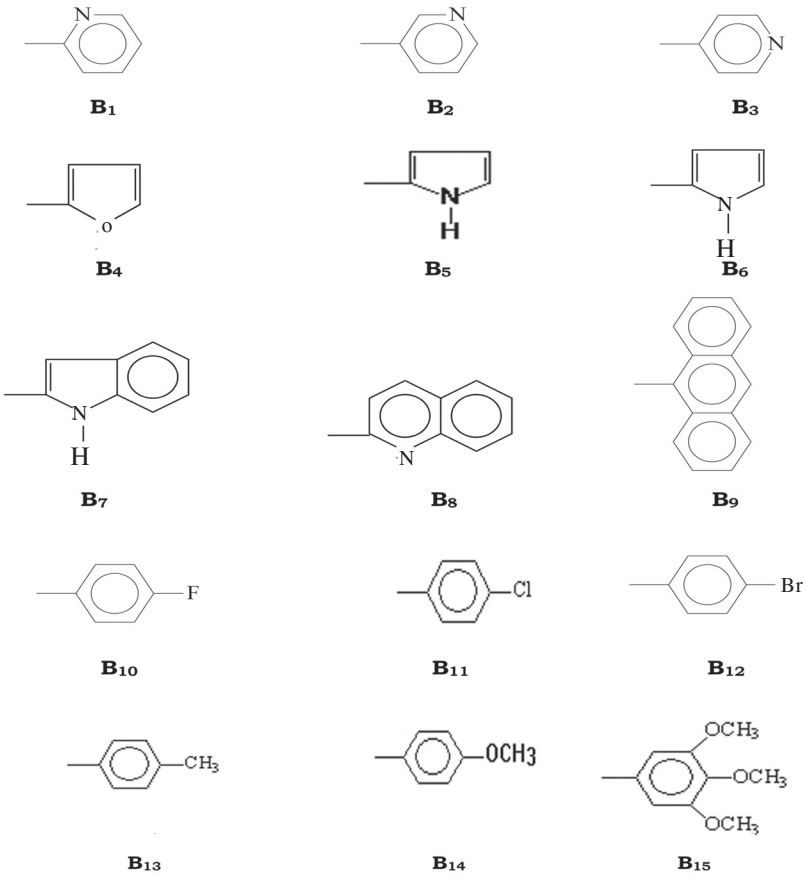
Physical Characterization Data of Chalcones

1-(3'–Methyl -4'-hydroxyphenyl)-3-(2''-pyridinyl)-2-propen-1-one (B_1)

Yield: 85%; **m.p.** 180-181^{0}C

IR (KBr, cm^{-1}): 3628 (O-H), 1651 (C=O), 1581 (C=N),

Physical Characterization Data of Chalcones



1504 (C=C Quadrant of Ar), 1464 (CH=CH)
^{1}H NMR ($CDCl_3$, äppm): 7.96 (1H,d,J=17Hz, =CH-Ar), 7.83 (1H,s,J=17Hz, -CO-CH=),7.92 (1H,s,C-2'-H),2.30 (3H,s,C-3'-CH_3),6.93 (1H,d,J=8.6Hz,C-5'-H)
7.80 (1H,d,J=8.6Hz,C-6'-H),8.50 (1H,d, J=7.2Hz,C-3''-H),
7.18 (1H,m,J=7.3Hz,C-4''-H),7.71 (1H,m ,C-5''-H),7.78 (1H,d,J=8Hz,C-6''-H)
Anal.Calcd.for $C_{15}H_{13}N0_2$: C, 75.301; H, 5.480; N, 4.852.
Found: C, 75.213; H, 5.434; N, 4.827.

1-(3'–Methyl -4'-hydroxyphenyl)-3-(3''-pyridinyl)-2-propen-1-one (B_2)
Yield: 82%; **m.p.** 78-82^{0}C
IR (KBr, cm^{-1}): 3627 (O-H), 1651 (C=O), 1582 (C=N),
1505 (C=C Quadrant of Ar), 1465 (CH=CH)
^{1}H NMR ($CDCl_3$, äppm): 7.71 (1H,d,J=17Hz, =CH-Ar), 7.32 (1H,d,J=17Hz , -CO-CH=), 7.89 (1H,s,C-2'-H),2.30 (3H,s,C-3'-CH_3),6.93 (1H,d,J=8.6Hz,C-5'-H),7.80 (1H,d,J=8.6Hz,C-6'-H),8.86 (1H,s,C-2''-H),8.65 (1H,d,J=7.3Hz,C-4''-H),7.38 (1H,m,J=8Hz,C-5''-H),7.96 (1H,d,J=8Hz, C -6''-H)
Anal.Calcd.for$C_{15}H_{13}N0_2$: C, 75.303; H, 5.483; N, 4.852.
Found: C, 75.183; H, 5.463; N, 4.802.

1-(3'–Methyl -4'-hydroxyphenyl)-3-(4''-pyridinyl)-2-propen-1-one (B_3)
Yield: 75%; **m.p.** 71-73^{0}C
IR (KBr, cm^{-1}): 3625 (O-H), 1650 (C=O), 1583 (C=N),
1503 (C=C Quadrant of Ar), 1466 (CH=CH)
^{1}H NMR ($CDCl_3$, äppm): 7.60 (1H,d,J=17Hz, =CH-Ar),7.74 (1H,d,J=17Hz, –CO-CH=),7.89 (1H,s,C-2'-H),2.30 (3H,s,C-3'-CH_3),6.93 (1H,d,J=8.6Hz,C-5'-H)
7.80 (1H,d,J=8.6Hz,C-6'-H),7.32 (1H,d,J=7.2Hz,C-2''-H),
8.52 (1H,d,J=7.2Hz,C-3''-H),8.65 (1H,d,J=7.2Hz,C-5''-H),
7.32 (1H,d,J=7.2Hz,c-6''-H)
Anal.Calcd.for$C_{15}H_{13}N0_2$: C, 75.303; H, 5.483; NFound: C, 75.245; H, 5.458; N, 4.833.

1-(3'–Methyl -4'-hydroxyphenyl)-3-(2''-furyl)-2-propen-1-one (B_4)
Yield: 63%; **m.p.** 162-163^{0}C
IR (KBr, cm^{-1}): 3626 (O-H), 1652 (C=O), 1505 (C=C Quadrant of Ar),
1463 (CH=CH), 1200 (C-O)
^{1}H NMR ($CDCl_3$, äppm): 7.59 (1H,d,J=17Hz, =CH-Ar),7.46 (1H,d,J=17Hz, -CO-CH=),7.85 (1H,s,C-2'-H),2.30 (3H,s,C-3'-CH_3),6.93 (1H,d,J=8.6Hz,C-5'-H)
7.81 (1H,d,J=8.6Hz,C-6'-H),6.66 (1H,d, J=7.2Hz,C-3''-H),
6.43 (1H,d,J=7.2Hz,C-4''-H),7.45 (1H,s,C-5''-H)
Anal.Calcd. for $C_{14}H_{12}0_3$: C, 73.673; H, 5.301.
Found: C, 73.583; H, 5.243.

1-(3'–Methyl -4'-hydroxyphenyl)-3-(2''-pyrrolyl)-2-propen-1-one (B_5)
Yield: 64%; **m.p.** 205-207^{0}C
IR (KBr, cm^{-1}): 3624 (O-H), 1653 (C=O), 1580 (C=N),
1504 (C=C Quadrant of Ar), 1465 (CH=CH)
^{1}H-NMR ($CDCl_3$, äppm): 7.71 (1H,d,J=17Hz,=CH-Ar),6.94 (1H,d,J=17Hz,-CO-CH=),7.81 (1H,s,C-2'-H),2.30 (3H,s,C-3'-CH_3),6.93 (1H,d,J=8.6Hz,C-5'-H)
7.79 (1H,d,J=8.6Hz,C-6'-H),6.25 (1H,d,J=7.2Hz,C-3''-H)
5.98 (1H,m,J=7.2Hz,C-4''-H),6.99 (1H,d,J=6.8Hz,C-5''-H)
Anal.Calcd.for$C_{14}H_{13}NO_2$: C, 73.993; H, 5.772; N, 6.161.
Found: C, 74.012; H, 5.742; N, 6.206.

1-(3'–Methyl -4'-hydroxyphenyl)-3-(2''-thienyl)-2-propen-1-one (B_6)
Yield: 73%; **m.p.** 192-196^{0}C
IR (KBr, cm^{-1}): 3625 (O-H), 1650 (C=O), 1505 (C=C Quadrant of Ar),

1462 (CH=CH), 650 (C-S)
^{1}H NMR ($CDCl_3$, äppm): 7.93 (1H,d,J=17Hz,=CH-Ar),7.18 (1H,d,J=17Hz,-CO-CH=),7.88 (1H,s,C-2'-H),2.30 (3H,s,C-3'-CH_3),6.93 (1H,d,J=8.6Hz,C-5'-H)
7.78 (1H,d,J=8.6Hz,C-6'-H),7.55 (1H,d,J=7.2Hz,C-3''-H)
7.16 (1H,m,J=7.2Hz,C-4''-H),7.61 (1H,d,J=6.8Hz,C-5''-H)
Anal.Calcd. for $C_{14}H_{12}S0_2$: C, 68.334; H, 5.004.
Found: C, 68.192; H, 5.041.

1-(3'–Methyl -4'-hydroxyphenyl)-3-(2''-indolyl)-2-propen-1-one (B_7)
Yield: 85%; **m.p.** 195-198^{0}C
IR (KBr, cm^{-1}): 3623 (O-H), 1655 (C=O), 1585 (C=N),
1506 (C=C Quadrant of Ar), 1467 (CH=CH)
^{1}H NMR ($CDCl_3$, äppm): 7.83 (1H,d,J=17Hz,=CH-Ar),7.35 (1H,d,J=17Hz,-CO-CH=),7.97 (1H,s,C-2'-H),2.30 (3H,s,C-3'-CH_3),6.93 (1H,d,J=8.6Hz,C-5'-H)
7.83 (1H,d,J=8.6Hz,C-6'-H),7.92 (1H,d,J=7.2Hz,C-2''-H),
7.93 (1H,d,J=7.8Hz,C-4''-H),7.19 (1H,d,J=7.8Hz,C-5''-H)
7.13 (1H,d,J=7.9Hz,C-6''-H),7.45 (1H,d,J=7.9Hz, C-7''-H)
Anal.Calcd.for$C_{18}H_{15}NO_2$: C, 77.962; H, 5.453; N, 5.052
Found: C, 77.712; H, 5.152; N, 4.983.

1-(3'–Methyl -4'-hydroxyphenyl)-3-(2''-quinolinyl)-2-propen-1-one (B_8)
Yield: 84%; **m.p.** 70-72^{0}C
IR (KBr, cm^{-1}): 3628 (O-H), 1654 (C=O), 1582 (C=N),
1504 (C=C Quadrant of Ar), 1464 (CH=CH)
^{1}H NMR ($CDCl_3$, äppm): 7.82 (1H,d,J=17Hz,=CH-Ar),7.75 (1H,d,J=17Hz,-CO-CH=),7.71 (1H,s,C-2'-H),2.30 (3H,s,C-3'-CH_3),6.93 (1H,d,J=8.6Hz,C-5'-H)
7.62 (1H,d,J=8.6Hz,C-6'-H),7.64 (1H,d,J=8.5Hz,C-3''-H)'
7.86 (1H,d,J=8.5Hz,C-4''-H),7.78 (1H,d,J=7.9Hz,C-5''-H)
7.69 (1H,t,J=7.9Hz,C-6''-H),7.63 (1H,t,J=7.9Hz, C-7''-H),
7.68 (1H,d,J=7.9Hz,C-8''-H)
Anal.Calcd.for$C_{19}H_{15}NO_2$: C, 78.803; H, 5.181; N, 4.395.
Found: C, 78.772; H, 5.223; N, 4.422.

1-(3'–Methyl -4'-hydroxyphenyl)-3-(9''-anthracenyl)-2-propen-1-one (B_9)
Yield: 86%; **m.p.** 103-105^{0}C
IR (KBr, cm^{-1}): 3627 (O-H), 1655 (C=O),
1507 (C=C Quadrant of Ar), 1463(CH=CH)
^{1}H NMR ($CDCl_3$, äppm): 8.19 (1H,d,J=17Hz,=CH-Ar),7.78 (1H,d,J=17Hz,-CO-CH=),7.99 (1H,s,C-2'-H),2.30 (3H,s,C-3'-CH_3),6.93 (1H,d,J=8.6Hz,C-5'-H)
7.84 (1H,d,J=8.6Hz,C-6'-H),8.40 (1H,d,J=8.2Hz,C-1''-H),
7.02 (1H,m,J=8.2Hz,C-2''-H),7.30 (1H,m,J=8.1Hz,C-3''-H)
7.99 (1H,d,J=8.1Hz,C-4''-H),7.99 (1H,d,J=8.1Hz,C-5''-H)
7.30 (1H,m,J=8.1Hz, C-6''-H),7.02 (1H,m,J=8.2Hz,C-7''-H)
7.82 (1H,d,J=8.2Hz,C-8''-H),7.80 (1H,s, C-10''-H)
Anal.Calcd. for $C_{24}H_{18}O_2$: C, 85.182; H, 5.362.
Found: C, 85.122; H, 5.242.

1-(3'–Methyl -4'-hydroxyphenyl)-3-(4''-fluorophenyl)-2-propen-1-one (B_{10})
Yield: 78%; **m.p.** 181-183^{0}C
IR (KBr, cm^{-1}): 3625 (O-H), 1656 (C=O), 1506 (C=C Quadrant of Ar),
1461 (CH=CH), 1120 (C-F)
^{1}H-NMR ($CDCl_3$, äppm): 7.83 (1H,d,J=17Hz,=CH-Ar),7.26 (1H,d,J=17Hz,-CO-CH=),7.89 (1H,s,C-2'-H),2.30 (3H,s,C-3'-

CH_3),6.93 (1H,d,J=8.6Hz,C-5'-H)
7.80 (1H,d,J=8.6Hz,C-6'-H),7.58 (1H,d,J=8.6Hz,C-2''-H)
6.95 (1H,d,J=8.8Hz,C-3''-H),6.96 (1H,d,J=8.8Hz,C-5''-H)
7.58 (1H,d,J=8.6Hz,C-6''-H)
Anal.Calcd. for $C_{16}H_{13}FO_2$: C, 75.001; H, 5.102.
Found: C, 74.209; H, 4.707.

1-(3'–Methyl -4'-hydroxyphenyl)-3-(4''-chlorophenyl)-2-propen-1-one (B_{11})
Yield: 75%; **m.p.** 213-214^{0}C
IR (KBr, cm^{-1}): 3628 (O-H), 1650 (C=O), 1506 (C=C Quadrant of Ar),
1462 (CH=CH), 855 (C-Cl)
^{1}H NMR ($CDCl_3$, äppm): 7.76 (1H,d,J=17Hz,=CH-Ar),7.47 (1H,d,J=17Hz,-CO-CH=),7.89 (1H,s,C-2'-H),2.30 (3H,s,C-3'-CH_3),6.93 (1H,d,J=8.6Hz,C-5'-H)
7.80 (1H,d,J=8.6Hz,C-6'-H),7.55 (1H,d,J=8.5Hz,C-2''-H)
7.57 (1H,d,J=8.5Hz,C-3''-H),7.53 (1H,d,J=8.5Hz,C-5''-H)
7.55 (1H,d,J=8.5Hz,C-6''-H)
Anal.Calcd. for $C_{16}H_{13}ClO_2$: C, 70.503; H, 4.802.
Found: C, 70.103; H, 4.104.

1-(3'–Methyl -4'-hydroxyphenyl)-3-(4''-bromophenyl)-2-propen-1-one (B_{12})
Yield: 74%; **m.p.** 55-58^{0}C
IR (KBr, cm^{-1}): 3624 (O-H), 1652 (C=O), 1507 (C=C Quadrant of Ar),
1464 (CH=CH), 1020 (C-Br)
^{1}H NMR ($CDCl_3$, äppm): 7.82 (1H,d,J=17Hz,=CH-Ar),7.29 (1H,d,J=17Hz,-CO-CH=),7.89 (1H,s,C-2'-H),2.30 (3H,s,C-3'-CH_3),6.93 (1H,d,J=8.6Hz,C-5'-H)
7.80 (1H,d,J=8.6Hz,C-6'-H),7.45 (1H,d,J=8.6Hz,C-2''-H)
7.59 (1H,d,J=8.5Hz,C-3''-H),7.56 (1H,d,J=8.5Hz,C-5''-H)
7.45 (1H,d,J=8.6Hz,C-6''-H)
Anal.Calcd. for $C_{16}H_{13}BrO_2$: C, 60.591; H, 4.132.
Found: C, 60.481; H, 4.072.

1-(3'–Methyl -4'-hydroxyphenyl)-3-(4''-methylphenyl)-2-propen-1-one (B_{13})
Yield: 85%; **m.p.** 121-123^{0}C
IR (KBr, cm^{-1}): 3629 (O-H), 1653 (C=O),
1506 (C=C Quadrant of Ar),
1463(CH=CH)
^{1}H NMR ($CDCl_3$, äppm): 7.74 (1H,d,J=17Hz,=CH-Ar),7.38 (1H,d,J=17Hz,-CO-CH=),7.89 (1H,s,C-2'-H),2.30 (3H,s,C-3'-CH_3),6.93 (1H,d,J=8.6Hz,C-5'-H)
7.80 (1H,d,J=8.6Hz,C-6'-H),7.47 (1H,d,J=8.1Hz,C-2''-H)
6.97 (1H,d,J=8.1Hz,C-3''-H),2.40 (3H,s,C-4''-CH_3)
6.94 (1H,d,J=8.1Hz,C-5''-H),7.47 (1H,d,J=8.1Hz, C-6''-H)
Anal.Calcd. for $C_{17}H_{16}0_2$: C, 80.934; H, 6.934.
Found: C, 80.453; H, 6.783.

1-(3'–Methyl-4'-hydroxyphenyl)-3-(4''-methoxyphenyl)-2-propen-1-one(B_{14})
Yield: 83%; **m.p.** 209-211^{0}C
IR (KBr, cm^{-1}): 3627 (O-H), 1652 (C=O), 1503 (C=C Quadrant of Ar),
1461 (CH=CH), 1170 (-O-CH_3)
^{1}H NMR ($CDCl_3$, äppm): 7.80 (1H,d,J=17Hz,=CH-Ar),7.37 (1H,d,J=17Hz,-CO-CH=),7.89 (1H,s,C-2'-H),2.30 (3H,s,C-3'-CH_3),6.93 (1H,d,J=8.6Hz,C-5'-H)
7.80 (1H,d,J=8.6Hz,C-6'-H),7.56 (1H,d,J=8.5Hz,C-2''-H)
6.92 (1H,d,J=8.5Hz,C-3''-H),3.87 (3H,s,C-4''-OCH_3)
6.92 (1H,d,J=8.5Hz,C-5''-H),7.56 (1H,d,J=8.5Hz, C-6''-H)
Anal.Calcd. for $C_{17}H_{16}0_3$: C, 76.102; H, 6.018.
Found: C, 76.001; H, 5.937.

1-(3'–Methyl -4'-hydroxyphenyl)-3-(3'',4'',5''- trimethoxyphenyl)-2-propen-1-

one (B_{15})
Yield: 80%; **m.p.** 116-119^{0}C
IR (KBr, cm^{-1}): 3625 (O-H), 1654 (C=O), 1505 (C=C Quadrant of Ar),
1465 (CH=CH), 1175 (-O-CH_3)
^{1}H NMR ($CDCl_3$, äppm): 7.70 (1H,d,J=17Hz,=CH-Ar),7.50 (1H,d,J=17Hz,-CO-CH=),7.89 (1H,s,C-2'-H),2.30 (3H,s,C-3'-CH_3),6.93 (1H,d,J=8.6Hz,C-5'-H)
7.80 (1H,d,J=8.6Hz,C-6'-H),6.80 (1H,s ,C-2''-H),3.85 (9H,s, 3 x -OCH_3)
6.80 (1H,s ,C-6''-H)
Anal. Calcd. for $C_{19}H_{20}O_5$: C, 69.502; H, 6.153.
Found: C, 69.431; H, 6.118.

Anti-Inflammatory activity

The anti-inflammatory activity of the synthesized chalcones was evaluated by using carrageenan-induced rat paw oedema method (10,11). Albino rats of either sex (150-200g) were used in the study. The animals were housed under standard environmental conditions (temperature of 22 ±1^{0}C with an alternating 12 hour light-dark cycle and relative humidity of 60±5%), fed with standard diet and water *ad libitum*.

Rats were divided into seventeen groups of six animals in each group. Inflammation was induced by injecting 0.05 ml of 1% carrageenan (Sigma) subcutaneously into the sub-plantar region of the right hind paw and 0.05ml saline was injected into the sub-plantar region of the left hind paw for all groups. One hour prior to carrageenan injection, the groups III – XVII treated with compounds (B_1–B_{15}) (10 mg/kg). 1% Sodium CMC gel (1ml/kg) was given to group I used as carrageenan treated control and the standard drug aceclofenac (2 mg/kg) was administered to group II. All the doses were administered orally. Anti-inflammatory activity was evaluated by measuring carrageenan induced paw oedema.

The thickness of the both paws of each rat, lower and upper surface was measured using Zeitlin's constant load level method consisting of a graduated micrometer combined with a constant loaded level system to magnify the small changes in paw thickness during the course of the experiment. The percent increase of paw oedema thickness was determined at 0, 0.5, 1, 2, 3, 4 and 6 hours after induction of inflammation.

The percent increase at each time interval was determined by using the formula: $Y_t - Y_o / Y_o$ x 100

Y_t = paw thickness at time t hours (after injection)

Y_o = paw thickness at time 0 hours (before injection)

The percent inhibition of paw oedema thickness was calculated by using the formula:

$$\text{Percentage inhibition} = \frac{1-y^1}{y^2} \times 100 = 100$$

Where Y_t = average increase in paw thickness in groups tested with chalcones and the standard

Y_c = average increase in paw thickness in control

The results and statistical analysis of anti-inflammatory activity of aceclofenac and the compounds tested are shown in Table 1.

All values are represented as mean ± SEM (n=6).
*P<0.01 compared to reference standard Aceclofenac. Student's t-test.
Dosage: Aceclofenac: 2 mg/kg
Test compounds: 10 mg/kg body weight of rat.

Table 1. Anti-Inflammatory activity of Chalcones

Compound	Ar	% inhibition ± SEM at various time intervals					
		0.5h	**1.0h**	**2.0h**	**3.0h**	**4.0h**	**6.0h**
B_1	2''-pyridinyl	6±1	7±1	37±1	45±1	76±1	78±1
B_2	3''-pyridinyl	6±1	7±1	36±1	45±1	75±1	77±1
B_3	4''-pyridinyl	6±1	7±1	35±1	46±1	74±1	78±1
B_4	2''-furyl	5±1	6±1	33±1	45±1	72±1	75±1
B_5	2''-pyrrolyl	13±1	15±1	52±1	64±1	93±1	97±1
B_6	2''-thienyl	7±1	9±1	39±1	50±1	79±1	80±1
B_7	2''-indolyl	14±1	17±1	54±1	65±1	95±1	98±1
B_8	2''-quinolinyl	7±1	8±1	38±1	48±1	77±1	79±1
B_9	9''-anthracenyl	10±1	12±1	45±1	56±1	85±1	90±1
B_{10}	4''-fluorophenyl	9±1	11±1	43±1	55±1	82±1	87±1
B_{11}	4''-chlorophenyl	8±1	11±1	42±1	53±1	81±1	85±1
B_{12}	4''-bromophenyl	8±1	10±1	40±1	52±1	80±1	82±1
B_{13}	4''-methylphenyl	12±1	13±1	49±1	58±1	90±1	94±1
B_{14}	4''-methoxyphenyl	11±1	13±1	47±1	57±1	88±1	91±1
B_{15}	3'',4'',5''-trimethoxyphenyl	13±1	14±1	51±1	61±1	92±1	95±1
	Aceclofenac (standard)	21±1	23±1	56±1	67±1	96±1	99±1

Results and Discussion

The anti-inflammatory activity of the some newly synthesized chalcones (B_1-B_{15}) has been evaluated by using carrageenan-induced rat paw oedema method. The results of the evaluation have been viewed by taking aceclofenac as the standard drug.

The results of anti-inflammatory activity revealed that the compounds B_1 to B_{15} exhibited moderate to considerable activity when compared with reference standard aceclofenac, but not at an identical dose level as the standard drug was tested at 2 mg/kg, whereas the chalcones were tested at a dose of 10 mg/kg.

From the results it is evident that all the chalcones showed some degree of anti-inflammatory activity. However, it is found that the chalcone having indole substituent (B_7) displayed significant anti-inflammatory activity from 3rd hour onwards and reached the maximum at the 6th hour and is comparable to that of the standard aceclofenac, but not at an identical dose level. This type of anti-inflammatory activity for this chalcone is understandable since indole derivatives are known to possess significant anti-inflammatory activity and a number of drugs belonging to this class are also being used as a NSAIDs. But what is worth noticing is the retention of such activity even in the case of chalcones bearing this moiety. This suggests chalcones can be prepared having other substituted indole moieties in order to enhance the activity further. The next compound in order of potency is the one having a pyrrole substituent (B_5). This was followed by compounds having

electron releasing groups as seen in the case of compound B_{15}, B_{13} and B_{14}. This clearly reveals the importance of electron releasing groups on the aromatic ring in enhancing the anti-inflammatory activity and hence attempts can be made to synthesize chalcones having a number of such electron releasing substituents at different positions of the aromatic ring as part of preparing more potent compounds.

However, the contributing physico-chemical properties for the anti-inflammatory activity of the chalcones need to be established by detailed QSAR studies, which may provide insights into the structural requirements of this class of molecules. Further studies are required to establish the mechanism of anti-inflammatory activity of these compounds, even though literature reports suggest the inhibition of COX-2 enzyme in some cases.

Conclusions

Chalcones with heterocyclic nucleus such as indole, pyrrole and also the substituents with electron releasing groups such as methoxy, methyl showed better anti-inflammatory activity. Compounds having pharmacophores such as fluoro, chloro, bromo groups have exhibited moderate anti-inflammatory activity. These results suggest that's the chalcone derivatives have excellent scope for further development as commercial anti-inflammatory agents.

References

1. Bohm, B.A. (1998) In: Introduction to Flavanoids, Harwood Academic Publishers, Amsterdam.

2. Kumar, S.K., Hager, E., Pettit, C., Gurulingappa, H., Davidson, N.E. and Khan, S.R. (2003) Design, synthesis and evaluation of novel boronicchalcone derivatives as antitumor agents. J.Med.Chem., 46: 2813.

3. Mukarami, S., Muramatsu, M., Aihara, H. and Otomo, S. (1991) Inhibition of gastric H^+, K^+-ATPase by the antiulcer agent Sofalcone. Biochem. Pharmacol., 42: 1447.

4. Viana, G.S., Bandeira, M. A. and Matos, F. (2003) Analgesic and anti-inflammatory effects of chalcones isolated from Myracrodruon urundeuva. J.Phytomedicine., 10:189.

5. Onyilagna, J.C., Malhotra, B., Elder, M. and Towers, G.H.N. (1997) Synthesis and antimicrobial activity of some chalcone derivatives. Can.J.Plant Pathol., 19: 133.

6. Liu, M., Wilairat, P. and Go, L.M (2001) Antimalarial alkoxylated and hydroxylated chalcones: structure-activity relationship analysis. J.Med.Chem., 44: 4443.

7. Chen, Z-H., Zheng, C-Ji., Sun, L-P. and Piao, H-R. (2010) Synthesis of new chalcone derivatives containing a rhodanine-3-aceticacid moiety with potential antibacterial activity. Eur. J. Med. Chem., 45: 5739.

8. Prasad, Y.R., Kumar, P.R., Deepti, Ch.A. and Ramana, M.V. (2007) Synthesis and antimicrobial activity of some chalcones of 3-acetylcoumarin and 2-hydroxy-1-acetonaphthone. Asian.J.Chem., 19: 4799.

9. Prasad, Y.R., Kumar, P.R., Srivani, N. and Rao, A.S. (2006) Synthesis and antimicrobial activity of some new chalcone derivatives. Int.J.Chem.Sci., 44: 905.

10. Turner, R.A. (1965) In: Screening Methods in Pharmacology, Demic Press, NewYork, p. 152.

11. Winter, C.A., Risley, E.A. and Nuss, G.W. (1962) Carrageenan induced edema in hind paw of the rat as an assay for anti-inflammatory drugs. Proc. Soc. Exp. Biol. Med., 111: 544.

Current Trends in Biotechnology and Pharmacy
Vol. 5 (1) 1029-1037 January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

Variations in quantity and quality of neemoil (*Azadirachta indica* A.juss) extracted by petroleum ether and ethanol in select areas of Guntur, Andhra Pradesh, India

T.Satyanandam, G.Rosaiah*, K. Babu, Y. Sambasiva Rao and K.R.S.Sambasiva Rao[1]
Department of Botany, Acharya Nagarjuna University, Nagarjunanagar, Guntur, A.P., India
[1]Department of Biotechnology, Acharya Nagarjuna University, Nagarjunanagar, Guntur, A.P., India
*For Correspondence – gorrepati_r@yahoo.co.in

Abstract

Neem oil extracted from seeds of neem trees (*Azadirachta indica* A.juss) growing in three soil types has showed variations, using petroleum ether and ethanol. Effect of temperature and the influence of solvent on the extraction were studied. Results showed that the maximum oil yield was 42.60% with petroleum ether and 39.17% with ethanol at 50ºC. It was observed that the increase in temperature, decreased the iodine value, whereas increased acid, and peroxide values, envisaging that the higher extraction temperature, though gives higher oil yield, decreased the quality of oil. It was evident that petroleum ether (bp 60-80° C) can be effectively used as a good solvent for the extraction of oil in solvent extraction process. Further it was observed that some significant variations in Azadirachtin (980 – 2420 mg/kg), seed protein (27.2 -32.8 %) and seed weight (16.8 – 18.9 g) were observed in trees growing in different soil types.

Key words: Neem oil, extraction, solvent type, oil quality.

Introduction

Azadirachta indica A.juss, native to the Indian subcontinent, commonly referred to as neem belongs to family Meliaceae and is found in the tropical and sub tropical climate. It is observed that this tree can survive in very dry and arid conditions. All parts of the neem tree such as leaves, bark, flower, seed and root have significance in medical treatment and industrial applications. Its leaves are being used as a component in drug formulations for diabetics, eczema and fever. Barks of neem have been used to brush the tooth. Neem roots have an ability to combat diseases and act against insects (1). The medicinal value of neem dates back to the Vedic times, as suggested in Ayurveda, by name Sarva-roga- nivarini (2).

Neem seed kernels consist of azadirachtin (3, 4) limonoids such as nimbin, salanin (5) and meliantriol (6, 7). Azadirachtin acts as an insect repellent, antifeedent, and analysis shows that it mimics hormones blocking the receptors for ecdysteroids, which are useful for hormonal balance and disrupt the metamorphosis of several insect larvae and larval development (8). Neem seed has high concentration of neem oil (approximately 30 – 50 %) compared to the other parts of the tree (1, 10). Neem oil is an edible oil, widely used as insecticide and lubricant apart from being used in making drugs to cure a variety of diseases such as diabetics and tuberculosis (1, 2, and 9). It is also being used for preparing cosmetics as well as Ayurvedic, Unani and other

traditional medicines, used in treatment of a wide range of afflictions. Use of Neem oil for human use has an extensive history in India and its surrounding regions for a variety of therapeutic purposes (10).

Formulations made of neem oil are being used as biopesticide for organic farming, as it repels a wide variety of pests (11). Neem oil can also control the black spot, powdery mildew, anthracnose and rust. The oil extraction using solvents has several advantages. It gives high yield and less turbid oil than the mechanical extraction whereas operating cost is also low when compared to supercritical fluid extraction.

Materials and Methods

Material preparation: Neem seeds, used in this study, were collected from trees of 13 to 20 years old growing in Black, Red and Sandy soils of Guntur district. The disease free, mature, ripe (deep yellow to light yellow) fruits were collected. The seeds were then manually depulped, cleaned under running tap water and then air dried. The mesocarp adhering to the hard epicarp was removed by scrubbing the seed on a rough surface, by using a sand paper and subsequently dried in oven at 50^0C until the seed reached to a constant moisture content.

Oil extraction by solvent extraction procedure, using two solvents i.e.,petroleum ether and ethanol at three temperature levels (30^0 C, 40^0 C and 50^0 C) was carried out by taking neem seed powder and solvent in a ratio of 1:5 in soxhlet apparatus (AOAC, 1975). The extraction was continued for about 6 hours and the filtrate obtained was heated and evaporated at 70^0 C to obtain solvent free neem oil. Further solvent traces were removed by putting the oil in a round bottomed flask and placed on a water bath for 12 hours at 60^0 C -70^0 C. Then the oil was weighed and its percentage was calculated.

The acid and peroxide values were determined using titrimetric method of Cox and Pearson (18). Acid value was expressed interms of free fatty acid in oil. The peroxides were determined by titration against thiosulphate in presence of potassium iodide with starch as an indicator. The iodine value was determined by titrimetric method described in AOAC (1975). The neem oil contains both saturated and unsaturated fatty acids. Iodine gets incorporated into the fatty acid chain wherever the double bond exist.

Seed protein was estimated adopting the microkjeldhal method described in AOAC (1970). Seed weight of 100 completely depulped seeds along with seed coat taken from each tree was measured. The amount of azadirachtin -A of neem seed kernels was estimated through HPLC (12). The seed kernels were made into fine powder and subjected to repeated extraction with *n*- hexane through soxhlet extraction apparatus for complete removal of fat. The residue was air-dried and the azadirachtin content was extracted by methanol. The precipitate obtained through methanol extraction was ground again with methanol and 25 ìl of this extract was utilized for quantifying azadirachtin- A (13). C8 columns were used with a mobile phase containing water, methanol and acetonitrile in the ratio of 50:35:15 with a flow rate of 1ml/min and the azadirachtin-A was detected at 215 nm.

Results and Discussion

Extracts isolated using both the solvents from the trees growing in black soil differed significantly in oil yield, acid value, peroxide value and iodine value and at all temperatures (Table 1- 4). However peroxide value with ethanol at 30^0C differed non significantly. Neem oil yield was high during extraction with both petroleum ether and ethanol at 50^0C compared to other two temperatures and it was more conspicuous with

Table 1. Oil yield and oil quality of black soil grown neem trees tested with two solvents at three temperature levels

Character →	Neem oil % with petroleum ether extraction			Neem oil % with ethanol extraction			Acid value of neem oil (mg/g) extracted with petroleum ether			Acid value of neem oil (mg/g) extracted with ethanol			Peroxide value of neem oil extracted with petroleum ether (mg/g)			Peroxide value of neem oil extracted with ethanol(mg/g)			Iodine value of neem oil extracted with petroleum ether (gr/100 g)			Iodine value of neem oil extracted with ethanol (gr/100 g)		
Tree↓	30°C	40°C	50°C	30°C	40°C	50°C	30°C	40°C	50°C	30°C	40°C	50°C	30° C	40°C	50°C	30°C	40°C	50°C	30°C	40°C	50°C	30°C	40°C	50°C
BT1	32.8	34.4	39.0	30.7	33.3	38.9	13.3	25.2	46.8	18.5	26.3	34.6	2.1	2.7	3.3	3.2	5.0	5.6	76.8	72.5	64.7	74.0	74.0	65.0
BT2	32.1	35.8	40.2	29.0	31.6	39.1	26.2	35.4	47.1	21.4	314	35.8	1.7	2.3	4.0	2.8	3.8	6.2	64.1	63.2	56.1	82.1	69.0	68.2
BT3	32.7	34.2	41.5	30.7	33.5	38.6	19.7	43.6	58.9	23.8	29.2	39.1	1.9	3.0	4.3	3.6	5.8	6.7	78.7	65.7	74.4	76.4	74.3	64.6
BT4	31.8	33.2	39.3	30.1	31.3	38.4	33.3	52.2	66.2	27.4	30.3	40.2	1.5	2.2	2.9	2.9	5.7	7.2	51.1	65.6	72.9	78.2	61.0	60.3
BT5	32.3	34.0	41.8	29.2	31.6	38.9	34.8	47.9	63.9	25.0	37.1	47.1	1.6	2.2	3.6	2.6	5.6	6.5	83.9	71.7	66.2	75.1	76.0	75.3
BT6	33.1	34.8	39.0	31.6	32.4	38.6	17.7	41.6	58.0	19.4	37.2	40.1	1.3	2.9	3.5	2.9	6.5	7.7	82.8	72.2	64.9	75.1	71.7	70.1
BT7	32.8	34.1	39.6	30.5	32.9	39.1	26.1	43.5	64.5	26.6	37.3	43.1	2.5	3.2	3.2	3.2	6.9	7.3	75.4	70.4	64.1	71.7	69.3	67.7
BT8	33.0	34.9	42.6	30.0	32.8	38.4	23.8	51.2	59.8	24.7	34.2	38.1	2.3	3.6	4.4	3.2	6.9	7.2	80.7	73.8	61.3	74.6	64.2	63.4
BT9	33.6	35.9	38.7	31.7	33.2	38.5	28.0	50.1	58.2	25.6	31.6	35.1	2.2	2.2	4.8	3.5	6.8	7.7	88.1	76.7	62.0	77.6	65.5	59.3
BT10	33.3	35.7	42.6	29.9	35.2	37.7	26.6	52.2	67.0	24.8	32.4	34.8	2.0	2.7	2.9	3.0	6.3	7.2	84.7	76.2	65.4	75.2	67.0	60.2
BT11	32.2	34.9	40.5	30.1	33.0	38.2	25.2	55.4	70.0	26.1	33.1	35.6	1.3	3.6	3.2	2.6	6.3	6.9	77.9	70.6	68.1	70.0	67.8	66.3
BT12	32.8	36.1	39.2	30.8	34.0	36.7	30.3	47.5	74.1	28.1	35.7	38.8	1.3	3.2	4.0	2.7	6.8	6.9	86.1	72.5	67.0	66.1	59.7	59.9
BT13	33.3	38.0	38.5	29.7	34.4	36.7	29.5	57.4	60.9	27.1	36.3	39.7	1.1	3.2	3.6	2.7	6.9	7.2	77.2	73.1	66.6	67.5	59.7	60.3
BT14	31.5	34.8	38.3	30.2	33.2	37.1	30.1	54.3	55.3	28.3	32.1	36.1	1.4	3.0	3.4	2.8	6.2	7.0	85.4	70.8	64.9	67.0	61.6	60.5
BT15	31.5	35.3	38.4	30.2	33.1	35.1	26.5	49.1	56.1	26.1	31.9	40.1	2.1	3.2	3.3	2.6	5.9	6.7	79.8	69.9	63.7	69.6	60.5	61.2
BT16	32.4	35.8	38.8	28.7	33.9	36.5	28.1	50.5	54.2	29.7	36.3	42.1	2.1	3.1	3.3	3.0	6.5	6.8	89.1	88.3	65.4	67.7	63.4	58.4
BT17	33.0	35.1	41.1	29.0	33.8	35.9	31.8	56.6	50.4	31.1	37.4	43.1	2.2	3.0	3.2	3.2	7.5	7.7	85.7	73.1	65.7	62.9	61.6	61.5
BT18	32.8	33.4	41.0	30.1	33.1	35.9	29.6	48.7	60.2	24.4	35.0	40.6	1.9	2.6	3.4	3.4	7.6	7.7	81.2	73.6	70.8	63.9	60.0	61.9
BT19	31.8	34.2	41.4	29.5	33.2	38.5	30.1	46.7	63.8	25.1	39.1	39.8	2.2	2.8	2.9	3.3	7.3	7.8	83.3	72.7	72.5	65.3	59.2	64.0
BT20	34.0	36.5	42.2	30.1	33.9	37.1	33.2	50.3	67.0	24.6	37.5	40.1	2.2	2.9	2.7	2.9	7.0	7.6	80.7	74.1	67.1	72.8	64.2	59.5
Mean	32.6	35.0	40.1	30.0	33.1	37.6	27.1	47.9	60.0	25.3	34.0	39.3	1.84	2.88	3.4	2.87	6.0	7.0	79.6	72.3	66.1	68.2	65.4	63.3
CD at 5% level	1.252	1.11	1.80	1.41	1.28	1.97	1.23	1.39	2.86	0.58	1.43	1.66	0.61	0.61	0.36	NS	0.61	0.77	13.60	NS	4.28	4.56	5.65	2.19
CV at 5% level	2.4	2.0	2.8	2.9	2.4	3.3	2.8	1.8	3.0	1.4	2.6	2.6	20.4	13.1	6.5	13.6	6.0	6.6	1.07	102.8	4.0	4.0	5.5	2.2

Table 2. Oil yield and oil quality of red soil grown neem trees tested with two solvents at three temperature levels

Character →	Neem oil % with petroleum ether extraction			Neem oil % with ethanol extraction			Acid value of neem oil (mg/g) extracted with petroleum ether			Acid value of neem oil (mg/g) extracted with ethanol			Peroxide value of neem oil extracted with petroleum ether (mg/g)			Peroxide value of neem oil extracted with ethanol(mg/g)			Iodine value of neem oil extracted with petroleum ether (g/100 g)			Iodine value of neem oil extracted with ethanol (g/100 g)		
Tree↓	30°C	40°C	50°C	30°C	40°C	50°C	30°C	40°C	50°C	30°C	40°C	50°C	30° C	40°C	50°C	30°C	40°C	50°C	30°C	40°C	50°C	30°C	40°C	50°C
RT1	32.4	32.5	39.5	30.7	32.7	38.1	27.6	37.9	48.2	25.7	27.7	35.2	1.3	2.6	2.9	2.8	4.2	5.7	71.1	67.7	63.1	73.9	68.2	64.7
RT2	31.6	33.9	38.4	30.4	22.4	36.3	30.9	40.7	46.1	25.3	25.7	32.9	1.5	2.5	2.8	2.8	4.6	6.5	70.5	64.7	61.6	70.8	65.5	62.0
RT3	31.0	32.2	40.0	31.6	30.8	38.3	36.6	37.3	53.8	30.8	31.5	38.5	1.6	3.0	3.2	3.3	4.2	7.1	72.6	64.4	62.9	69.3	64.0	62.2
RT4	33.4	32.9	38.6	31.2	31.0	38.4	34.6	35.1	54.3	29.5	30.3	35.9	1.5	3.2	3.4	2.8	4.3	6.4	70.2	69.6	60.2	67.1	69.0	63.2
RT5	31.4	34.1	39.2	30.2	32.7	38.8	31.0	42.3	55.8	31.5	31.2	39.0	1.4	3.0	3.6	3.4	4.4	6.6	74.4	67.7	63.2	73.1	66.9	62.4
RT6	33.4	34.1	39.0	30.6	32.7	37.7	33.0	38.4	59.0	32.7	33.8	40.5	2.3	3.4	3.7	3.7	4.6	7.0	74.2	64.8	66.5	69.0	69.1	66.9
RT7	33.9	35.0	40.5	30.4	33.2	38.0	38.5	46.4	60.6	34.6	34.7	47.1	1.4	3.1	4.4	3.8	5.1	7.4	71.2	71.0	62.9	66.7	64.0	63.9
RT8	34.0	33.8	39.9	31.0	32.3	34.7	30.5	47.0	62.7	35.1	33.1	42.5	1.6	2.6	3.0	3.7	5.8	6.8	75.2	69.6	66.1	71.3	64.0	63.0
RT9	31.8	33.4	40.8	30.7	32.8	36.9	27.7	44.2	58.0	32.4	32.2	42.8	2.2	3.2	3.2	3.4	6.2	6.5	74.1	72.8	60.8	73.3	65.0	62.4
RT10	31.9	35.3	39.1	29.2	33.2	36.4	28.9	49.6	58.8	34.1	30.9	39.9	2.5	3.3	3.3	2.9	6.0	7.5	79.9	71.3	64.8	72.9	66.0	68.0
RT11	33.2	35.0	40.5	30.5	34.1	36.1	29.7	49.0	62.5	29.7	31.2	39.2	1.6	3.1	3.8	3.0	6.4	7.6	79.2	69.8	63.2	70.2	63.2	65.0
RT12	31.9	34.8	39.1	30.7	32.7	35.5	24.9	52.7	63.1	31.5	30.9	41.3	2.3	2.8	3.2	3.6	6.2	7.3	76.2	72.1	68.5	66.8	65.0	66.2
RT13	30.8	35.0	38.8	30.7	32.3	38.2	31.3	51.7	71.9	35.9	37.6	40.8	1.6	3.0	3.0	3.6	6.2	7.3	76.6	69.5	63.9	72.1	65.9	65.6
RT14	28.5	35.8	38.7	28.5	32.1	36.7	28.9	57.3	66.1	32.1	34.8	44.2	2.0	3.2	3.9	3.4	6.0	7.3	74.1	68.7	63.0	70.9	66.9	66.0
RT15	31.4	34.4	38.8	30.9	32.6	3.60	29.6	54.3	67.3	30.9	33.8	39.1	22	3.0	4.0	4.5	7.2	7.2	81.1	63.7	64.6	72.3	64.6	63.2
RT16	31.1	33.9	38.6	31.2	33.6	35.0	30.3	49.6	62.5	34.3	35.8	38.0	1.9	3.6	4.1	3.3	7.3	7.6	76.2	69.9	60.5	69.6	65.7	62.9
RT17	31.9	34.0	38.2	28.1	32.0	36.5	30.0	54.7	65.6	28.8	32.4	46.4	1.2	2.8	2.9	3.6	7.1	7.4	73.8	64.2	59.5	68.8	63.7	65.1
RT18	30.4	33.4	39.1	28.5	32.6	37.6	34.0	55.0	60.9	32.9	30.3	43.5	1.7	2.9	3.4	3.3	7.5	7.0	73.0	64.7	58.6	68.7	67.2	65.3
RT19	32.2	33.6	40.2	23.7	32.2	37.8	29.7	54.2	63.0	32.9	33.4	42.7	2.6	3.3	3.6	3.6	6.9	7.3	73.3	68.5	63.1	66.3	67.9	65.7
RT20	29.2	33.7	38.9	30.0	32.0	37.9	30.3	57.6	65.0	31.0	32.3	40.4	2.0	3.4	3.3	4.2	7.2	7.5	76.6	66.6	63.0	69.3	67.4	63.0
Mean	31.7	34.0	39.2	29.9	32.0	37.0	30.9	46.3	60.2	31.5	32.1	40.4	1.82	3.05	3.95	3.43	5.87	7.05	74.6	68.0	63.0	70.1	65.9	64.3
CD at 5% level	1.15	1.24	1.59	1.56	NS	NS	3.25	2.56	3.61	4.12	2.95	3.01	0.61	0.52	0.67	0.58	0.76	0.82	3.49	2.59	3.53	3.57	NS	NS
CV at 5% level	2.3	2.3	2.5	3.3	12.6	4.5	6.6	3.4	3.7	8.1	5.7	4.7	20.7	10.7	12.1	10.6	8.1	7.3	2.9	2.4	3.5	3.2	3.7	3.7

Table 3. Oil yield and oil quality of sandy soil grown neem trees tested with two solvents at three temperature levels

Character →	Neem oil % with petroleum ether extraction			Neem oil % with ethanol extraction			Acid value of neem oil (mg/g) extracted with petroleum ether			Acid value of neem oil (mg/g) extracted with ethanol			Peroxide value of neem oil extracted with petroleum ether (mg/g)			Peroxide value of neem oil extracted with ethanol(mg/g)			Iodine value of neem oil extracted with petroleum ether (g/100 g)			Iodine value of neem oil extracted with ethanol (g/100 g)		
Tree↓	30°C	40°C	50°C	30°C	40°C	50°C	30°C	40°C	50°C	30°C	40°C	50°C	30° C	40°C	50°C	30°C	40°C	50°C	30°C	40°C	50°C	30°C	40°C	50°C
ST1	28.3	33.3	38.3	30.3	32.0	37.4	24.0	33.9	58.7	20.2	25.7	35.5	2.3	2.3	3.5	1.5	5.3	6.2	67.8	71.8	67.0	70.0	67.2	62.9
ST2	30.2	32.1	36.7	30.1	31.6	36.1	21.7	35.8	59.4	21.9	28.0	37.6	2.5	2.3	3.4	1.2	5.4	6.8	70.7	69.5	62.8	70.8	66.0	63.3
ST3	33.4	36.2	38.1	29.6	32.3	37.3	25.1	36.8	63.8	15.2	31.2	36.3	2.7	2.8	3.6	1.6	5.9	7.3	73.7	68.5	65.4	65.7	69.5	68.5
ST4	30.3	31.1	37.6	29.6	31.2	37.5	27.0	40.6	59.8	18.5	31.4	40.3	2.6	3.2	3.3	2.1	5.3	7.2	69.5	63.8	63.9	68.4	70.0	70.8
ST5	30.3	33.6	37.2	29.5	33.6	35.7	24.9	40.2	64.4	23.1	31.9	41.8	2.5	2.8	3.4	1.3	6.0	7.3	73.7	66.8	64.2	74.8	71.4	66.6
ST6	33.4	35.5	38.4	30.5	31.3	36.8	24.1	38.7	70.1	22.4	31.6	42.6	3.4	3.4	3.4	2.1	6.0	7.1	78.7	67.5	64.8	80.7	67.6	66.4
ST7	32.6	33.4	38.7	30.4	30.9	37.6	29.0	45.4	72.1	23.0	34.5	42.2	3.6	3.3	4.0	2.4	6.5	7.3	83.0	67.4	69.5	68.6	68.9	63.5
ST8	30.5	32.6	38.2	28.5	32.9	37.9	30.7	38.9	68.2	21.2	35.5	38.9	2.7	2.8	3.1	1.9	6.7	6.8	69.3	67.8	66.0	65.1	65.5	63.1
ST9	31.9	32.9	38.1	29.8	32.2	37.6	29.4	33.7	61.2	24.3	32.0	41.0	2.7	2.8	3.5	2.2	6.3	6.6	73.2	67.9	65.7	74.6	66.6	60.5
ST10	29.5	33.5	39.0	30.0	32.2	37.5	31.2	41.5	68.7	24.7	32.4	42.9	2.8	3.0	3.3	1.8	5.9	6.9	79.3	70.7	69.5	78.8	68.7	54.6
ST11	31.3	31.8	38.9	30.4	31.9	36.7	32.7	53.9	68.8	26.3	33.1	45.3	3.5	3.3	3.6	1.9	6.2	7.1	83.7	71.9	68.9	76.4	66.4	57.2
ST12	29.6	34.2	37.5	30.6	33.1	37.9	34.1	48.0	62.9	26.9	24.4	48.2	2.9	3.2	4.0	2.4	6.1	7.0	83.7	72.5	62.1	69.9	67.8	60.3
ST13	29.6	32.6	37.6	28.1	32.3	37.1	29.3	53.1	63.4	23.3	33.0	42.1	3.5	2.8	3.5	2.2	6.8	7.1	83.4	68.9	60.5	68.4	64.2	62.0
ST14	31.7	32.9	38.2	29.6	32.8	38.2	31.9	40.0	59.2	24.4	29.8	47.1	2.2	3.4	3.8	1.7	6.2	6.9	77.8	67.8	63.0	70.4	66.9	61.9
ST15	30.4	32.3	37.9	28.1	31.3	35.8	31.5	42.9	57.1	24.2	30.6	44.6	3.4	2.4	3.7	1.8	7.6	7.0	85.8	68.7	64.0	68.3	65.5	64.2
ST16	30.7	33.9	37.7	28.9	32.7	35.9	27.3	44.4	60.8	24.1	32.8	42.6	2.4	2.4	3.2	1.9	6.6	7.4	76.8	68.5	62.4	66.8	65.6	60.2
ST17	30.6	31.0	37.2	29.8	29.0	37.1	32.0	40.0	57.4	24.6	34.2	41.7	2.2	3.2	3.4	1.5	6.4	7.3	74.5	65.3	63.9	68.7	66.7	57.6
ST18	31.5	32.8	38.7	29.1	32.5	36.2	30.0	30.3	69.0	25.2	34.5	43.7	3.2	3.6	3.5	1.6	6.4	7.0	76.8	72.6	63.8	71.5	67.2	57.5
ST19	30.2	32.9	38.4	28.3	31.9	35.4	30.9	54.1	65.8	22.5	36.1	29.7	2.9	3.1	3.7	1.9	6.1	7.2	77.2	67.9	64.7	72.0	67.4	56.4
ST20	28.5	31.9	39.0	28.0	31.8	34.2	29.0	39.5	70.4	23.1	32.6	42.9	3.2	3.4	4.4	1.7	7.0	6.8.	75.9	72.1	67.3	69.6	69.1	61.4
Mean	30.7	33.0	38.0	29.4	31.9	36.7	28.7	41.5	64.0	22.9	31.7	41.2	2.8	2.9	3.5	1.8	6.2	6.6	76.7	68.8	64.9	70.9	67.4	61.9
CD at 5% level	1.87	2.14	NS	NS	NS	NS	2.52	8.43	7.96	5.11	NS	NS	0.57	0.71	NS	NS	0.97	NS	3.78	4.69	4.80	4.67	NS	11.49
CV at 5% level	3.8	4.1	2.4	4.2	4.7	3.8	5.5	12.7	7.8	13.9	14.2	12.9	12.6	1.22	14.3	22.6	9.8	7.1	3.1	4.3	4.6	4.1	6.0	11.9

Table 4. Variation between different characters of neem trees growing in three soil types

Character→ Tree↓	Black soil			Red soil			Sandy soil		
	100 Seed weight (g)	Seed protein (%)	Azadirachtin (mg/kg)	100 Seed weight (g)	Seed protein (%)	Azadirachtin (mg/kg)	100 Seed weight (g)	Seed protein (%)	Azadirachtin (mg/kg)
T1	18.7	33.2	2700	13.6	30.0	1755	17.3	28.5	870
T2	25.0	32.1	2600	18.3	33.7	1865	17.0	31.2	960
T3	23.2	35.2	2300	18.3	30.3	1929	18.8	30.0	980
T4	21.0	32.3	2420	19.2	28.5	1681	17.2	30.3	1000
T5	19.3	33.2	2200	18.5	26.2	2010	16.4	28.8	973
T6	25.0	31.0	2350	19.3	29.2	1774	21.9	27.8	982
T7	18.5	29.4	2480	16.1	25.1	1900	19.7	21.5	982
T8	19.1	32.2	2390	17.6	31.1	1870	21.0	29.6	995
T9	18.6	33.2	2450	18.6	30.5	1830	17.8	30.4	991
T10	22.2	33.4	2342	13.7	29.5	1955	20.5	28.8	979
T11	20.0	30.9	2368	19.6	31.5	1950	18.3	26.3	986
T12	17.7	34.3	2412	19.4	32.6	1791	20.6	24.6	975
T13	19.4	34.7	2632	2.35	31.5	1856	19.7	25.7	992
T14	18.4	32.9	2580	16.0	34.4	1942	18.4	27.9	998
T15	19.1	33.9	2430	16.4	34.9	1991	17.7	28.5	920
T16	16.4	32.3	2392	16.0	31.8	1853	18.8	30.4	965
T17	17.2	30.1	2200	17.7	31.7	1894	17.6	31.3	970
T18	18.2	34.6	1870	17.8	29.5	1950	17.4	31.2	980
T19	22.4	32.8	1990	16.5	28.8	1940	20.7	32.1	993
T20	19.1	34.3	2800	18.5	27.5	1780	22.3	30.9	989
Mean	18.9	32.8	2420	16.8	30.4	1900	18.9	27.2	980
CD at 5% level	0.283	0.805	193.071	NS	2.505	96.287	1.463	1.771	NS
CV at 5% level	0.9	1.5	5.1	490.8	5.1	3.2	4.8	3.8	5.4

Table 5. Overall mean values of oil yield and oil quality characters of trees growing in three soil types

Soil type →	Black soil						Red soil						Sandy soil					
Character↓	Petroleum ether			Ethanol			Petroleum ether			Ethanol			Petroleum ether			Ethanol		
	30º C	40º C	50º C	30º C	40º C	50º C	30º C	40º C	50º C	30º C	40º C	50º C	30º C	40º C	50º C	30º C	40º C	50º C
Oil Yield (%)	32.6	35.0	40.1	30.0	33.1	37.6	31.7	34.0	39.2	29.9	32.0	37.0	30.7	33.0	38.0	29.4	31.9	36.7
Acid value (mg/g)	27.1	47.9	60.0	25.3	34.0	39.3	30.9	47.7	60.2	31.5	32.1	40.4	28.7	41.5	64.0	22.9	31.7	41.2
Peroxide value (mg/g)	1.8	2.8	3.4	2.8	6.0	7.0	1.8	3.0	3.2	3.4	5.8	7.0	2.8	2.9	3.5	1.8	6.2	6.6
Iodine value (g/100 g)	79.6	72.3	66.1	68.2	65.4	63.3	74.6	68.0	60.0	70.1	65.9	64.3	76.7	68.8	64.9	70.9	67.4	61.9

petroleum ether. Among the 20 trees tested with both the solvents in three different temperatures (Table 1) BT8 (42.6), BT10 (42.6) and BT20 (42.2) recorded high oil yield with petroleum ether at 50^{0}C. Seed protein, Azadirachtin and seed weight were differed significantly among the trees growing in Black soil. Of the 20 trees screened for these parameters, BT20 (2800), BT1 (2700) and BT13 (2632) contained high Azadirachtin (Table 4). The tree designated as BT20 has been identified as a good oil yielding tree apart from containing higher Azadirachtin content (Table 4). Similar trend was also observed in the trees growing in red soil. In ethanol extracts obtained at 40^{0}C and 50^{0}C, the oil yield and iodine value recorded non significantly. Among the 20 trees tested with two solvents at different temperatures, RT9 (40.8), RT7 and RT11 (40.5) recorded high oil yield with petroleum ether at 50^{0}C. Oil yield of trees grown in sandy soil is nonsignificant with petroleum ether at 50^{0}C, and with ethanol at all the temperatures. Acid value of sandy soil trees is significant in petroleum ether extracts at 30^{0}C, 40^{0}C and 50^{0}C, but it is insignificant in ethanol extracts at 40^{0}C and 50^{0}C. Peroxide value differed significantly among the trees of sandy soil in petroleum ether extracts at 30^{0}C and 40^{0}C and in ethanol extracts at 40^{0}C, and iodine value differed significantly in both solvent extracts obtained at all temperatures except with ethanol at 40^{0}C.

The study envisaged that the neem trees growing in three soil types differed significantly among themselves in oil yield and quality. It was clearly evident that all the twenty trees growing in a black soil are found superior when compared to the trees growing in red and sandy soils suggesting that black soil supports the trees for expression of their full genetic potential (Table 5).

Acid, peroxide and iodine values of oil content are used as indicators of oil quality. In the present study the Acid, peroxide values were increased with increasing temperature in all the tress of different soils. Acid value represents the amount of free fatty acid present in oil. Hence increase in acid value with the increases of temperatures might be due to increased rate of hydrolysis of neem oil into free fatty acid and glycerol by lipase enzyme (10, 11, 14, and 15). Peroxide value, depends on the levels of aldehydes, ketones and their oxidation, might result in rancidity, it was observed to be increasing with increased temperatures there by causing the oil to turn rancid, which reflected its status of preservation (17, 18). Iodine value is an indicator for determining the quality of oil particularly with respect to the amount of unsaturated fatty acids in the oil (16). The decrease in iodine value indicating the presence of less unsaturated fatty acids with increasing temperature. Similar results of increased acid and peroxide values and decreased iodine values were observed at higher temperature by many workers (19, 20).

Among the two solvents used for extraction of neem oil, petroleum ether was found to be a

better solvent and it is suggested as a good solvent for extraction of neem oil. Among the trees grown in three soil types, black soil grown trees are proved to be higher oil yielding trees, and the tree numbered as BT20 recorded higher oil yield coupled with high Azadirachtin content indicating the need for its conservation studies.

Conclusion

Based on physicochemical analysis, it can be concluded that the increasing temperature has decreased iodine value but increased the acid and peroxide values suggesting that even though the oil yield was more at high temperatures, extracting oil at these temperatures was not advisable because it affects the oil quality. Among the different trees grown in three soil types black soil trees are proved to be higher oil yielding trees, and the tree numbered as BT 20 recorded higher oil yield coupled with high azadirachtin content indicating the need for conservation of this tree type. Of the two solvents used for extraction of neem oil, petroleum ether was found to be a better solvent.

Acknowledgements

Financial assistance from Department of Biotechnology, Ministry of Science and Technology, New Delhi is gratefully acknowledged.

References

1. Puri, H. S. (1999). Neem –The divine tree. Hardwood Academic Publishers. Singapore.pp 9-23
2. Govindachari, T. R. (1992). Chemical and Biological investigations on *Azadirachta indica* (The Neem Tree). Current Science 63: 117-122.
3. Butterworth, J. H. and Morgan, E. D. (1960). Isolation and structural studies on a locust feeding inhibitor from neem seeds. Ph.D thesis, University of Keele, UK.
4. Schroeder, R. and Nakanishi, K. (1987). A simplified isolation procedure for azdirachtin. Journal of Natural Products. 50: 241-244.
5. Henderson, R., McCrindle, R., Overton, K. H. and Matera, A. (1964). Salannin. Tetrahedron lett. 52:3969-3974
6. Lavie, D. and Jain, M.K. (1967). Tetranotriterpenoids from Melia azadirach L. Chem. Commum.,pp 278-280.
7. Zanno, P.R., Miara,E.,Nakanaishi, K. and Elder, D.L. (1975). Structure of the insect phagorepellent azadirachtin ,application of PRFT/CWD carbon 13 nuclear magnetic resonance. Journal of American Chemical Society. 97:1975-1977.
8. Biswas, S.A.S., Singh, P. and Chandra, S. (1995). Neem (*Azadirachta indica*A.juss)-A versatile multipurpose tree. Indian for.,121:1057-1060
9. Johnson, S. Margon, E. D. and Peiris. C. N. (1996). Development of the Major Triterpenoids and

   oil in the Fruit and Seeds of Neem (*Azadirachta indica*). Journal of Annals Botany. 78: 383-388.
10. Kaushik, N. and Vir. S. (2000). Variations in fatty acid composition of neem seeds collected from the Rajasthan state of India; Biochemical Society Transactions. 28:6.
11. Schmutterer, H. (1990). Properties and potential of natural pesticides from the neem tree, *Azadirachta indica*. Ann. Rev. Entomol.35, 271-297
12. Ubel, E.C., Warthen, J.D.J. and Jacobson, M. (1979). Preparative reversed phase liquid chromatographic isolation of azadirachtin from neem kernels. Journal of Liquid Chromatogra.2:875-882.

13. Jyothi Pattnaik K.S., Raghupathi Rao,N.D. and Parvathi Chary (2006). Ecomorphometric markers reflect variations in Azadirachtin –A content of *Azadirachta indica* A.jus (Meliaceae) in select regions of Andhra Pradesh, India. Current Science 91:628-636.

14. Adeeko, K. A. and Ajibolao. O. O. (1990). Processing factors affecting yield and quality of Mechanically expressed Groundnut oil. Journal of Agric. Engineering. Vol 45: 31-43.

15. Khraisha, Y. H. (2000). Retorting of oil shale followed by solvent extraction of spent shale: Experiment and Kinetic Analysis. Journal of Enegry Sources. 22: 347-355.

16. Horowitz, W. (1975). Official Methods of Analysis of AOAC Association of official Analytical Chemists Washington (12th Ed). p 488.

17. Mongkholkhajornsilp, D, Douglas, S., Douglas, P. L., Elkamel, A., Teppaitoon, W. and Pongamphai, S. (2005). Supercritical CO_2 extraction of Nimbin from Neem seeds – A Modeling Study. Journal of Food Engineering. 71: 331-340.

18. Cox, H. E and Pearson, D. (1962). The Chemical Analysis of Foods Chemical Publishing Co Inc New York. p 421.

19. Maria Yuliana Liauw, Natan, F.A. Widiyanti, P. Ikasari, D. Indraswati N. and. Soetaredjo. F. E. (2008). Extraction of Neem oil (*Azadirachta indica A.juss*) using n-hexane and ethanol: Studies of oil quality, Kinetic and Thermodynamic. Vol 3, No 3, 49-54.

20. Felycia EdiSoetaredjo, Budijanto, G. M. Prasetyo, R. I. and Indraswati, N. (2008). Effectes of pre treatment condition on the yield and quality of neem oil obtained by mechanical pressing. ARPN Journal of Engineering and Applied Sciences. Vol 3, No 5, 45-49. *(2E,4Z,6Z)-*

Current Trends in Biotechnology and Pharmacy
Vol. 5 (1) 1038-1042 January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

Extraction, Isolation, Molecular Modeling and Optimization of Antimicrobial Agents from *Coriandrum sativum*

K. Venkateswara Swamy[1], Supriya Kore[2], Rachna Pandey[2], Madhukar Khetmalas[2*]

[1]Department of Bioinformatics and Computer Science, Dr. D. Y. Patil Biotechnology and Bioinformatics Institute, Pune, India

[2] Department of Molecular Biology, Genetics and Pharmacology, Dr. D. Y. Patil Biotechnology and Bioinformatics Institute, Pune, India.

*For Correspondence - venkateswara.swamy@gmail.com

Abstract

During the recent years the identification of active biomolecules against microbial/viral pathogen are becoming increasingly important, particularly from the medicinal plants. The CCl_4 fraction of *Coriandrum sativum* was tested against nine bacterial strains (*Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Erwinia sp, Proteus vulgaris*) and one fungal strain (*Candida albicans*) to determine antimicrobial activity. The CCl_4 extract of *C. sativum* (analyzed using GC/MS) showed eight compounds based on retention time and physico chemical properties. All the compounds were modeled and optimized with VlifeMDS™ software under specified parameters. These molecules will be more important to design for best antibacterial agents based on computational tools.

Key words: *C. sativum*, antimicrobial activity, antimicrobial agents, molecular modeling and optimization, VlifeMDS

Introduction

The need for new antimicrobial agents is greater than ever because of the emergence of multidrug resistance in common pathogens, the rapid emergence of new infections, and the potential for use of multidrug-resistant agents in bioweapons (1). Evaluation of synthetic compounds and natural products for potential antimicrobial activity continues to be an important strategy for the initial identification of new drugs with possible clinical values (2, 12).

Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial properties (3). Despite increasing antimicrobial resistance and multiple drug resistance in clinical isolates of both Gram-positive and Gram-negative bacteria, there are few novel antimicrobial agents in development. The few new agents that have been recently licensed have tended to have narrow spectra of activity, focused on Gram-positive pathogens, especially methicillin-resistant *Staphylococcus aureus* (MRSA)(4). Chemical principles from natural sources have become much simpler and have contributed significantly to the development of new drugs from medicinal plants (4). Vital plant extracts are playing an important role in antimicrobial activity against pathogenic bacteria and fungal diseases (5). Today, there are at least 120 distinct chemical substances derived from plants that are considered as important drugs currently in use in one or more countries in the

world. Among all 120 drugs, seven plant-derived anticancer drugs have received Food and Drug Administration (FDA) approval for commercial production like taxol / paclitaxel (isolated from *Taxus brevifolia*), vinblastine, vincristine (isolated from Madagascar Periwinkle), Topotecan, irinotecan (isolated from *Camptotheca acuminata*), etoposide, teniposide isolated from *Podophyllum peltatum*). The *in vitro* antibacterial activities of a total of 46 extracts from dietary spices and medicinal herbs were investigated by agar – well diffusion method against five food borne bacteria (6). Disk diffusion and minimum inhibitory concentration determination assays coriander oil exhibited the strongest antimicrobial activity against the tested strains (7).

In our work, different parts of *Coriandrum sativum* (roots, leaves etc.) were extracted with the help of n-hexane, chloroform, Methanol, Ethanol, Acetone, cold water, hot water, toluene as solvents. *C. sativum* seed fractions, collected with column chromatography with carbon tetra chloride (CCl_4) as solvent showed most effective antimicrobial activity against all Gram positive and Gram negative bacteria tested (8). The phytochemical analysis suggested the presence of bioactive compounds as diverse as flavanoids, glycosides, terpenoids, steroids and tannins. The present study indicates the strong possibility of exploring *C. sativum* CCl_4 extract as potential antimicrobial source for future drug development and to combat the emerging threat of antibiotic resistance in microbes (9). All the antimicrobial agents were modeled and minimized under physiological conditions with VlifeMDS. These molecules can be used for further analysis to design in the form of drugs.

Methodology

C. sativum plant seed extract was applied on various nine bacterial strains (*Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Erwinia sp, Proteus vulgaris*) and one fungal strain (*Candida albicans*) to determine antimicrobial activity. Several phytochemical tests were carried out to ascertain the presence of the active chemical constituents such as alkaloids, glycosides, terpenoids, steroids and flavanoids, reducing sugar and tannin by standard procedures (10). The Kirby-Bauer and Strokes method was found suitable for antimicrobial susceptibility testing, with the Kirby Bauer method being recommended by the NCCLS (National Committee for Clinical Laboratory Standards). Single isolated colony from each bacterial species was used for the inoculation of flask containing 20 ml nutrient broth medium. The flask was incubated at 200 rpm in the incubated shaker at 37^0C for 24 hrs. Overnight grown culture was used for the disc diffusion assay. The zone of inhibition was measured using a scale. In the final step of analysis, CCl_4 fraction was analyzed by Gas chromatography Mass spectrometry (GC/MS) method. GC analyses were carried out on a Varian 300 GC with a DB-5 fused column (25 m×0.25 mm; film thickness 0.25 µm). The operating condition were as follows: Carrier gas was helium with a flow rate of 1.5 ml/min, the oven temperature was programmed 5 min. isothermal at 60° C then from 60 to 280°C at 3°C/min, injector and detector temperature were set at 280° C.GC/MS was run on a Finnegan MAT Incos-50 instrument and the DB-5 capillary column and the GC conditions were the same as above. Mass spectrometer conditions were as follows: Ionization potential: 70eV ionization current: 2A source temperature: 150°C; resolution: 1000. Antimicrobial compounds of *C. sativum* were collected based on retention time and analyzed based on molecular weight. Molecular structural mining was done with

Pubchem database (http://pubchem. ncbi.nlm.nih.gov/) and ZINC database (http://zinc.docking.org/) based on common name and molecular weight. Structures of antimicrobial agents were converted to pdb from sdf file format with Vlife MDS™

Molecules were energy minimized with USF force filed to understand conformational structures. With the application of Universal Force Field (UFF) molecules were optimized (11). We have selected broad range of 1000 cycles for better optimization and included convergence criteria for RMS (Root Mean Square) as 1.0. Energy minimization process was carried out till molecules have reached to their optimum in terms of bond, angle, torsion, van der waal's energies of each molecule in *C. sativum* crude extract from CCl_4.

Results

The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. The CCl_4 fraction of *C. sativum* showed most effective antibacterial activity against all Gram positive and Gram negative bacteria tested. We have done structural mining with Pubchem (http://pubchem.ncbi.nlm.nih.gov) database and Zinc database (http://zinc.docking.org/choose.shtml). Important physicochemical properties were listed in Table. 1 and structures represented in Fig. 1. The zone of inhibition by *C. sativum* extract from CCl_4, acetone, $CHCl_3$ and H_20 fraction of column chromatography on *S. aureus ATCC* was observed. Concentration of CCl_4 and zone of inhibition are shown in Fig. 2 and Fig. 3 respectively.

Molecular structure of Pentadec-2-yn-1-ol has the two fractions based on retention time (RT). Molecular weight of all the extracted agents is ranging from 200 to 540. Heptatriaconta-1-ol and octadecyl hexadecnoate has above 500 remaining within range of 200 to 540 only. Pentadec-2-yn-1-ol and heptatriacontan-1-ol have the hydroxyl group as functional group so it can donate or accept one hydrogen atom in biochemical reactions. Octadecyl hexadecanoate can accept two hydrogen atoms due to the presence of two oxygen atoms in its structure. Structure of chemicals were drawn on workspace of VlifeMDS™ and converted to 3D

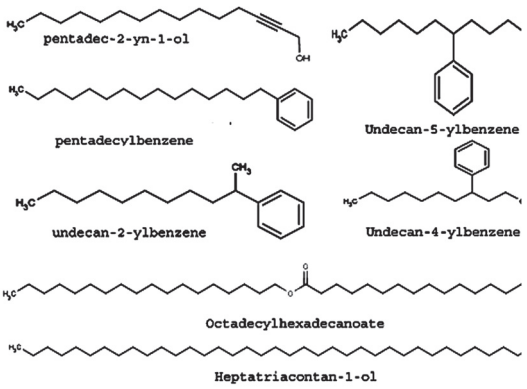


Fig. 1. Compounds of CCl_4 extract of *C. sativum* from GC/MS analysis.

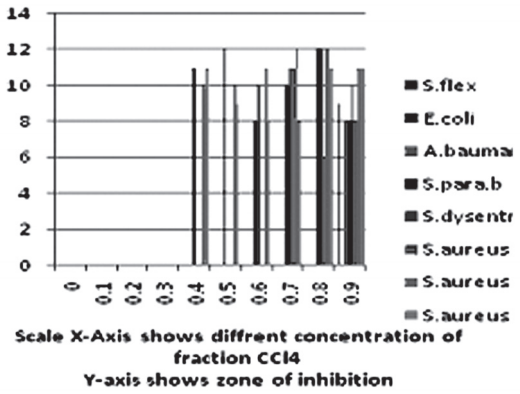


Fig. 2. The zone of inhibition against eight different drug resistant bacterial isolates tested.

Table 1 . Chemical properties of antimicrobial agents of *C. sativum* extracted with CCl_4 solvent and retention time (RT) of compounds by GC/MS analysis.

S. No	Compound ID (pubchem)	IUPAC name	Molecular weight (g/mol)	Molecular formula count	heavy atom	H-donor	H-acceptor	RT
1.	534429	pentadec-2-yn-1-ol	224.382	$C_{15}H_{28}O$	16	1	1	34.61
2.	16476	pentadecylbenzene	288.510	$C_{21}H_{36}$	21	0	0	36.53
3	20661	undecan-5-ylbenzene	232.404	$C_{17}H_{28}$	17	0	0	36.69
4	20654	undecan-4-ylbenzene	232.404	$C_{17}H_{28}$	17	0	0	37.08
5	534429	pentadec-2-yn-1-ol	224.382	$C_{15}H_{28}O$	16	1	1	38.48
6	20656	undecan-2-ylbenzene	232.404	$C_{17}H_{28}$	17	0	0	39.33
7	537071	heptatriacontan-1-ol	536.998	$C_{37}H_{76}O$	38	1	1	60.36
8	75778	octadecyl hexadecanoate	508.902	$C_{34}H_{68}O_2$	36	0	2	65.6

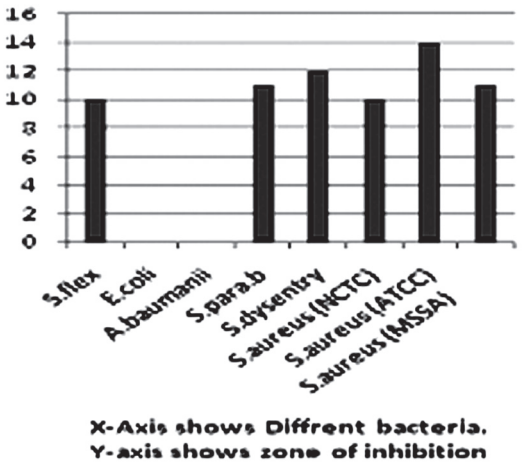


Fig. 3. Effect of CCl_4 fraction of *C. sativum* in the formation of zone of inhibition against eight different pathogenic bacterial isolates tested. (Bar heights are measured in mm).

structures with optimization parameters. Optimization of molecules requires the force filed, which is a functional representation of the energy of a molecular system, as a function of its atomic co-ordinates and expressed in the form of bonded and non-bonded energy terms. The terms in a force field are parameterized based on experimental or quantum mechanical data. Force field optimization feature in VLifeMDS allows obtaining minimum energy conformation of molecules. Almost all the molecules were energy minimized within 50 iterations of 1000 cycles. The optimized molecular structure details are listed in the Table. 2

Conclusion

Medicinal plant *C. sativum* has been investigated for the compounds available for the secondary plant products that may have antimicrobial activity against pathogenic bacteria. The compounds were analyzed through bond angle, torsion, vander waal's energies by VifeMDS algorithms.

The GC/MS analysis of *C. sativum* extract has resulted in compounds that have antimicrobial activity compounds. Based on the total energy of the compounds the aromatic compounds (pubchem ID 16476, 20661, 20654, and 20566) has less energy as compared to aliphatic compounds (Pubchem ID 537071 and 75778). These results conclude that compounds can be used to target specific protein in pathogen bacterial system to design new drug molecules. This analysis will help to design new antimicrobial agents which contribute for pharmacological applications in future research work.

Table 2. Calculated energy values of antimicrobial agents of *C. sativum* with Vlife MDS algorithms.

S.No	Compound ID (Pubchem)	Bond energy (kcal/mol)		Angle energy (kcal/mol)		Torsion energy (kcal/mol)		vwd energy (kcal/mol)		No. of iterations	Total energy (kcal/mol)	
		Initial	final	Initial	final	Initial	final	Initial	final		Initial	Final
1	534429	2.960	1.442	2.429	1.121	0.060	0.031	6.204	5.522	10	11.654	8.1178
2	16476	3.884	4.144	8.187	2.741	1.154	1.163	16.191	15.353	25	29.416	23.410
3	20661	3.810	4.317	7.655	3.322	1.132	1.117	20.007	15.255	48	32.609	24.020
4	20654	3.818	4.370	6.047	3.309	1.184	1.223	20.647	15.395	44	31.698	24.302
5	20656	3.329	3.702	4.638	2.717	1.135	1.162	20.105	15.901	29	29.210	23.486
6	537071	50.293	3.088	0.000	1.946	0.000	0.020	29.732	22.484	6	80.024	27.539
7	75778	58.497	3.343	0.000	2.827	0.000	0.023	32.077	24.780	15	90.574	30.972

Acknowledgements

The authors are grateful to Dr. D. Y. Patil Vidyapeeth, Pune, India for financial assistance and completion of the investigation.

References

1. Brad Spellberg, John, H., Powers, Eric, P., Brass, Loren, G., Miller, John, E. and Edwards Jr. (2004). Trends in Antimicrobial Drug Development: Implications for the Future. Clinical Infectious Diseases 38: 1279–1286.
2 Mishra, P., Lukose, T. and Kashaw, S. K. (2007). Synthesis and antimicrobial evaluation of some novel 2-imino-3-(4'-carboxamido pyridyl)-5-arylidene-4-thiazolidinones and their brominated derivatives. Indian J Pharm Sci 69: 665-668.
3. Marjorie, M. C. (1999). Plant products as anti-microbial agents. Clin Microbiol Rev. 12: 564-582.
4. French G. L (2008). What's new and not so new on the antimicrobial horizon? Clinical Microbiology and Infection, 14: 19 – 29.
5. Cox, P. and Balick, M. J. (1994). The ethanobotanical approach to drug discovery. Sci American.270 (1): 82 -87.
6. Pongsak, R. and Parichat, P. (2010). Potential of Coriander (Coriandrum sativum) oil as a natural antimicrobial compound in controlling Campylobacter jejuni in raw meat. Bioscien. Biotechnol Biochem 74 9 (1): 31-35.
7. Binshan Yi-Zhong Cai, John. D. Brooks, and Harold Corke (2007). The in vitro-antibacterial activity of dietary spice and medicinal herbs extracts.Int. of Food Microbiology 117:112-119.
8. Venkatesh, S., Sailaxmi, K., Reddy, B. M. and Ramesh, M. (2007) Antimicrobial activity of *Helicteres isora* root. Indian J Pharm Sci 69: 687-689.
9. Saeed, S. and Tariq P. (2007) Antibacterial activities of *Emblica officinails* and *Coriandrum sativum* against Gram negative and positive urinary pathogens.Pak J. Pharmacol Vol 20 (1): 32-35.
10. Nair, R and Chanda, S. (2007). In-vitro antimicrobial activity of *Psidium guajava* leaf extracts against clinically important pathogenic microbial strains Braz.J. Microbiol. 38 (3):452-458.
11. Rappe, A, K., Casewit, C J., Colwell, K. S, Goddard III, W. A. and Skiff, W. M. (1992). UFF, a full periodic table force field for molecular mechanics and molecular dynamics simulations. Am. Chem. Soc. 114 (25),pp 10024–10035.
12. Elizabeth, A., Mazzio, Karam, F. A. and Soliman. (2009). *In Vitro* screening for the tumoricidal properties of internainal medicinal herbs. Phytother Res. 23 (3): 385-398.

Current Trends in Biotechnology and Pharmacy
Vol. 5 (1) 1043-1053 January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

Isolation and Characterization of *Streptomyces sp.* from Durg District of Chhattisgarh for Antimicrobial activity

N. Singh* and V. Rai
Microbiology and Biochemistry Lab, School of Studies in Life Sciences
Pt. Ravishankar Shukla University, Raipur-492010, Chhattisgarh, India
*For Correspondence - nevi0007@gmail.com

Abstract:

The present work was carried out in order to establish a suitable protocol for screening program for antimicrobial producing *Streptomyces* sp. from soil sample of Durg District of Chhattisgarh, INDIA. Fifty seven *Streptomyces* isolates with distinct characteristics were isolated and purified by serial dilution method on starch casein nitrate agar medium. These isolates were screened for their antimicrobial activity against twenty one test organisms obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), IMTECH, Chandigarh. Morphological, physiological and biochemical characteristics indicated that these isolates belong to the genus *Streptomyces* and characterized as *Streptomyces rimosus, Streptomyces chromogenus, Streptomyces rouchi*-3, *Streptomyces antibioticus*-3, *Streptomyces filipinensis, Streptomyces atroolivaceus, Streptomyces exfloliatus*-2, *Streptomyces halstedii, Streptomyces cyaneus, Streptomyces violaceus, Streptomyces oliovaceoviridis, Streptomyces lydicus, Streptomyces phaeochromogenes, Streptomyces chromofuscus* . Among fifty seven isolates, nineteen exhibited antimicrobial activity against test pathogens, and out of these nineteen isolates, 11 isolates exhibited both antibacterial and antifungal activity while 8 exhibited only antibacterial activity.

Key words: *Streptomyces* sp., Antimicrobial activity, Screening, Characterization

Introduction

The genus *Streptomyces* was described for the first time by Waksman and Henrici in the year 1943 (1). *Streptomyces* are filamentous, aerobic spore formers and omnipresent (1, 2). Presence of L, L isomer of 2, 6-Diaminopimelic acid (L,L-DAP) and absence of any diagnostic sugar in the cell wall is a salient feature of the genus *Streptomyces* (3). *Streptomycetes* proved to be a rich source of novel bioactive, commercially significant compounds (4, 5, 6). Though genus *Streptomyces* is being studied from last many decade still there is very little documented information of the occurrence of *Streptomyces sp.* from the different soil habit (5,7,8,9). *Streptomyces* is the largest antibiotic-producing (almost 75% of commercially and medically useful antibiotics) genus in the microbial world discovered so far (2). The number of antimicrobial compounds reported from the species of this genus per year has increased almost exponentially for about two decades. Reports show that this group of microorganisms still remains an important source of antibiotics (2), because they are readily biodegradable, specific and generally have low toxicity (10). In the present study, *Streptomyces* isolated from soil of Durg District of Chhattisgarh were screened for their antimicrobial activity

against bacterial and fungal pathogens and the selected isolates were characterized on the basis of their morphological, physiological and biochemical characteristics.

Materials and Methods

Collection of soil samples: Soil samples were collected from various locations i.e. rhizosphere of plants, agricultural soil, preserved areas and forest soils of Durg District of Chhattisgarh. The samples were taken from up to 20 cm depth, after removing approximately 3 cm of the soil surface. The samples were placed in sterile polyethylene bags, closed tightly and stored in a refrigerator at 4°C until further analysis.

Isolation of Actinomycetes: Soil samples were incubated at 37°C for 4 days then suspended in sterile ringer solution. Test tubes containing a 10^{-2} to 10^{-6} dilution of samples were placed in a water bath (SONAR, INDIA) at 45°C for 16 h to separate spores from vegetative cells. *Streptomyces* were isolated by spread plate technique by serial dilution of soil samples on starch casein nitrate agar plates containing cycloheximide and nystatin, each at concentration of 50 ìg/ml of medium to inhibit the fungal growth (11, 25). The plates were incubated at 28°C until the sporulation of *Streptomyces* colonies occurred. Pure cultures were obtained from selected colonies by repeated sub culturing on starch casein nitrate agar slants.

In-vitro screening of isolates for antagonism: Primary screening of isolates were done by using of starch casein nitrate agar plates and inoculated with *Streptomyces* isolate by a single streak of inoculum in the center of the Petri dish. After 4 days of incubation at 28°C the plates were seeded with test organisms by a single streak at a 90° angle to the *Streptomyces* isolates. The microbial interactions were analyzed by determination of the size of the inhibition zone (12). Isolates showing activity against test organisms were grown in 250 ml flasks containing 50 ml of starch casein nitrate broth medium. Seven-day-old culture grown on starch casein nitrate agar media was used to inoculate the flasks. These flasks were incubated in a rotary shaker at 100 rpm in 28°C for seven days. The resulting culture broth (approximately 50 ml) was separated from the mycelium by centrifugation at 8000rpm for 15 min. The supernatant obtained was used for extracellular antimicrobial activity by agar well diffusion method (13). Appropriate agar plates were seeded with the test organisms and wells were made by using a sterile cork borer (6mm). One hundred micro liter of supernatant of each isolate was loaded in each well. Plates were kept in refrigerator for about 1h to allow the diffusion of produced antimicrobial metabolites. The diameters of inhibition were measured after 24 h of incubation at 37°C for bacteria, for 48 h of incubation at 28°C for yeasts and filamentous fungi. Each experiment was repeated three times and means value of inhibition zones was calculated.

Test organisms used: Test organisms used for screening of antimicrobial activity of isolates, were obtained from Microbial Type Culture Collection and gene bank (MTCC), IMTECH Chandigarh. they are *Bacillus subtilis* (MTCC 1789), *Bacillus megaterium* (MTCC 1684), *Bacillus cereus* (MTCC 1305), *Staphylococcus aureus* (MTCC 96*)*, *Staphylococcus epidermis* (MTCC 435) *Salmonella typhi* (MTCC 531), *Proteus vulgaris* (MTCC 1771), *Klebsiella pneumoniae* (MTCC 2405), *Escherichia coli* (MTCC 1667), *Candida albicans* (MTCC 1637), *Aspergillus niger* (MTCC 872), *Alterneria alternate (*MTCC 1779*), Penicillium citrinum* (MTCC 1751), from Jawaharlal Nehru Medical College (JNMC), Raipur, *Staphylococcus aureus* (JNMC),

Serratia liquefaciens (JNMC), *Pseudomonas aureginosa* (JNMC), *Penicillum crysogenum* (JNMC), *Candida albicans* (JNMC-1), *Candida albicans* (JNMC-2) and from ITRC (Indian Toxicology Research Center, Lucknow) *Bacillus cereus* (ATCC 10876), *Escherichia coli* (ATCC 35218). Bacterial cultures were grown at 37°C on nutrient agar medium and fungi were grown at 28°C on Sabouraud's dextrose agar. All the cultures were preserved at 4°C and sub-cultured regularly.

Morphological, Physiological and biochemical characteristics: The potent *Streptomyces* isolates selected after primary screening was characterized by morphological, biochemical and physiological studies (Table III). They were then observed for their mycelia structure, and spore arrangements on the mycelia under light microscope at 1000X resolution by using Gram staining. The observed morphology of the isolates was compared with the actinomycetes morphology provided in Bergey's Manual for the presumptive identification of the isolates. The biochemical tests performed were catalase, lecithinase (egg yolk rection), oxidase, citrate utilization, nitrate reduction, starch hydrolysis, tween-20 hydrolysis (lipolysis), urea hydrolysis, gelatin hydrolysis, ability to produce H_2S, degradation of elastin, arbutin, pectin, guanine, tyrosine, esculin and xanthine. Carbon utilization such as D-glucose, D-fructose, D-galactose, D-xylose, D-mannitol, D-sorbitol, D-lactose, D-arabinose, D-cellubiose, *meso*-inositol, dextran, melibiose, melezitose, xylitol, L-rhamnose, L-raffinose was determined on plates containing basal salt agar media (KH_2PO_4 2.64 g, $K_2HPO_4.3H_2O$ 5.65 g, $(NH4)_2SO_4$ 2.64 g, trace salt solution ($CuSO_4.5H_2O$ 0.64 g, $FeSO_4.7H_2O$ 0.11 g, $MnCl_2.4H_2O$ 0.7 g, $ZnSO_4.7H_2O$ 0.15 g, distilled water 100ml) 1ml, agar 20 g and distilled water 1000 ml) to which carbon sources were added to a final concentration of 1.0% (14). The plates were incubated at 28°C and growth was assessed after 7, 14, 21 days using glucose as positive control and carbon source free medium as negative control. The ability to utilize nitrogen sources such as L-cystein, L-valine, L-leucine, L-asparagine, L-hydroxyproline, L-serine, L-phenylalanine and L-histidine was determined in a basal medium (glucose 1.0 g, $MgSO_4.7H_2O$ 0.05 g, $FeSO_4.7H_2O$ 0.001 g, K_2HPO_4 0.01 g, NaCl 0.05 g, agar 2.0 g and distilled water 100 ml) to which 0.1% nitrogen sources were added. The plates were incubated at 28°C and growth was recorded after 7, 14, 21 days using L-asparagine as positive control and a medium without nitrogen source as a negative control (16). Determination of cell wall L, L-diaminopimelic acid was performed by the method of Becker *et al.,* (15). Antibiotic sensitivity against the different antibiotics (Himedia, Mumbai, India) gentamycin (G^{30}), chloromphenicol (C^{10}), linezolid (LZ^{30}), ciprofloxacin (CF^5), amoxicillin (AM^{10}), methicillin(M^{10}), nalidixic acid (NA^{30}), novobiocin (Nv^5), tetracycline (T^{10}), oleondamycin (OL^{15}), streptomycin (S^{25}), vancomycin (VA^5), kanamycin (K^{30}), polymixim B (PB^{100}), neomycin (N^{30}), rifamycin (R^5), erythromycin (E^{15}), cephalosporin (CR^{30}), penicillin G (P^{10}), amphotericin B (AP^{30}), fluconazole (F^{10}), miconazole (MIC^{30}) were performed using Muller Hinton agar medium by paper disc method (16).

Results

The genus *Streptomyces*, which is the most abundant and a recoverable *actinomycete* group isolated from the soil. Total fifty seven *Streptomyces* isolates were recorded from the soil of Durg District of Chhattisgarh according to differences in colony morphology (17) on starch casein nitrate agar media (Fig. 1). Out of 57 isolates, only 19 isolates showed the antimicrobial activity. Among this 19 isolates, 11 showed both

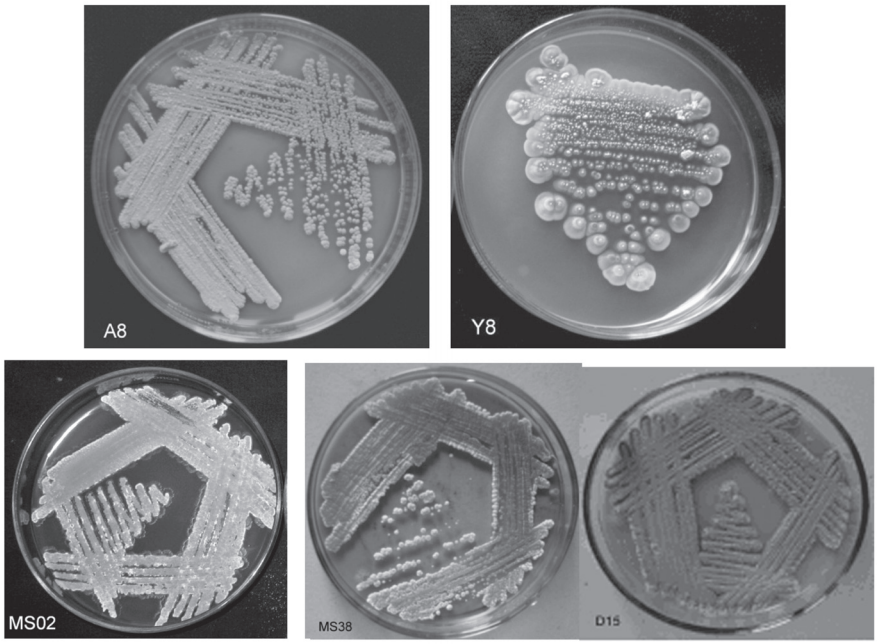


Fig. 1. Growth pattern of bioactive *Streptomyces* isolates on starch casein nitrate agar media from various location of Durg.

antibacterial and antifungal activity while 8 exhibited only antibacterial activity. The diameter of zone of inhibition (in millimeter) showed by the culture filtrate of 19 isolates is represented in table (1). In present study results indicated that gray colored isolates were showing activity against the most of the test organisms when subjected in to submerged culture. The yellow colored isolates (Y8, MS38) and grey colored isolates (A8, MS02 and D15) exhibited highest antibacterial and antifungal activity (Fig. 3). Among these isolate Y8 (yellow) recorded to show broad spectrum activity against all gram positive bacteria, gram negative bacteria and fungi. Results of antibiotic sensitivity test was presented in table-2 showed that *Bacillus cereus* (MTCC 1305) was the most sensitive organism inhibited by the extract of 14 isolates followed by *Bacillus subtilis* (MTCC 1789), *Salmonella typhi* (MTCC 531), *Escherichia coli* (MTCC 1667), *Bacillus cereus* (ATCC 10876), *Staphylococcus aureus* (MTCC 96), *Proteus vulgaris* (MTCC 1771), *Candida albicans* (MTCC 1637), *Klebsiella Pneumoniae* (MTCC 2405), *Penicillium citrinum* (MTCC 1751), *Staphylococcus epidermis* (MTCC 435), *Alternaria alternate* (MTCC 1779), *Escherichia coli* (ATCC 35218), *Aspergillus niger* (MTCC 872), *Staphylococcus aureus* (JNMC), *Serratia liquefaciens* (JNMC) *Penicillium crysogenum* (JNMC), *Candida albicans* (JNMC-1), *Candida albicans* (JNMC-2), *Bacillus megaterium* (MTCC 1684), *Pseudomonas aureginosa* (JNMC). Antibiotic sensitivity profile of isolates is shown table-(II) Most of the isolates showed resistance to the amoxicillin (AM^{10}), methicillin (M^{10}), nalidixic acid (NA^{30}), cephalosporin (CR^{30}), penicillinG (P^{10}), amphotericin B (AP^{30}), fluconazole (F^{10}) and showed sensitivity towards the gentamycin (G^{30}), linezolid (LZ^{30}), streptomycin (S^{25}),

vancomycin (VA^5), kanamycin (K^{30}), novobiocin (Nv^5),tetracycline (T^{10}), neomycin (N^{30}), erythromycin (E^{15}). Only one isolate Y8 showed the resistance against the tetracycline (T^{10}) and kanamycin (K^{30}).

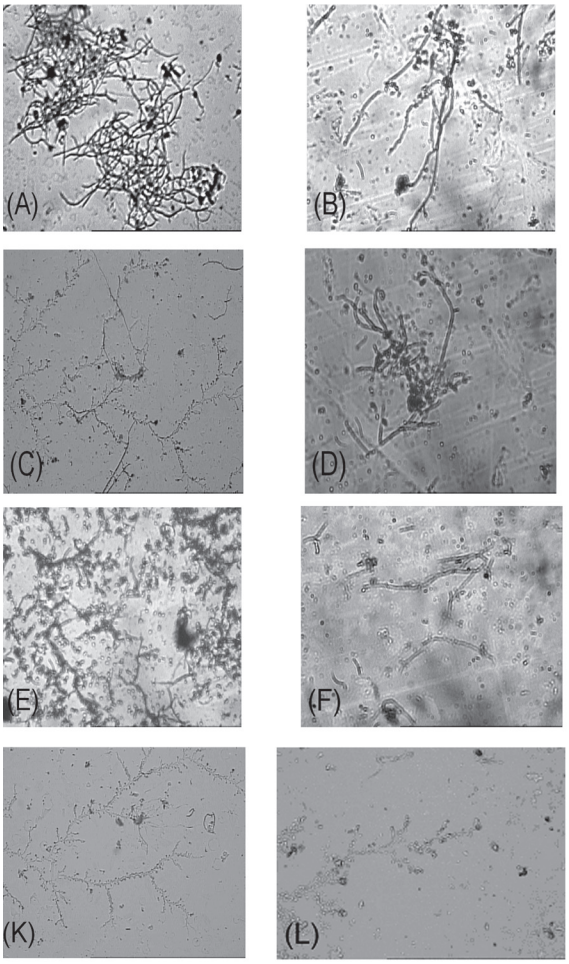


Fig. 2. The microscopic observation of *Streptomyces* isolates under the light microscope (1000X) (A) Y8 (*Streptomyces rimosus*) (B) A8 (*Streptomyces antibioticus*) (C) D13 (*Streptomyces antibioticus*) (D) M8 (*Streptomyces lydicus*) (E) MS02 (*Streptomyces antibioticus*) (F) M1 (*Streptomyces cyaneus*) (G) M9 (*Streptomyces phaeochromogenes*) (F) MS41 (*Streptomyces filipinensis*) (H) MS03 (*Streptomyces exfloliatus*) (I) D15 (*Streptomyces rouchi*) (J) M7 (*Streptomyces chromofuscus*) (K) P2N (*Streptomyces rouchi*) (L) MS14 (*Streptomyces atroolivaceus*)

The identification of *Streptomyces* isolates was carried out by using morphological, physiological and biochemical characteristics (Table-3). All the *Streptomyces* were found to gram positive when subjected to Gram staining. Fig. 2 showed the microscopic observation (1000X) of *Streptomyces* isolates. Probabilistic identification matrix for *Streptomyces* that based on various morphological, physiological and biochemical characteristic, antibiosis and resistance to antibiotics, was calculated by using PIBWin software, Bacterial Identification Program Version: 1.9.2 (18). Most active isolate Y8 was identified as *Streptomyces rimosus* with the ID score 0.99, three isolates No A8, MS02 and D13 were identified as *Streptomyces antibioticus* with ID score 0.99, 1.0 and 1.0 respectively. Isolate MS38 was identified as *Streptomyces chromogenus* with ID score 1.0, isolate No D15, MS09 and P2N were identified

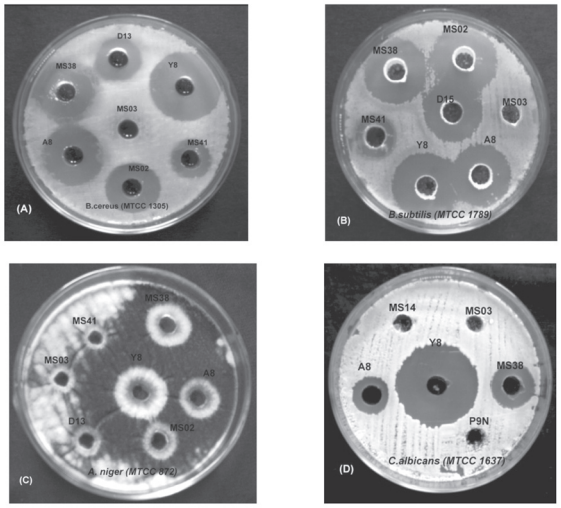


Fig. 3. Antimicrobial activity of *Streptomyces* isolates (A) A8, MS02, MS41, Y8, D13, MS38 showing activity against *B.subtilis* (MTCC 1789) (B) A8, MS02, MS41, Y8, D15, MS38 showing activity against *B.cereus* (MTCC 1305) (C) Y8, MS38, A8, MS02 showing activity against *A. niger* (MTCC 872) (D) A8, Y8, MS38 showing activity against *C.albicans* (MTCC 1637) by agar well diffusion method.

Table 1. Screening of *Streptomyces* isolates for their antimicrobial activity against Gram positive, Gram negative bacteria and fungi:

No of isolates/ Zone of inhibition (mm)

Test organisms	Y8 (Y)	A8 (G)	MS 02 (G)	MS 38 (Y)	D 15 (G)	MS 41 (W)	M S 14 (G)	MS 03 (Gr)	MS 09 (G)	D9 (G)	D 13 (G)	P9N (G)	P2 N (G)	M1 (W)	M 11 (G)	M 12 (G)	M8 (G)	M9 (G)	M7 (G)
B. subtilis (MTCC 1789)	30	30	29	25	18	20	26	-	26	25	16	30	-	-	-	-	-	-	26
B. megaterium (MTCC 1684)	29	-	-	22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. cereus (MTCC 1305)	40	40	35	30	24	13	20	-	26	25	15	40	10	-	-	-	10	-	21-
S. aureus (MTCC 96)	38	22	27	26	31	-	19	-	25	14	-	20	-	-	-	-	-	-	-
S. epidermis (MTCC 435)	34	32	15	25	-	-	11	-	-	-	14	22	-	-	-	-	-	-	-
S. typhi (MTCC 531)	40	30	27	30	20	32	24	-	40	40	-	25	-	21	-	-	-	26	-
S. aureus (JNMC)	20	26	20	-	-	-	-	-	16	-	-	-	-	-	-	-	-	-	-
P. vulgaris (MTCC 1771)	30	30	20	-	10	-	30	22	-	22	-	-	-	-	19	20	-	26	-
K. Pneumoniae (MTCC 2405)	26	29	35	-	20	-	-	11	-	26	-	20	-	13	-	-	-	-	-
E. coli (MTCC 1667)	30	34	32	32	-	25	-		34	-	16	30	-	20	-	11	-	-	-
P. aureginosa(JNMC)	21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. cereus(ATCC 10876)	35	35	35	28	24	-	22	12	25	26	-	-	19	-	-	-	-	-	-
E.coli (ATCC 35218)	30	34	-	22	24	-	22	-	-	-	-	-	-	-	-	-	-	-	-
S. liquefaciens (JNMC)	25	26	20	-	-	-	-	-	-	-	-	-	-	-	-	-	18	-	-
C. albicans (MTCC 1637)	30	22	-	20	20	12	-	-	14	-	20	-	-	-	-	26	-	-	-
A. niger (MTCC 872)	29	25	20	10	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-
*A. alternate(*MTCC 1779)	35	22	17	16	-	-	-	-	-	-	20	10	-	-	-	-	-	-	-
P. citrinum (MTCC 1751)	30	21	17	20	20	17	-	-	-	-	-	20	-	-	-	-	-	-	23
P. crysogenum (JNMC)	28	-	-	12	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. albicans (JNMC-1)	30	10	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. albicans (JNMC-2)	20	20	15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2. Antibiotic sensitivity of *Streptomyces* isolates.
No of isolates

Antibiotic (µg/disc)	Y8	A8	MS02	MS38	D15	MS41	MS14	MS03	MS09	D9	D13	P9N	P2N	M1	M11	M12	M8	M9	M7
gentamycin (G^{30}),	S	S	S	S	S	S	I	S	S	S	S	S	S	S	S	S	S	S	S
chloromphenicol (C^{10})	I	R	I	I	R	S	S	S	I	I	I	I	I	S	S	S	S	I	S
linezolid (LZ^{30})	I	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
ciprofloxacin (CF^{5})	S	S	S	R	I	S	S	R	S	S	S	S	R	S	S	S	S	S	S
amoxicillin (AM^{10}),	R	I	R	I	R	I	R	S	R	R	I	R	R	S	R	R	R	R	R
methicillin(M^{10}),	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R
nalidixic acid (NA^{30})	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
novobiocin (Nv^{5})	S	S	I	R	I	I	S	S	S	S	I	S	S	S	S	S	S	S	S
tetracycline (T^{10})	R	I	I	I	I	S	S	S	I	S	S	I	S	S	S	S	S	S	S
oleondamycin (OL^{15})	S	I	I	R	I	S	S	S	I	S	S	S	S	S	S	S	I	S	I
streptomycin (S^{25})	S	S	S	I	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
vancomycin (VA^{5})	S	I	S	I	S	S	S	S	I	I	S	S	S	S	S	S	S	S	S
kanamycin (K^{30}),	R	S	S	I	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
polymixim B (PB^{300})	S	I	I	I	I	S	S	S	I	I	I	S	S	S	S	S	S	S	S
neomycin (N^{30}),	S	I	S	I	S	I	S	S	S	S	S	S	S	S	S	S	S	S	S
rifamycin (R^{5}),	I	R	R	I	R	I	R	S	R	S	S	R	R	I	S	S	I	I	I
erythromycin (E^{15}),	S	I	I	R	I	S	S	S	S	S	I	I	S	S	S	S	S	S	S
cephalosporin (CR^{30}),	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R
penicillinG (P^{10})	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Amphotericin B (AP^{30})	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
fluconazole (F^{10})	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
miconazole (MIC^{30})	I	I	I	I	I	I	I	S	I	I	I	I	I	I	I	S	I	I	I

as *Streptomyces rouchi* with the ID score 0.99, 0.98, 0.98, isolate MS03 and P9N were identified as *Streptomyces exfloliatus* whereas isolates No MS41, MS14, D9, M1, M11, M12, M8, M9, M7 were identified as *Streptomyces filipinensis, Streptomyces atroolivaceus, Streptomyces halstedii, Streptomyces cyaneus, Streptomyces violaceus, Streptomyces oliovaceoviridis, Streptomyces lydicus, Streptomyces phaeochromogenes, Streptomyces chromofuscus* with the ID score 0.99, 0.99, 0.99, 1.0, 0.99, 0.99, 0.99, 1.0, 0.99, 1.0 respectively.

Discussion

From the above study, it is concluded that all the isolates identified having antimicrobial activity belongs to *Streptomyces* group and this confirms the report that most of the actinomycetes isolated from the soil that produce antibiotics are from the genus *Streptomyces* (19). genus *Streptomyces* were found to produce more then 1, 00,000 bioactive compounds that are of biological interest (20). Out of thousands of microbial metabolites produced from actinomycetes about 150-160 (0.2-0.3%)

Table 3. Morphological, physiological and biochemical characteristics of *Streptomyces* isolates

Characteristics	Y8	A8	MS02	MS38	D15	MS41	MS14	MS03	MS09	D9	D13	P9N	P2N	M1	M11	M12	M8	M9	M7
Spore chains rectiflexiles	-	+	+	-	-	-	+	+	+	+	+	+	+	-	+	-	-	+	+
Spore chains Spirales	+	-	-	+	+	+	-	-	-	-	-	-	-	+	-	+	+	-	-
Spore mass red	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Spore mass gray	-	+	+	-	+	-	+	-	+	+	+	+	+	-	-	+	+	-	-
Spore mass yellow	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mycelial pigment red/orange	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-
Diffusible pigment produced	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-
Diffusible pigment yellow/brown	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Melanine on Tyrosine agar	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	-
Diaminopimelic acid	+	+	+	+	+	-	+	-	+	+	+	+	+	-	-	+	+	+	+
Coagulation of milk	+	+	+	-	+	+	+	-	+	+	+	-	+		+	+	+	+	-
Catalase test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H2S Production	-	+	-	+	+	+	+	-	-	-	+	+	-	+	+	+	-	+	+
Nitrate reduction	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	-
Citrate utilization	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hydrolysis of																			
Starch	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lipid(Tween 80)	+	+	-	+	-	+	+	+	+	-	+	+	+	-	+	+	-	+	+
Gelatin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Egg yolk(lecithin)	+	-	-	+	-	+	-	+	+	-	-	+	+	-	+	-	+	-	-
Urease	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
Degradation of																			
Guanine	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+
Xanthine	+	+	+	-	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-
Pectin	-	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	+	-
Arbutin	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-
Adenine	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-
Esculin	-	+	+	-	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+
Utilization of																			
Adonitol	+	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	+	-
D-Xylitol	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
Sucrose	-	+	+	-	+	+	-	-	+	+	-	-	+	+	-	+	+	+	-
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	-	-	+	+	+	+	-	+	+	+	+	-	-	-	-	-	+	-
D-fructose	+	+	+	+	+	+	+	-	-	+	-	+	-	-	+	-	+	+	-
D-Mannitol	+	+	+	+	+	+	+	-	+	+	+	-	+	+	-	+	+	+	+
D-Sorbitol	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+
D-Lactose	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+
D-Maltose	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	+	+	+	+	+	+	-	-	+	+	-	-	+	+	+	+	+	+	-
L-Rhamnose	-	+	+	-	+	-	+	+	+	+	+	+	+	+	-	+	-	+	+
Cellubiose	+	+	+	+	+	+	+	-	+	+	-	+	+	-	-	-	-	-	+
L-Arabinose	-	+	-	+	+	-	+	+	+	+	+	+	+	-	+	-	+	+	-
meso-inositol	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+
Dextran	-	l	-	-	l	-	-	-	-	-	l	-	-	l	-	-	-	l	l
D-melezitose	+	+	+	-	+	+	-	-	+	+	-	-	+	+	+	-	+	+	-
D-milibiose	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	+	+	-
L-Cycteine	-	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	-	+
L-Histidine	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
L-Valine	+	-	+	-	-	+	-	-	+	+	+	+	+	+	+	+	-	+	-
L-Hydroxyproline	-	-	-	-	-	+	-	+	-	-	-	+	-	-	+	-	+	+	-
L-Arginine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Asparginine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Tyrosine	-	-	-	-	-	+	-	+	-	-	+	-	-	+	+	+	-	+	+
L-Serine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Phenylalanine	+	-	-	+	-	+	+	+	-	-	+	+	-	+	+	+	+	+	-
DL-a-Amino-n-butyric acid	-	-	-	+	-	-	+	+	-	-	+	+	-	-	+	+	-	+	+

compounds were practically proved as successful lead compounds and are continues to provide new bioactive products that lead to the discovery of many novel strains that produce useful secondary metabolites. There is a broad range of *actinomycetes* products such as antibiotics like streptomycin and novobiocin are firmly cemented these chemically prolific bacteria in the centre stage of natural products drug discovery research (21). The perspective of rapid emergence of drug resistance among bacterial pathogens shows that the potencies of prevalent antibiotics are decreasing steadily, leading to reduced useful period of drugs. This condition creates a demand for the investigation of new, safe and effective antimicrobial compounds for replacement with existing antimicrobials or use in antibiotic rotation programs (22,23,24). In present study *Streptomyces* represent a major portion of actinomycetes in soil of Durg District of Chhattisgarh with significant biological activity. These isolates which show the both antibacterial and antifungal activity, may be produced either a broad spectrum antimicrobial compound or several compound with multitude of activity. The present finding highlighted the importance of work for further investigation towards the goal of obtaining a reliable methodology to isolate rare and unusual *actinomycetes*, to reduce the re-isolation of strains producing known bioactive compounds and to improve the quality of natural products screened isolated from Durg District of Chhattisgarh. INDIA.

Acknowledgement

Authors are thankful to the School of Studies in Life Sciences, Pt. Ravishankar Shukla University, Raipur, for providing lab facilities and financial support to carry out this work.

References

1. Kim, H.J., Lee, S.C. and Hwang, B.K. (2006) *Streptomyces cheonanensis sp. nov*., a novel *Streptomycete* with antifungal activity. International Journal System Evolution Microbiology 56: 471-475.
2. Watve, M.G., Tickoo, R., Jog, M.M. and Bhole, B.D. (2001). How many antibiotics are produced by the genus *Streptomyces*? Archives Microbiology 176: 386-390.
3. Lakshmipathy, D. and Kannabriran, K. (2010) Isolation and characterization of antagonistic *Actinomycetes* from marine soil. Journal of Microbial Biochemical Technology 2(1): 1-6.
4. Labeda, D.P., Lechevalier, M.P. and Testa, R.T. (1997) *Streptomyces stramineus* sp. nov., a new species of the verticillate *Streptomycetes*. Integral Journal System Bacteriology 47: 747–753.
5. Dietera, A., Hamm, A., Fiedler, H.P., Muller, W.E.G., Brun, R., Beil, W., Kim, S.B. and Goodfellow, M. (2002) *Streptomyces avermitilis* sp. nov., nom. rev., a taxonomic home for the avermectin-producing *Streptomycetes*. International Journal of System Evolution Microbiology 52: 2011–2014.
6. Berdy, J. (2005) Bioactive microbial metabolites. Journal of Antibiotics (Tokyo) 58: 1–26.
7. Saintpierre, D., Amir, H., Pineau, R., Sembiring, L. and Goodfellow, M. (2003) *Streptomyces yatensis* sp. nov., a novel bioactive streptomycete isolated from a New-Caledonian ultramafic soil. Antonie van Leeuwenhoek 83: 21–26.
8. Huang, Y., Li, W., Wang, L., Lanoot, B., Vancanneyt, M., Rodriguez, C., Liu, Z., Swings, J. and Goodfellow, M. (2004) Streptomyces glauciniger sp. nov., a novel mesophilic streptomycete isolated from soil

in south China. International Journal of System Evolution Microbiology 54: 2085–2089.

9. Xu, C., Wang, L., Cui, Q., Huang, Y., Liu, Z., Zheng, G., and Goodfellow, M. (2006). Neutrotolerant acidophilic Streptomyces species isolated from acidic soils in China: *Streptomyces guanduensis* sp. nov., *Streptomyces paucisporeus* sp. nov., *Streptomyces rubidus* sp. nov. and *Streptomyces yanglinensis* sp. nov. International Journal of System Evolution Microbiology 56: 1109–1115.

10. Newman, D.J., Cragg, G.M. and Snader, K.M. (2003). Natural products as a source of new drugs over the period 1981-2002. A detailed analysis and description of current natural product derived therapeutic agents. Journal National Prod. 66: 1002-1037.

11. Williams, S.T. and Davies, F.L. (1965) Use of antibiotics for selective isolation and enumeration of Actinomycetes in soil. Journal of General Microbiology 38: 251-261.

12. Madigan, M.T., Martinko, J.M. and Parker, J. (1997) Antibiotics: isolation and characterization. In:Brock Biology of Microorganisms, 8th (eds), Prentice-Hall International Inc. *New Jersey*, pp: 440-442.

13. Grammer, A. (1976) Antibiotic sensitivity and assay test. In: Collins, C.H. and P.N. Lyne, editors. Microbiological Methods. *London: Butterworths* 235

14. Shirling, E.B., and Gottieb, D. (1966) Methods, classification, identification and description of genera and species. The Williams and Wilkins Company, *Baltimore*, 2: 61-292.

15. Becker, B., Lechevalier, M.P., Gordon, R.E. and Lechevalier, H.A. (1964) Rapid differentiation between Nocardia and streptomyces by paper chromatography whole cell hydrolysates. Applied Microbiology 12: 421-423.

16. Williams, S.T. Goodfellow, M. Alderson, G., Wellington, E.M., Sneath, P.H. and Sackin, M.J. (1983) Numerical classification of Streptomyces and related genera. Journal of General Microbiology 129: 1743-1813.

17. Moncheva, P., Tishkov, S., Dimitrova, N. and Chipeva, V. (2002) Characteristics of soil actinomycetes from Antarctica. Journal of Culture Collection 3: 3-14.

18. Augustine, S.K., Bhavasar, S.P. and Kapadnis, B.P. (2005) Production of growth dependent metabolite active against dermatophytes by *Streptomyces rouchi* AK 39. Indian Journal of Medical Research 121: 164-70.

19. Mustafa, S.A., Tamer, U.A. and Azer, C. (2004) Antibacterial activity of some actinomycetes isolated from farming soil of turkey. African Journal Biotechnology 3: 441-446.

20. Thakur, D., Mazumadar, S., Gogoi, D.K. and Bora, T.C. (2003) Isolation and screening for biologically active metabolites. In: Bora, R.C. Unni, B. G. Deka, P. C. (Eds.), Bioprospecting of commercially important plants. Proceedings National Symposium. ISAB-JC., 202-206.

21. Jensen, P.R., Mincer, T.J. and Finical, W. (2003) The true potential of the marine microorganisms. Current Drug Discovery 17-19.

22. Gerding, D.N., Larson, T.A. and Hughes, R.A. (1991). Aminoglycoside resistance and aminoglycoside usage: ten year of

experience in one hospital. Antimicrobial Agents Chemotherapy 35:1284-1290.

23. Neiderman, M.S. (1997). Is "crop rotation" of antibiotics the solution to a "resistant" problem in the ICU? American Journal of Respiratory Critical Care Medicine 156: 1029-1031.

24. Quale, J., Landman, D., Atwood, E., Kreiswirth, B., Willey, B.M., Ditore, V., Zaman, M., Patel, K., Saurina, G., Huang, W., Oydna, E., and Burney, S. (1996). Experience with a hospital-wide outbreak of vancomycin-resistant enterococci. American Journal of Infection Control 24 (5): 372-379.

25. Porter, J.N., Wilhelm, J.J., Tresner, H.D. (1960). A method for the preferential isolation of actinomycetes from soil. Journal of Applied Microbialogy 8: 174-178.

Current Trends in Biotechnology and Pharmacy
Vol. 5 (1) 1054-1059 January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

Investigations on Microbial Resistance among Bacteria Dwelling in Indian Soils

C. Ganesh Kumar*, Joveeta Joseph and Ahmed Kamal
Chemical Biology Laboratory,
Indian Institute of Chemical Technology
Uppal Road, Hyderabad 500 607, India
*For correspondence - cgkumar@iict.res.in

Abstract

There is growing evidence that bacteria that living in diverse soil environments are gaining multi-drug resistance. Recent functional screens and the growing information of metagenomic databases are also revealing the presence of an unexpected density of resistance genes in the environment, the antibiotic resistome. To explore this reservoir, we isolated 15000 morphologically diverse microorganisms directly from soil samples, and constructed a diverse library of 264 resistant isolates. Without exception, all the 264 isolates were found to be resistant to at least one antibiotic. Multi-drug resistance was observed for more than 3 antibiotics in 83% of the isolates, while five strains showed resistance to more than 10 antibiotics of the 15 antibiotics tested in the library. These results indicate that soil bacteria are a reservoir of antibiotic resistance with greater genetic diversity than previously accounted for.

Keywords: antibiotic; resistance; soil isolates; bacteria; India

Introduction

Bacterial infections continue to be one of the leading causes of morbidity and mortality worldwide, despite the availability of diverse arsenal of chemotherapies, attributed in part to the evolution and dissemination of antibiotic resistance genes (1). Traditional approaches to antibiotic resistance have involved extensive research on human pathogens, limiting efforts to only clinically identified mechanisms. It is now becoming increasingly evident, however, that environmental forces have greatly impacted the determinants that have emerged clinically. Among the first to be recognized publicly was the impact of the agricultural use of antibiotics as animal growth promoters (1). Since 1940s, the extensive use of antimicrobials (2) and disposal of health-care products, etc. contain resistance determinants that induce resistance in microorganisms towards various antibiotics (3). This use of antibiotics in agriculture has resulted in the spread of strains such as vancomycin-resistant enterococci in both farm animals exposed to antimicrobials and humans in contact with the animals (4), and has been directly linked to the development of drug-resistant infections (1).

Considering the growing body of evidence suggesting that clinical resistance is intimately associated with mechanisms found environmentally, there is a clear need to expand the focus to include non-pathogenic organisms in antibiotic research. In doing so, it may be possible to establish strategies to predict resistance before it emerges clinically as well as develop diagnostic techniques and therapeutic strategies to

Vol. 5 (1) 1054-1059 January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

counteract resistance. For example, Vancomycin was discovered in the 1950's and did not show any resistance until the mid 1980's. After which certain nosocomial pathogens developed resistance and by early 1990's molecular studies demonstrated that this mechanism prevailed in the soil microorganisms proving that an early intervention would have prevented this emergence. A recent study by Knapp *et al*. (5) expressed concern that increased antibiotic resistance in soils could have broad consequences to public health through potential exposure through water and food supplies. Their results suggested that there may be a progressively increasing chance of encountering organisms in soils collected from in and around Netherlands exhibiting resistance to antimicrobial therapy. They concluded that further studies need be performed globally so that the scope and possible ramifications of their results could be better understood. Considering these facts, we performed these studies with Indian soils to prove this hypothesis and generate baseline data under Indian context on antibiotic resistance in bacteria living in soils collected from different habitats.

Materials and Methods

Raw materials **:** Different antibiotics like amoxicillin, ampicillin, ciprofloxacin, erythromycin, penicillin G, tetracycline, vancomycin, amikacin, chloramphenicol, gentamicin, gatifloxacin, kanamycin, neomycin, streptomycin, cycloheximide and rifamycin, and different media such as Muller Hinton Agar were procured from HiMedia Laboratories Pvt. Ltd., Mumbai, India.

Collection of soil samples and isolation of bacteria : Soil samples of about one gram were collected with sterile metal spatulas from a depth of 1–5 cm from different habitats in and around Hyderabad, India, like residential garden soil, farm soil, public park soil, agricultural field soils, composting manure soils and soils near factories and lakes in sterile collection bottles. The entire contents of each tube were suspended in 50 ml sterile water and vortexed on a shaker. Dilutions of 1:10 and 1:100 were prepared from the suspensions. Amounts of 1 ml from the original suspension and from each of the dilutions were pipetted into sterile Petri dishes containing 15 ml of Muller Hinton agar. Fungal growth was prevented by the addition of 30 µg/ml of cycloheximide to the medium. The Petri dishes were incubated at 35°C for 18–24 h in an incubator until the colonies were fully formed. Pure colonies were obtained after repeated sub-culturing and the pure colonies were maintained on nutrient agar slants. Preliminary characterization of the isolates was based on colony morphology and Gram staining.

Antibiotic susceptibility testing : Target isolates were tested for susceptibility to specified antibiotics using a standard agar disc diffusion method. Isolated colonies were initially cultured in nutrient broth at 35 ± 2°C for 24 hours and the cell density was adjusted to McFarland 0·5 turbidity standard, resulting in a suspension containing $1–2 \times 10^8$ cfu ml^{-1}. Within 15 min after adjusting the turbidity, a sterile swab was dipped into the adjusted suspension for inoculation and then uniformly streaked over the entire surface of a Mueller–Hinton agar (MHA) plate and then the drug-impregnated discs were placed on the surface of these inoculated MHA plates. Testing for antibiotic susceptibility was performed following the testing and quality assurance practices outlined in the M2-A6 NCCLS Document (NCCLS, 1997a). The panel included amoxicillin (Amx), ampicillin (Amp), ciprofloxacin (Cip), erythromycin (Ery), penicillin G (Pen), tetracycline (Tet), vancomycin (Van), amikacin (Amk), chloramphenicol (Chl), gentamicin (Gen), gatifloxacin (Gat), kanamycin (Kan), neomycin (Neo), streptomycin (Str) and rifamycin (Rif). The

plates were then inverted and incubated at 35 ± 2°C for 20–24 h. After the specified incubation period, the zone of inhibition around each of the discs was measured to the nearest whole millimetre. The zone margin was defined as that area showing no obvious, visible growth detected by the unaided eye. Interpretation of these zone sizes was based on Table 2 of the M100-S9 NCCLS document (NCCLS, 1999).

Results and Discussion

The concept of soil as a location of antibiotic resistance determinants, particularly those harboured in antibiotic producers as self protection mechanisms has been acknowledged for decades. Around 57 soil samples collected from different habitats (urban and agricultural) within Hyderabad, India were processed and about 15,000 morphologically diverse collection of bacteria colonies were isolated from these soil samples and about 264 target organisms (at least minimum one target bacterial colony type was picked up from each soil sample) were selected for further phenotypic characterization based on Gram staining and antibiotic susceptibility testing. The phenotypic diversity of the 264 target isolates based on gram staining is shown in Table 1. It was observed that of the total isolates, 57 belonged to the genus *Bacillus*, 37 were Pseudomonads, 30 belonged to Enterobacteriaceae and 126 represented to the group of Staphylococci. A closer look at the distribution of the recovered isolates indicated that only 14 actinomycetes were recovered from these soil samples.

These 264 strains were subsequently screened against 15 antibiotics, including natural products (such as penicillin, vancomycin and erythromycin), and completely synthetic molecules (such as ciprofloxacin and gatifloxacin). The antibiotics encompassed all major bacterial targets and included drugs that have been on the market for decades as well as several antibiotics that have only recently been clinically approved (such as gatifloxacin). This study provides an analysis of the antibiotic resistance potential of soil microorganisms. The frequency of resistance is high as observed in the study to antibiotics that have for decades served as gold-standard for treatment, as well as those only recently approved for human use. No class of antibiotic was spared with respect to bacterial target or natural or synthetic origin. A broad survey of all the 264 isolates, without exception suggested that every strain in the library was found to be resistant to at least one antibiotic. The multi-drug resistance for more than 3 antibiotics was observed in 219 out of the 264 isolates (83%), with five strains being resistant to more than 10 antibiotics of the 15 antibiotics tested (Table 2). A comparative

Table 1. Phenotypic density of the 264 target bacteria isolated from the 57 soil samples

Diversity of isolates	No of isolates
Actinomycetes	14
Staphylococci	126
Bacillus	57
Pseudomonads	37
Enterobacteriaceae	30

Table 2. Antibiotic resistance spectrum depicting maximum number of isolates having multi-drug resistance of the soil isolates

Number of antibiotics resistant to	% of isolates
≤2	14.78
3	17.05
4	20.54
5	19.32
6	14.78
7	6.81
8	1.13
9	1.51
≥10	1.89

assessment of antibiotic resistance to one or more antibiotics in the standard panels for all bacterial isolates indicated that 96.6% of the total isolates were resistant to ampicillin (Table 3). Only four isolates were sensitive and four were intermediate to ampicillin. In addition, 79.5% resistance was observed towards penicillin G and 78% isolates exhibited resistance to rifampicin. The least resistance recorded for some antibiotics included ciprofloxacin, erythromycin, gentamicin, gatifloxacin and neomycin (Table 3). A comparative assessment of antibiotic resistance to one or more antibiotics in the standard panels for all bacterial isolates is presented in Table 4.

A study conducted by D'Costa *et al.* (6) reported a high density of multi-drug resistance among the 480 diverse spore-forming microorganisms they had isolated. The phenotypic density of resistance and diversity of the resulting profiles were greater than ever anticipated, with strains resistant to an average of seven to eight antibiotics. In addition, the work of D'Costa and co-workers identified a wealth of antibiotic-inactivating enzymes, including novel mechanisms of resistance to the recently approved antibiotics, telithromycin and daptomycin (6). They suggested that these microorganisms may represent a path through which resistance genes can find their way into human pathogens. In 1973, two molecular mechanisms of aminoglycoside resistance in soil-dwelling actinomycetes from the genus *Streptomyces* were determined to be identical to

Table 3. Antibiotic resistance profiling against each antibiotic of interest of the soil isolates

Antibiotic type	**No of resistant isolates**	**% of resistant isolates**
Amoxycillin	171	64.77
Ampicillin	256	96.96
Chloramphenicol	31	11.74
Ciprofloxacin	10	3.78
Erythromycin	9	3.4
Gentamicin	9	3.4
Gatifloxacin	9	3.4
Kanamycin	35	13.26
Neomycin	10	3.78
Penicillin G	210	79.55
Rifampicin	206	78.03
Streptomycin	24	9.09
Tetracycline	56	21.21
Vancomycin	128	48.48
Amikacin	11	4.16

Table 4. Comparative antibiotic resistance in soil-derived bacterial isolates according to standard susceptibility test panels

Resistance pattern*	**Amx**	**Amp**	**Chl**	**Cip**	**Ery**	**Gen**	**Gat**	**Kan**	**Neo**	**Pen**	**Rif**	**Str**	**Tet**	**Van**	**Amk**
R	171	256	31	10	9	9	9	35	10	210	206	24	56	128	11
I	18	4	51	25	102	8	5	64	57	34	25	35	79	2	9
S	75	4	82	239	153	247	250	165	197	20	33	205	129	134	244

*R-Resistant, I-Intermediate, S-Sensitive.

those in clinical pathogens (7). These strains, producers of the aminoglycosides: kanamycin and neomycin, were capable of drug modification by acetylation and phosphorylation, respectively, as a means of self-protection (7).

In the late 1990s, Gerard Wright and his team demonstrated that the mechanism of vancomycin resistance in vancomycin-producing soil bacteria was identical to the mechanism that emerged in vancomycin-resistant enterococci (VRE) in the late 1980s (8). Hence they suggested extending the scope of studies on antibiotic resistance to include bacterial species that are never pathogens and may not even be in the "human" sphere. Although this study does not provide evidence for the direct transfer of resistance elements from the soil resistome to pathogenic bacteria, it identifies a previously under appreciated density and concentration of environmental antibiotic resistance. The level and diversity of resistance uncovered in this work is likely to be substantially higher than what this study reveals as the present study is restricted exclusively to culturable bacteria, which represent only a fraction of soil-dwelling bacteria. For example, a recent soil metagenome analysis uncovered several aminoglycoside resistance genes in uncultured organisms (9). As a whole, the study of resistance in soil bacteria is rapidly gaining recognition as an important reservoir from which many clinical parallels can be drawn. This unexpected conclusion should have a paradigm shifting impact on our understanding of the judicious use of antibiotics and the drug discovery process. The present study also raises questions about protein evolution and gene transfer among bacteria. However, these are early days for studies on resistome and there are yet major problems to be tackled.

Extensions of this work will include analysis of soils from diverse geographical locations and resistance to other antibiotics. In addition, studies of the genetic diversity and structure of bacterial antibiotic resistance proteins may eventually lead to the design of compounds that inhibit resistance mechanisms, thus extending the useful lifetime of currently available antibiotics. Secondly, as genes for antibiotic resistance are often clustered with genes for antibiotic biosynthesis (10), antibiotic resistance studies may lead to the discovery of biosynthetic pathways encoding potentially novel antibiotics (11). It is not known whether antibiotic resistance genes move readily from environmental reservoirs to clinical settings, but future work should consider the potential contributions of soil bacteria to the problem of antibiotic resistance. The survey of antibiotic resistance mechanisms can assist the elucidation of novel mechanisms that may emerge clinically, as well as serve as a foundation for new antibiotic development. An emphasis has therefore been placed on educating doctors and national governments relative to restricting antibiotic usage to those cases where human health is threatened by virulent pathogens.

Acknowledgements

The authors wish to thank the help rendered by Ms. Aruna during the initial isolation of the bacteria used in the study. The authors gratefully acknowledge the funding received in the form of DST-SERC FAST Track Scheme (NO. SR/FT/LS-143/2008).

Competing interests: None declared

Ethical approval: Not required

References

1. D'Costa, V.M., Griffiths, E., Wright, G.D. (2007). Expanding the soil antibiotic resistome: exploring environmental diversity. Curr. Opin. Microbiol., 10: 481-89.
2. Bager, F., Madsen, M., Christensen, J., Aarestrup, F.M. (1997). Avoparcin used as a growth promoter is associated with the

occurrence of vancomycin-resistant *Enterococcus faecium* on Danish poultry and pig farms. Prev Vet Med, 31: 95–112.

3. Lu, K., Asano, R., Davies, J. (2004). Antimicrobial resistance gene delivery in animal feeds. Emerg Infect Dis, 10: 679–83.

4. Hammerum, A.M., Fussing, V., Aarestrup, F.M., Wegener, H.C. (2000). Characterization of vancomycin-resistant and vancomycin-susceptible *Enterococcus faecium* isolates from humans, chickens and pigs by riboprinting and pulsed-field gel electrophoresis. J Antimicrob Chemother, 45: 677–80.

5. Knapp, C.W., Dolfing, J., Ehlert, P.A.I. and Graham, D.W. (2010). Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940**.** Environ Sci Technol, 44: 580–7.

6. D'Costa, V.M., McGrann, K.M., Hughes, D.W., Wright, G.D. (2006). Sampling the antibiotic resistome. Science*,* 311: 374–77.

7. Benveniste, R., Davies, J. (1973). Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. Proc Natl Acad Sci USA, 70: 2276–80.

8. Wright, G.D. (2007). The antibiotic resistome: the nexus of chemical and genetic diversity. Nat Rev Microbiol, 5: 175–86.

9. Riesenfeld, C.S., Goodman, R.M., Handelsman, J. (2004). Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. Environ Microbiol, 6: 981–9.

10. Descheemaeker, P., Chapelle, S., Lammens, C., Hauchecorne, M., Wijdooghe, M., Vandamme, P., et al. (2000). Macrolide resistance and erythromycin resistance determinants among Belgian *Streptococcus pyogenes* and *Streptococcus pneumoniae* isolates J Antimicrob Chemother, 45: 167-73.

11. Witte, W., Klare, I. (1995). Glycopeptide-resistant *Enterococcus faecium* outside hospitals: a commentary. Microb Drug Resist, 1: 259-63.

Current Trends in Biotechnology and Pharmacy
Vol. 5 (1) 1060-1063 January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

Factor XI Gene (Plasma thromboplastin antecedent) Deficiency in Karan Fries Cattle

M. S. Azad, I. D. Gupta*, Archana Verma, Vikas Bohra, S. Rajesh Kumar and Kawardeep Kour

Molecular Genetics Lab, Dairy Cattle Breeding Division,
National Dairy Research Institute, Karnal, Haryana -132001, India
*For Correspondence - idgdcb59@yahoo.co.in

Abstract

The PCR optimization for FXI gene Exon 12 was found to be consistent and specific. PCR analysis of FXI gene Exon 12 revealed single band of 244 bp in all the KF cattle studied. No polymorphism was detected at FXI gene exon 12 of Karan Fries cattle. One non synonymous nucleotide change from G to A at position 105 in Karan Fries was observed when compared with *Bos taurus* resulting into a change of amino acid in second codon from Arginine (R) in *Bos taurus* to Glutamine (Q) in Karan fries. The study revealed that the screened Karan Fries cattle were free from Factor XI gene deficiency. As there was no polymorphism at FXI gene exon 12 of Karan Fries cattle, it was not feasible to explore association with repeat breeding.

Key words: FXI gene, Karan Fries cattle, Polymorphism

Introduction

Factor XI or plasma thromboplastin antecedent is the zymogen form of factor XI which is one of the enzymes of the coagulation cascade. Like many other coagulation factors, it is a serine protease. In humans, Factor XI is encoded by the F11 gene. Factor XI (FXI) is produced by the liver and circulates as a homo-dimer in its inactive form. FXIa along with FVIIIa are responsible for conversion of FX to its activated form FXa which results in conversion of prothrombin to thrombin. This reaction makes formation of fibrin clot (8) FXI gene of cattle is 19150 bp in length (17607721 - 17626871 nt). It has 15 exons and 14 introns and is located on BTA 27.

Studies done at international level showed that FXI gene deficient cattle were highly susceptibility to infectious diseases, showed increased frequencies of repeat breeding and lower rate of fetal and calf survival (2, 3, 7). FXI gene deficiency in cattle is caused by 76-bp insertion of an imperfect poly-adenine (Poly-A) tract occurring at 188 bp position in exon 12 [AT$(A)_{28}$TAAAG$(A)_{26}$GGAAATAATAATTCA]. This insertion introduces a stop codon that results in a FXI protein lacking the functional protease domain encoded by exons 13, 14 and 15 (4, 8). Liptrap *et al., (7)* found that FXI-deficient Canadian Holstein cattle have 50% greater prevalence of repeat breeding. FXI gene deficiency has been observed in Holstein cattle in Canadian USA, Polish and British herds. in Canada (2, 7), USA (8), Poland (5), Britain (1), Japan (4), India (11), Turkey (9) and in Japanese Black cattle also (6, 10). As very little work has been done on FXI gene deficiency on crossbred cattle in India, so the present study was carried out to screen cattle of the National Dairy

Research Institute (NDRI) for presence of FXI deficiency.

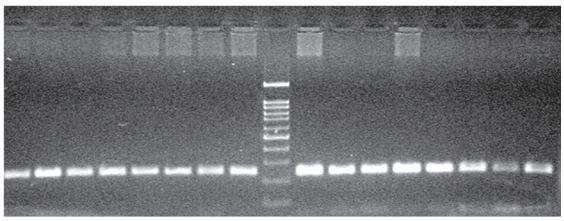
Materials and Methods

Blood samples were collected from randomly selected 200 Karan Fries cattle (Holstein Friesian x Tharparkar crossbred) maintained at cattle yard of NDRI, Karnal. DNA extraction was done using Phenol-chloroform method as described by Sambrook *et al*. (12) with few modifications. Gene-specific oligonucleotide primers (Forward 5' CCC ACT GGC TAG GAA TCG TT 3' ; Reverse 5' CAA GGC AAT GTC ATA TCC AC 3') for bovine factor XI gene were synthesized as described by Marron *et al.*, (8) to amplify exon 12 (244 and/or 320 bp) of FXI gene

The PCR amplification was performed in programmed Thermal cycler (MJ research). 22 ul of PCR reaction mixture was mixed thoroughly by vortexing with 3 ul template DNA (50ng/ul) and PCR amplification was performed in a final volume of 25 ul. The PCR reaction mixture was incubated in thermal cycler initially at 94 °C for 2 minutes followed by 30 cycles of 94 °C for 30 seconds, 55 °C for 1 minute, 72 °C for 1 minute and a final extension of 72 °c for 10 minutes to obtain PCR product of amplified DNA. The PCR products were checked by electrophoresis on 1.5% agarose gel at 100 volts for 30 minutes to verify the amplification of target region. Genotyping of animals of animals was carried out by 2.5% Agarose gel horizontal electrophoresis at 100 volts for 40 minutes. The gels were stained with 1% ethidium bromide solution (@ 1µl/100 ml of gel) and scored for the presence of single and double bands for homozygous and heterozygous individual respectively. The gel photograph was taken by gel documentation system

Result and Discussion

The genotyping of Karan Fries cattle revealed only one band of 244 bp in all the animals as shown in Plate 1 indicating that there is no 76-bp insertion in exon 12, similar results were obtained in Indian crossbred cattle by Patel *et al*., (11). However, they found two Holstein cattle as carrier with 77bp insertion within exon 12 of the FXI gene (Genbank accession numbers DQ438908 and DQ438909). The representative samples of Karan Fries obtained by PCR analysis were custom sequenced. Sequence data was analyzed using Chromas (Ver.5, http://www.technelysium.com.au/chromas.html). Multiple sequence alignments were performed to study nucleotide and amino acid changes among Karan fries and reference sequence of *Bos taurus*, which also indicated that there was no 76-bp or 77bp insertion in exon 12. There was neither homozygous mutant nor heterozygous carrier individual, hence, it is concluded that there



Lane 1-16 : 244 bp monomorphic
Lane M : 100 bp ladder

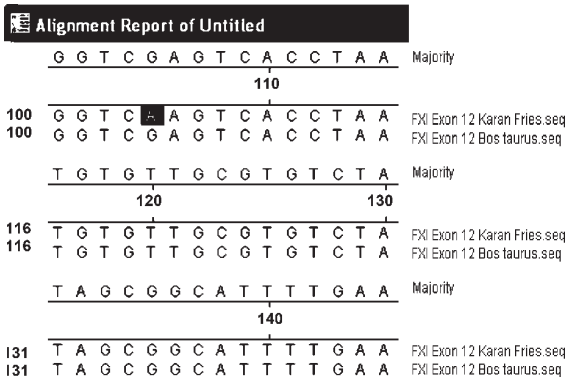


Fig. 1. Multiple sequence alignment of nucleotides of FXI Exon 12 of Karan Fries and *Bos taurus*

is no polymorphism of FXI gene in the studied animals of Karan Fries breed of cattle maintained at NDRI, Karnal. However, one nucleotide change from G to A at position 105 in Karan Fries was observed when compared with *Bos taurus* reference sequence and similar substitution was also seen in chromatograph (Figure 1 and 2). This non synonymous mutation resulted into a change of amino acid from Arginine (R) in *Bos taurus* to Glutamine (Q) in Karan fries in second codon (CGA TO CAA) as revealed by *in silico* nucleotide translation and the alignments of Karan fries and *Bos taurus* (Figure 3). The contigs containing exon 12 of Factor XI gene were subjected to basic local alignment search (BLAST) to know the sequence similarity with the corresponding regions of other species.

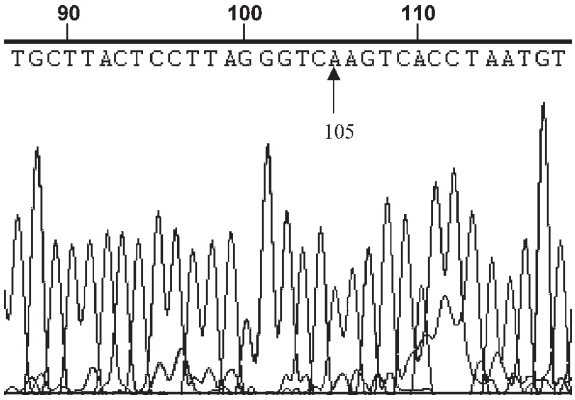


Fig. 2. Chromatogram of FXI Exon 12 showing nucleotide change

Fig. 3. Multiple sequence alignment of *in silico* translated amino acids sequences of FXI exon 12 of Karan Fries and *Bos taurus*

BLAST analysis of Factor XI gene for exon 12 regions of Karan Fries cattle showed 96 to 98% sequence identity with buffalo and *Bos Taurus*

References

1. Brush, P. J., Anderson, P. H. and Gunning, R. F. (1987). Identification of factor XI deficiency in Hostein-Friesian cattle in Britain. Vet. Rec., 121: 14–17.

2. Coomber, B. L., Galligan, C. L. and Gentry, P. A. (1997). Comparison of in vitro function of neutrophils from cattle deficient in plasma factor XI activating and from normal animals. Vet. Immunol. Immunopathol., 58: 121–131

3. Gentry, P. A. and Ross, M. L. (1993). Coagulation factor XI deficiency in Holstein cattle: Expression and distribution of factor XI activity. Can. J. Vet. Res., 58: 242–247.

4. Ghanem, M. E. and Nishibori, M. (2008). Genetic Description of Factor XI Deficiency in Holstein Semen in Western Japan. Reprod. Domestic. Anim., 44 (5): 792-6.

5. Gurgul, A., Rubioe, D. and Sota, E (2009). Identification of carriers of the mutation causing coagulation factor XI deficiency in Polish Holstein-Friesian cattle. J. Appl. Genet., 50 (2): 149-152.

6. Kunieda, M., Tsuji, T., Abbasi, A. R., Khalaj, M., Ikeda, M Miyadera, K., Ogawa, H. and Kunieda, T. (2005). Mamm. Genome 16: 383–389

7. Liptrap, R. M., Gentry, P. A., Ross, M. L. and Cummings, E. (1995). Preliminary findings of altered follicular activity in Holstein cows with coagulation factor XI deficiency. Vet. Res. Commun. 19: 463-471.

8. Marron, B. M., Robinson, J. L., Gentry, P. A. and Beever, J. E. (2004). Identification of a mutation associated with factor XI deficiency in Holstein cattle. Anim Genet., 35: 454-456.

9. Meydan, H., Yildiz, M. A., Ozdil, F., Gedik, Y. and Ozbeyaz, C. (2009). Identification of factor XI deficiency in Holstein cattle in Turkey. Acta Veterinaria Scandinavica, 51:5-9

10. Ohba Y., Takasu M., Nishii N., Takeda E., Maeda S., Kunieda T., and Kitagawa H. (2008). Pedigree analysis of factor XI deficiency in Japanese black cattle J Vet Med Sci.70 (3):297-9

11. Patel, R. K., Soni, K. J., Chauhan, J. B., Singh, K. M. and Sambasiva Rao K. R. S. (2007). Factor XI deficiency in Indian *Bos Taurus, Bos indicus, Bos Taurus* × *Bos indicus* crossbreds and *Bubalus bubalis*. Genetics and Molecular Biology, 30, (3): 580-583

12. Sambrook, J. E., Fritsch, E. F. and Maniatis, T. (1989). Molecular cloning: A laboratory manual. 2nd edn. Cold Spring Harbor Laboratory (CSH), Cold Spring Harbor, NY, USA.

Current Trends in Biotechnology and Pharmacy
Vol. 5 (1) 1064-1072 January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

Survival Selections Reveal Altered Pharmacological Phenotypes in *Nicotiana tabacum* var. SR1 Activation Tagged Mutants

S. K. Gunjan[1]*, T. Rogers[1,2], J. Czarnecki[1], J. Lutz[1] and J. Littleton[1,2]
[1]Kentucky Tobacco Research and Development Center, [2]Department of Pharmaceutical Sciences, University of Kentucky, Lexington, Kentucky 40546-0236, USA
*For correspondence - samir.gunjan@uky.edu

Abstract

This study demonstrates the utility of survival-selection strategies for identifying novel secondary metabolic profiles and accompanying pharmacological activity in activation-tagged mutants generated from *Nicotiana tabacum* var. SR1. Leaf discs were infected by *Agrobacterium tumefaciens* strain 3850 harboring an activation tagging vector pPCVICEn4HPT in which four copies of enhancer sequences are located at the right border of T-DNA. After *Agrobacterium* infection and co-cultivation, the transformed leaf discs were grown on shoot regeneration media containing either ethanol (200 mM) or 4-methyltryptophan (4-MT, 50 mM). The biochemical analysis of ethanol resistant mutants showed the presence of high level of antioxidants in plants. Similarly, the mutants were selected on 4-MT containing media revealed 3-5 fold higher nicotine content compared to wild-type control plants. These studies demonstrate the utility of survival selection for identifying individual mutants with novel pharmacological phenotypes from a large activation-tagged mutant population. Similar selection strategies may be applied to plants of pharmaceutical importance to identify novel phytochemical drug leads, or possibly improve yields of commercially important secondary metabolites.

Key words: 4-methyltryptophan, activation tagging mutagenesis, antioxidants, ethanol, *Nicotiana tabaccum.*

Introduction

The elucidation of plant genomic information offers tremendous potential for expanding use of plants as drug discovery and manufacturing resources. Generation of novel plant compounds or increasing concentrations of existing economically valuable plant secondary metabolites is one immediate application of our increased understanding of plant genomics. One strategy for applying the advances in our understanding of plant genomic is to use mutagenesis to produce large mutant populations followed by metabolic profiling to identify novel metabolites. Walden and colleagues developed a directed way to induce gain-of-function mutations in plants (1) that uses four copies of enhancer elements from cauliflower mosaic virus 35S gene cloned into T-DNA. This approach, known as activation tagging, can cause transcriptional activation of genes near its point of insertion into a plant genome. When combined with gene rescue techniques, activation tagging can be a powerful tool for identifying plant mutants and corresponding genes. The functions of several genes have

been characterized using an activation tagging approach. For example, a HIS kinase involved in cytokinin signalling isolated from *Arabidopsis* has been characterized (2) and genes conferring disease resistance have also been identified using this approach (3).

Activation tagging is especially useful in manipulating plants with complex, relatively intractable metabolic pathways, such the biosynthetic pathways found in many species of medicinal plants. For most laboratories, conventional biochemical analyses of the hundreds of thousands of mutants required for effective saturation of the majority of any given plant genome using ATM is prohibitive. In some cases, physical traits, such as flower color have been used to identify prospective mutants of interest. Overproduction of the plant pigment anthocyanin in arabidopsis flowers was used to identify mutants resistant to the lethal effects of ultra-violet radiation (4). Unfortunately, most valuable plant metabolites are "secondary" and confer no obvious survival advantage within a population, thus survival selection strategies are difficult to apply to large mutant plant populations, and most of the genes involved in plant secondary metabolism remain undiscovered.

Ethanol metabolism is not known to be a direct precursor to known secondary metabolite production in *Nicotiana* species. In this attempt to alter secondary metabolites using survival selections, we exposed a large population of activation tagged leaf disc cultures to high concentrations of ethanol. In both plants and mammals, ethanol is metabolized by alcohol dehydrogenase. Alcohol dehydrogenase activity in plants is increased by anaerobic conditions (5) and this metabolic adaptation is important in various stages of the life cycle such as flower formation, or adaptation to stressful conditions such as cold tolerance during early seedling development. The byproduct of ethanol, acetaldehyde, is toxic to plants and animal cells via oxidative damage. We hypothesize that ATM (activation tagged mutagenesis) *Nicotiana* mutants resistant to ethanol will contain elevated levels of antioxidants compared to wild-type plants and ultimately found that mutant survivors of ethanol selection contain higher antioxidants activity compared to non-mutant control plants.

Activation tagging mutagenesis has been used in *Catharanthus roseus* to isolate a master regulatory gene involved in terpenoid indole alkaloid synthesis (TIAs), *ORCA3*. Over-expression of *ORCA3* results in increased transcription of several genes directly involved in the *Catharanthus* TIA pathway (6). This study used resistance to the toxic substrate 4-MT (4-methyltryptophan) of the TIA biosynthetic enzyme tryptophan decarboxylase to identify mutants with enhanced TIA synthesis and presumably possessing the metabolic capacity to detoxify 4-MT. Because *Nicotiana* possesses a relatively large genome, activation tagging mutagenesis might uncover quiescent genes that may impact upon tryptophan metabolism and reveal novel synthetic routes not apparent in the native plant. These altered synthetic routes may be revealed in activation tagged mutants surviving exposure to toxic tryptophan analogs such as 4MT. In this study we hypothesize that survivors of 4-MT toxicity may possess increased nicotine levels compared to non-mutant controls. We found that mutant survivors of these selections contain 3-5 fold greater nicotine concentrations compared to control plants.

Vol. 5 (1) 1064-1072 January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

Materials and Methods

***Plant material and activation tagging by Agrobacterium infection*:** *Nicotiana tabacum* var. SR1 was used for all studies detailed here. Leaf discs from 3 week old magenta box-grown plants were transformed with *Agrobacterium tumefaciens* 3850 harbouring the activation tagging T-DNA construct pPCVICEn4HPT. Agrobacterium was cultured in Luria-Bertani (LB) medium containing 100 mg l^{-1} Ampicillin. Bacterial culture was initiated by inoculating single colony of *A. tumefaciens* into 25 ml flask containing 5 ml of liquid media and kept at 200 rpm on a rotary shaker at 28ºC for one day. Subsequently, 2 ml of overnight grown bacterial culture was transferred to 500 ml flask containing 200 ml of liquid media. When the optical density (OD) of the culture reached around 0.6 at 660 nm, 100 µM acetosyringone was added. Bacterial culture was spun down in a centrifuge at 2500 *g*, when culture reached around 1.0 OD. The pellet was gently resuspended in 2 ml MS medium and used as bacterial stock for infection. A final concentration of 100 µM acetosyringone was added to the bacterial culture and leaf explants were infected for approximately 20 min. After infection, explants were blot-dried onto sterile filter paper and transferred onto plates containing solid MS media. The infected explants were co-cultivated for 2 days in the culture room set at 25 ºC and then transferred onto shoot induction media. Ethanol (200mM) or 4-MT was added directly to culture media. Regenerated shoots were excised and transferred to rooting medium (MS medium without hormones) containing either ethanol or 4-MT along with hygromycin and cefotaxime. The resistant mutants were transferred to compost substrate and grown in a greenhouse.

***Antioxidant assays*:** 200 mg of fresh leaf tissue was ground to a fine powder under liquid nitrogen. The powder was homogenized in 2 mL of 2% (w/v) metaphosphoric acid and 2 mM ethylenediaminetetraacetic acid (EDTA), and transferred to eppendorf tubes. The supernatant was collected after centrifugation at 14,000x*g* for 10 min at 4 ºC. The supernatants were then split into two tubes of 900 uL each and neutralized with 600 uL of 10% sodium citrate (7). Total AsA was analysed with 500 uL of neutralized extract by measuring the change in spectrophotometric absorbance before and after adding ascorbate oxidase enzyme (8). Similarly, a spectrophotometric assay was used for measuring total GSH (7).

***ROS measurement*:** About 300 mg of leaf tissue was ground in liquid nitrogen and homogenized with 1 mL of TRIS (hydroxymethylaminomethane) buffer (pH 7.2). The supernatant was collected after centrifugation at 14,000x*g* for 10 min at 4 ºC. 800 uL of this extract was mixed with 1 mM 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and incubated in darkness for 10 min at room temp (7,9). Fluorescence was measured using a Victor Fluorescence plate reader (Perkin Elmer). Total protein was quantified using Bradford dye (Bio-Rad).

***Gas chromatography-mass spectrometry (GC-MS) analysis*:** Tobacco alkaloid content was determined using an Agilent 6890 GC/Agilent 5973 mass-selective detector (MSD) with an Agilent 7683 Autosampler. The GC was fitted with a 30m x 0.2 mm X 0.25 µm HP-5MS glass capillary column and a helium flow rate of 1.0 ml/ min. Extracts from survivors selected from mutant populations at a concentration of 100 mg dried material/ ml

methanol were auto-injected at a volume of 1.0 ìl and a split ratio of 1:20 (10). The temperature of the injection port, MS interface, electron impact source and mass filter were 280°C, 280°C, 230°C, and 150°C, respectively. The GC oven was programmed to hold at 120°C for 2 min, then heated to 160°C at a rate of 10°C/min. After holding the oven temperature at 160°C for 1 min, the oven was heated to 275°C at a rate of 6°C/min and held at this temperature for 10 min in order to elute possible interfering high boiling point impurities from the column.

Results and Discussion

Formation of endogenous ethanol is a common feature of plant metabolism and it is also produced in hypoxic conditions such as flood, cold and ozone stress (11). Ethanol is converted to acetaldehyde by alcohol dehydrogenase (ADH) and finally oxidized into acetate by acetaldehyde dehydrogenase (ALDH). The accumulation of NADH produced during ethanol metabolism can imbalance redox state of cells and cause oxidative stress (12) whereas acetaldehyde interacts with different bioactive molecules and impairs the normal function of cells (13). Overproduction of acetaldehyde in plant tissue causes necrosis (14). Thus, lethal concentrations of ethanol may be considered an elicitor of endogenous reactive oxygen species (ROS) in plant tissues. We treated *Nicotiana* leaf discs with different concentrations of ethanol ranging from 50 mM to 250 mM to identify the threshold of resistance to ethanol in wild type plants. We concluded that the minimum lethal concentration of ethanol was 175 mM. Thus, after activation tagging of leaf-discs through *Agrobacterium*, the explants were immediately subjected to ethanol selection pressure at the concentration of 200 mM. Few explants produced shoots in regeneration media or formed roots in the presence of ethanol in the hormone-free media. Finally, three mutants were able to grow in continuous ethanol selective media and produced shoots and roots. Extracts from these survivors were analyzed for antioxidant activity.

Ascorbate (AsA) and glutathione (GSH) are key components of the non-enzymatic defense system of plants (15-17). The cellular recycling of ascorbate/glutathione molecules to quench the ROS is interconnected (18,19). The ascorbate deficient mutant of *Arabidopsis thaliana* vtc1 shows 14-fold increased accumulation of ascorbate in leaves when supplemented with 10 mM ascorbate, without significant change in redox state (20). Previously, we have seen that AsA redox state was not favorably poised for scavenging induced ROS generated by ozone fumigation on Arabidopsis whereas total GSH content increased three- fold (9). The activation tagged mutants that are resistant to ethanol (200 mM) showed high glutathione and ascorbate contents compared to control plants (Fig.1). The mutant ET-R1 and ET-R3 have total GSH almost equal to control whereas ET-R2 and ET-R4 have two- and six-fold higher GSH concentration, respectively. The total AsA contents in these mutants are also variable ranging from 2-6 folds higher than control except in mutant ET-R1 (Fig.1a).

In *Arabidopsis* mutants, tocopherol-deficient lines have been reported with increased pools of ascorbate and glutathione (21). It is possible that the expression of connecting regulator of AsA/GSH cycle is enhanced in ET-R2 and ET-R4 mutants due to activation tagging and the over

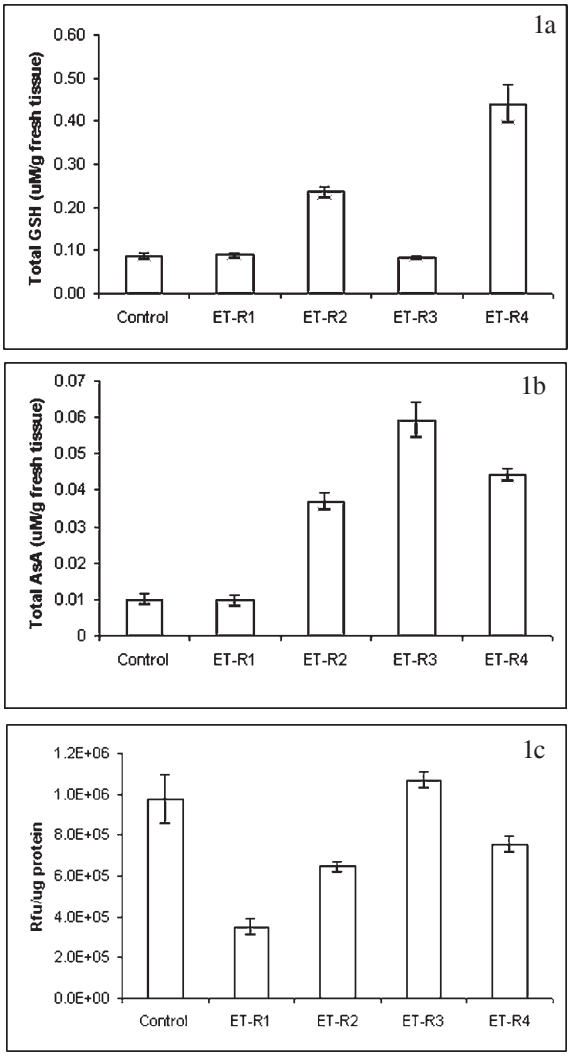


Fig.1. Comparison of antioxidants and ROS levels in activation tagged mutants resistant to ethanol (200 mM) and in control (wild type) of *Nicotiana tabaccum* var. SR1. About 200 mg of fresh leaf tissue was used to analyse total AsA by measuring the change in spectrophotometric absorbance before and after adding ascorbate oxidase enzyme. Similarly, a spectrophotometric assay was used for measuring total GSH. a) Glutathione (GSH), b) Ascorbate (AsA), c) Reactive oxygen species (ROS) was measured using general ROS substrate 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). Fluorescence was measured using a Victor Fluorescence plate reader (Perkin Elmer). Total protein was quantified using Bradford dye (Bio-Rad). RFU is relative fluorescence units. Error bars represent the SD from three biological replicates.

accumulation of glutathione also increased the ascorbate pool due to connected cycles. The GSH content of ET-R3 is almost equal to wild-type plants, but total AsA is six-fold higher (Fig. 1b). This may be due to enhanced expression of a regulatory component of AsA pathway in response to stress. It is well documented that plants produce large amounts of ROS in response to various biotic and abiotic stresses (22,23) and it is believed that the amount of different antioxidants strongly increases to scavenge ROS under stressful conditions (24,25). Thus, we compared the ROS level in these mutants with wild-type control plants. The general ROS substrate 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) was used with the extracts of plant samples (9). Our results show the ROS levels in mutants are either equal or less than control plant samples (Fig. 1c). Interestingly, the mutant ET-R1 showed ROS levels 3- fold less than wild type plant but almost similar antioxidants profile.

The advent of plant molecular biology has brought renewed interest in the understanding of the physiological and ecological roles of secondary metabolites. Plants coordinately regulate primary and secondary metabolism. Coordinate regulation in some plants is regulated by the stress hormone methyljasmonate (26). In *Catharanthus roseus*, this mechanism is modulated by a single stress-responsive transcription factor, ORCA 3. Likewise, alkaloid synthesis in *Nicotiana* is regulated by a host of developmental and environmentally responsive factors (26,27). A number of studies have demonstrated jasmonate-mediated signaling leads to increased nicotine biosynthesis following wounding or herbivore attack but at present, little is known regarding the gene regulatory mechanisms involved in

biosynthesis of nicotine, or most other plants alkaloids. The discovery of novel regulatory mechanisms may improve prospects for metabolic engineering of plants for drug discovery or production purposes, which are at present hampered by the lack of information regarding molecular control of secondary metabolism. Activation tagging mutagenesis coupled with gene recovery techniques are useful tools for discovery of such mechanisms of metabolic regulation, and may hold a distinct advantage compared to traditional biochemical elucidation in the potential for discovery of genuinely novel regulatory motifs. However, biochemical and genetic analyses of hundreds of thousands of individual mutants required to survey the full genomic potential of a given species is prohibitive in most instances. Survival selection strategies are commonly employed to identify transgenic individuals with desirable phenotypes, but this strategy is difficult to apply in the case of identifying phenotypes with altered secondary metabolic profiles. Selection of mutant *Catharanthus roseus* cultures using survival in the presence of 4-MT has been previously reported to result in mutants with altered levels of the secondary metabolic products of the TIA pathway. Similarly, when activation tagged mutants of *N. tabaccum* were selected on 4-MT containing media, the survival mutants showed high nicotine contents in their leaves (Fig. 2a-c). In tobacco, the principal secondary metabolic pathway is the nicotinic synthetic pathway. The main precursor in nicotine synthesis is putrescine, derived directly from ornitine in a reaction catalysed by ornithine decarboxylase, or indirectly from arginine beginning with arginine decarboxylase (28). The predominant route for nicotine synthesis is not known, and putrescine is also the principal precursor in spermine and

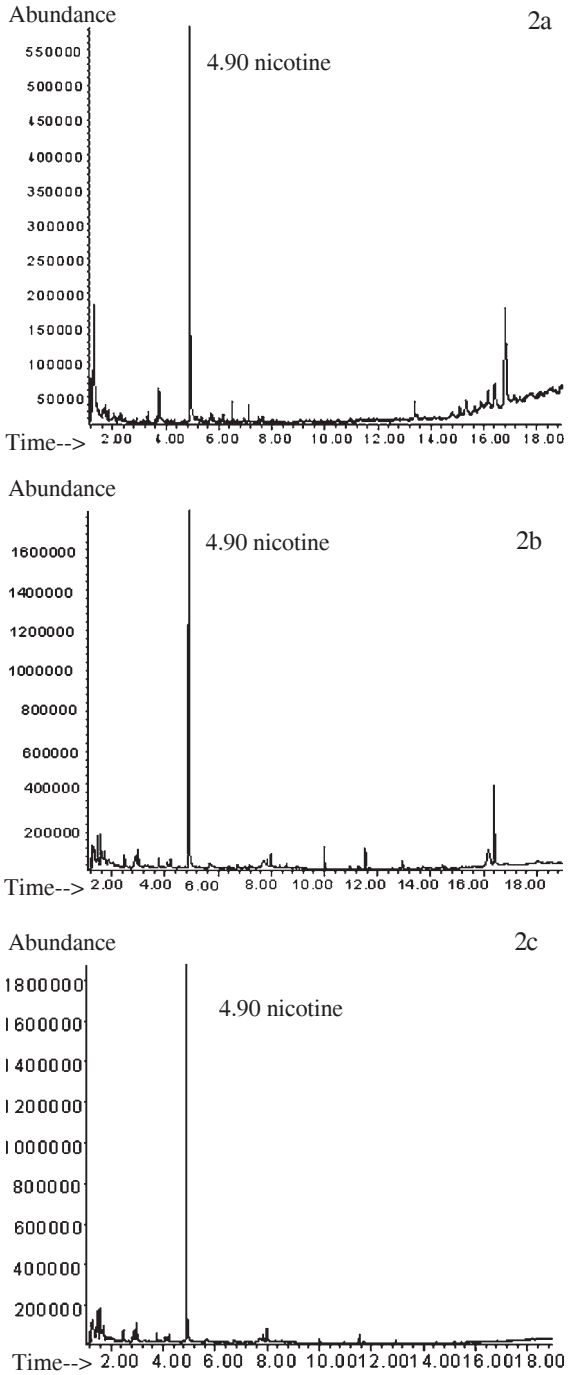


Fig. 2. Alkaloid profile analysis by GC-MS using leaf extracts from greenhouse grown plants. The ion chromatogram shows nicotine detection at 4.9 min.:
a) Wildtype, b) 4-MT Mutant 1, c) 4-MT Mutant 2

spermidine synthesis. Other amino acids and related compounds such as tyrosine, nicotinic acid and tryptophan also serve as minor biosynthetic precursors to some of the tobacco alkaloids. Thus, toxic tryptophan analogs may potentially select mutants with altered tryptophan metabolism along several points, but most of these pathways converge toward alterations in nicotine synthesis. This hypothesis is supported by the significantly increased nicotine content found in extracts from mutant survivors of 4-MT selection.

Acknowledgement

This work was supported by NIAAA (STTR Phase 1- 3R41AA014555-01, 2R42AA014554-01, STTR Phase 2- 242AA014554-02). The Kentucky Science and Technology Consortium (R&D Voucher 145-402-12, KSTC-184-512-024) and the Kentucky Tobacco Research and Development Center, Lexington, KY, USA.

References

1. Hayashi, H., Czaja, I., Lubenow, H., Schell, J. and Walden, R. (1992). Activation of a Plant Gene by T-DNA Tagging: Auxin-Independent Growth in Vitro. Science, 258: 1350-1353.
2. Kakimoto, T. (1996). CKI1, a histidine kinase homolog implicated in cytokinin signal transduction. Science, 274: 982-985.
3. Wiegel, D., Ahn, J.H., Blazquez, M.A., Borevitz, J.O., Christensen, S.K., Fankhauser, C., Ferrandiz, C., Kardailsky, I., Malancharuvil, E.J., Neff, M.M., Nguyen, J.T., Sato, S., Wang, Z.Y., Xia, Y., Dixon, R.A., Harrison, M.J., Lamb, C.J., Yanofsky, M.F. and Chory, J. (2000). Activation Tagging in Arabidopsis. Plant Physiology, 122: 1003-1013.
4. Mol, J., Grotewold, E. and Koes, R. (1998). How genes paint flowers and seeds? Trends Plant Science 3: 212-217.
5. Keyhani, E. and Keyhani, J. (2004). Hypoxia/anoxia as signaling for increased alcohol dehydrogenase activity in saffron (*Crocus sativus* L.) corm. Annal NY Acad Sci., 130: 449-57.
6. Fits, L van der, Hilliou, F. and Memelink, J. (2001). T-DNA activation tagging as a tool to isolate regulators of a metabolic pathway from a genetically non-tractable plant species. Transgenic Res.10:513-21.
7. Mahalingam, R., Jambunathan, N., Gunjan, S.K., Faustin, E., Weng, H. and Ayoubi, P. (2006). Analysis of oxidative signaling induced by ozone in *Arabidopsis thaliana*. Plant, Cell and Environment, 29: 1357-1371.
8. Rao, M.V. and Ormrod, D.P. (1995). Ozone exposure decreases uvb sensitivity in a uvb-sensitive flavonoid mutant of A*rabidopsis*. Phytochemistry and Photobiology, 61: 71-78.
9. Joo, J.H., Wang, SY., Chen, J.G., Jones, A.M. and Federoff, N.V. (2005). Different signaling and cell death roles of heterotrimeric G protein alpha and beta subunits in the *Arabidopsis* Oxidative stress response to ozone. Plant Cell, 17: 957-970.
10. Wei, X., Sumithran, S.P., Deaciuc, A.G., Burton, H.R., Bush, L.P., Dwoskin, L.P., Crooks, P.A. (2005). Identification and synthesis of novel alkaloids from the root system of *Nicotiana tabacum*: affinity for neuronal nicotinic acetylcholine receptors. Life Sciences, 78: 495-505.
11. Donaldson, R.P., Soochan, P. and Zaras, A. (1985). Anaerobic stress in germinating castor bean, ethanol

metabolism, and effect on subcellular organelles. Plant Physiology, 77: 978-983.

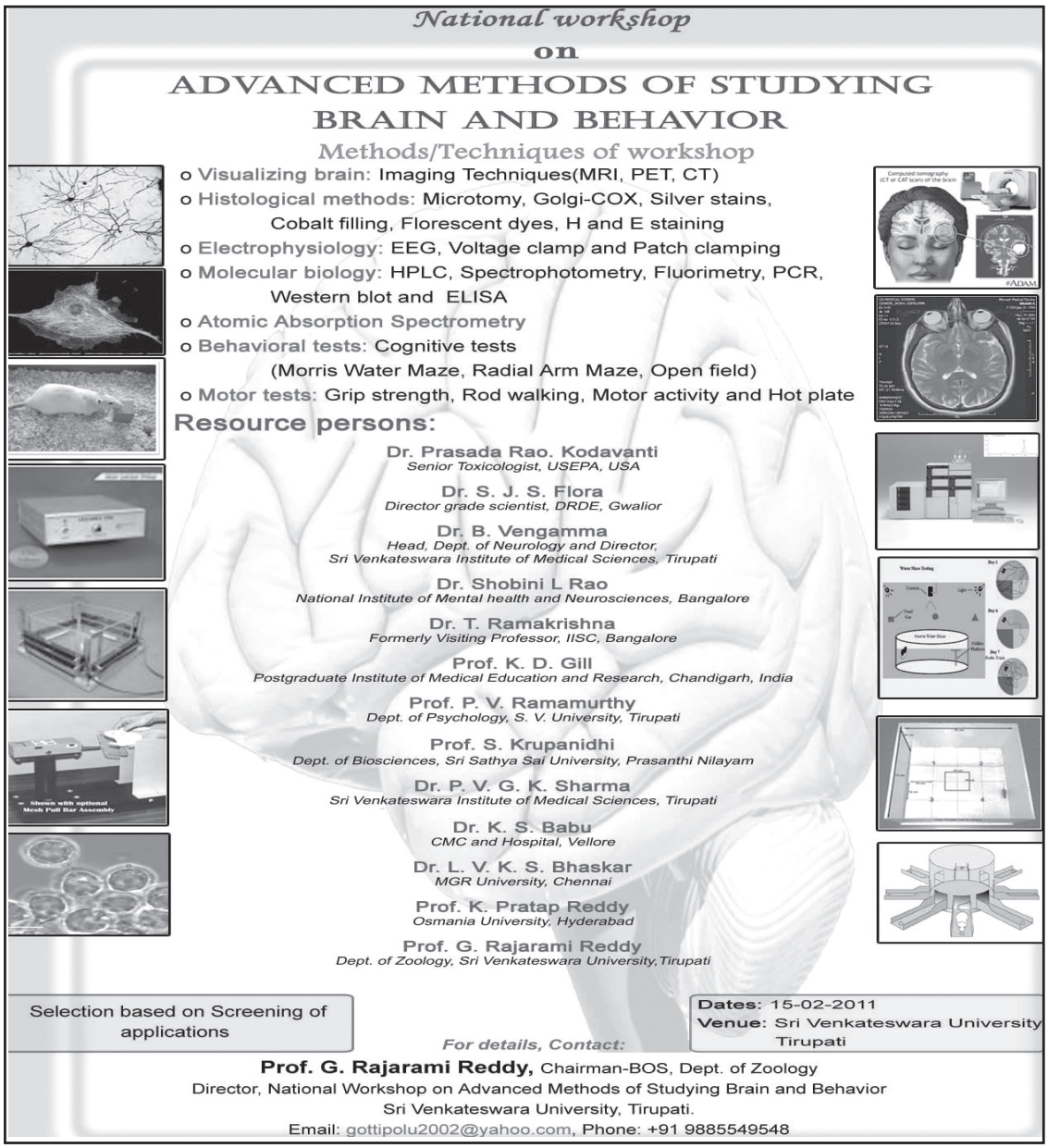
12. Fernandez-Checa, J.C., Kaplowitz, M.D., Colell, A. and Garcia-Ruiz, C. (1997) Oxidative stress and alcoholic liver disease. Alcohol Health & Research World, 21: 321-324.
13. Clements, D.I., Forman, A., Jerrells, T.R., Sorrell, M.F. and Tuma, D.J. (2002) Relationship between acetaldehyde levels and cell survival in ethanol metabolizing hepatoma cells. Hepatology, 35: 1196-1204.
14. Tadge, M., Bucher, M., Stahli, W., Suter, M., Dupuis, I. and Kuhlemeier, C. (1998) Activation of plant defense responses and sugar efflux by expression of pyruvate decorboxylase in potato leaves. Plant J., 16: 661-671.
15. Ball, L., Accotto, G.P. and Bechtold, U. (2004). Evidence for a direct link between glutathione biosynthesis and stress defense gene expression in *Arabidopsis*. Plant Cell, 16: 2448-2462.
16. Rentel, M.C. and Knight, M.R. (2004). Oxidative stress-induced calcium signaling in *Arabidopsis*. Plant Physiology, 135: 1471-1479.
17. Foyer, C.H. and Noctor, G. (2005). Redox homeostatis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. Plant Cell, 17: 1866-1875.
18. Smirnoff, N. (1995). Antioxidant systems and plant response to the environment. Environment and Plant metabolism: Flexibility and Acclimation. (N. Smirnoff ed), Bios Scientific, Oxford, 217-243.
19. Gossett, D.R., Banks, S.W., Millhollon, E.P. and Lucas, M.C. (1996). Antioxidant response to NaCl stress in a control and a NaCl-tolerant cotton cell line grown in the presence of paraquat, buthionine sulfoximine, and exogenous glutathione. Plant Physiology, 112: 803-809.
20. Kiddle, G., Pastori, G.M., Bernard, S., Pignocchi, C., Antoniw, J., Verrier, P.J. and Foyer, C.H. (2003) Effects of leaf ascorbate content on defense and photosynthesis gene expression in *Arabidopsis thaliana.* Antioxid. Redox Signal, 5: 23-32.
21. Kanwischer, M., Porfirova, S., Bergmuller, E. and Dormann, P. (2005) Alteration in tocopherol cyclase activity in transgenic and mutant plants Arabidopsis affect tocopherol content, tocopherol composition, and oxidative stress. Plant Physiology, 137: 713-723.
22. Allan, A.C. and Fluhr, R. (1997) Two distinct sources of elicited reactive oxygen species in tobacco epidermal cells. Plant Cell, 9: 1559-1572.
23. Scheel, D. (2002) Oxidative burst and the role of reactive oxygen species in plant-pathogen interactions. In Oxidative Stress in Plants (eds D. Inze & M. Van Montagu), pp. 137-153. Taylor & Francis, London, UK.
24. Noctor, G. and Foyer, C.H. (1998) Ascorbate and Glutathione: keeping active oxygen under control. Annu. Rev Plant Physiol Plant Mol Biol, 49: 249-279.
25. Collakova, E. and DellaPenna, D. (2003). The role of homogentistate phytyl-transferase and other tocopherol pathway enzymes in the regulation of tocopherol

synthesis during abiotic stress. Plant Physiology, 133: 930-940.

26. Wu, J., Wang, L. and Baldwin, T. (2008). Methyl jasmonate-elicited herbivore resistance: does MeJA function as a signal without being hydrolyzed to JA? Planta, 227(5): 1161-1168.

27. Facchini, P.J. (2006) Regulation of alkaloid biosynthesis in plants. Alkaloids Chem Biol. 63: 1-44.

28. Chou, W.M. and Kutchan, T.M. (1998). Enzymatic oxidations in the biosynthesis of complex alkaloids. Plant J. 15(3): 289-300.



Survival selection of *N. tabaccum* activation tagged mutants

Current Trends in Biotechnology and Pharmacy
Vol. 5 (1) 1073-1082 January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

The Protective role of *Luffa acutangula* fruit Methanolic Fraction against *t*-BHP Induced Oxidative damage in Human Erythrocytes

Boreddy Purushotham Reddy*, S. Venkata Mohan and P. N. Sarma

Bioengineering and Environmental Centre, Indian Institute of Chemical Technology Hyderabad 500 607, India
*For correspondence - poory_boreddy@yahoo.com

Abstract

The present study was designed to examine the ability of partially purified fractions of *Luffa acutangula (Cucurbitaceae)* fruit, to attenuate *t*-BHP induced oxidative damage in human erythrocyte as an *in vitro* model. Initially, we investigated the antioxidant property of the hexane, methanolic and aqueous extracts (FHE, FME and FAE, respectively) by DPPH free radical method and found that fruit methanolic extract was showing higher antioxidant activity (71.4±4.46% at 1 mg/ml) compared to other extracts (FHE&FAE were 13.93±1.3 and 51.84±3.76%, respectively). Hence, this extract was further partially purified chromatographically and out of these fractions (F1, F2, F3, F4, F2-1, F2-2, F2 -3 and F2-4) F2-3 showed significant antioxidant activity (73.96±6.4% at 25 µg/ml). This fraction was further tested for its effect on lipid peroxidation, superoxide dismutase, catalase and glutathione in *t*-butyl hydroperoxide (*t*-BHP) treated-erythrocytes. Pretreatment with fraction F2-3 significantly inhibited lipid peroxidation in a dose and time dependent manner compared to control (40.6±3.2, 27.9±2.4 and 75.5±5.2 nmol MDA/gHb, at 30, 90 min and control, respectively). Catalase, SOD and GSH levels were also brought up in a dose and time dependant manner compared to control (treated and control were CAT: 100.7±4.7 and 51.3±3.2 µMH_2O_2/gHb/min, SOD: 9.68±0.87 and 1.15±0.12 IU/g Hb, GSH: 21.3±1.23 and 6.0±0.91 µM/g Hb, respectively). These results establish that *L. acutangula* fruit aqueous fraction F2-3 possesses beneficial role in mitigating *t*-BHP induced oxidative stress in erythrocyte.

Key words: Fruit methanolic extract; Reactive oxygen species , Superoxide anion, Hydroxyl radical; Malondialdehyde; Superoxide dismutase

Introduction

Hydrogen peroxide (H_2O_2), organo peroxide, superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical ($^{\cdot}OH$), etc. are reactive oxygen species (ROS) generated by aerobic metabolism in biological system and also by exogenous sources such as drugs, ultra violet light, ionizing radiation and pollution. Under normal conditions, generated ROS are neutralized by inbuilt and default antioxidant enzymes present in the body such as catalase, superoxide dismutase, glutathione peroxidase, etc. (1, 2). Non-enzymatic antioxidant molecules such as ascorbic acid, glutathione and uric acid also play a key role in detoxifying the free radicals (3). Antioxidants are a type of complex compounds found in our diet that act as a protective shield for our body against several acute diseases (4). Increased oxygen flux conditions (i.e. exercise) or failure of antioxidant

mechanism leads to over production of free radicals that may exceed system capacity to remove them. This situation ultimately culminates into damage of macromolecules such as proteins, lipids and nucleic acids followed by oxidative stress (5).

Several compounds such as phenolic, flavonoids etc. obtained from different plants have strong antioxidant capacity (4). Dietary phenols are present in plant foods as bioactive molecules and data supporting the proposal that health benefits associated with vegetables and fruits are probably linked to the phenol antioxidants they contain. Phenols are present in a variety of plants utilized as important components of both human and animal diets (6, 7, 8). A general consensus has been reached during the last few years that diet has a major role in the development of chronic diseases, such as cancer, coronary heart disease, obesity, diabetes type 2, hypertension and cataract (9, 10). This consensus suggests that a predominantly plant-based diet rich in fruits and vegetables, pulses and minimally processed starchy staple foods reduces the risk for development of these diseases significantly (11, 12).

The recommendations are mainly based on epidemiological studies, are thus, that fruits, vegetables and less processed staple foods provide the best protection against the development of disease with little or no merit in recommending vitamin or other micronutrient supplements for disease prevention. This is a safe principle that promises to provide for improved public health. In recent years, search for novel type of antioxidants from several plant parts has achieved huge attention. Management of diseases with minimal side effects is still a complicated medical challenge. There is an increasing demand to use the natural products to prevent the free radical induced diseases (13, 14). In this regard, we selected *L.acutangula* to evaluate its antioxidant property because it is the popular in Indian traditional medicine. *Luffa acutangula* is a tropical running vine with rounded leaves and yellow flowers belongs to Cucurbitaceae family. Tea of these leaves is used as a diuretic, seeds have laxative properties and juice of the fruit is used against internal hemorrhage in traditional medicine. The results suggest that free radicals might play a role in the development of brain injury following brain hemorrhage (4). The fruit of the *Luffa acutangula* is rich in phenolic contents. Presently we made a hypothesis that *Luffa acutangla* may have potential antioxidant activity due to which it is being used against internal hemorrhage in traditional medicine. None of its therapeutic potentials are scientifically evaluated except robisome inactivating activity (15). Therefore we set out to determine the effect of various doses of *L.acutangula* fruit extract on lipid peroxidation, glutathione (GSH) and Superoxide dismutase (SOD), Catalase (CAT) activities in erythrocytes in a time and dose dependent manner using Tertiary butyl hydroperoxide *t*-BHP induction. It has been shown that human RBCs may compose up a potent system that can be used as an *in vitro* experimental model to investigate the antioxidant potential of dietary foods (16). *t*-BHP) is a well known cytotoxin and oxidative stress inducer, induces oxidative damage in different organs like liver (17) testes (18), etc. mainly by mobilization of arachidonic acid (AA) from membrane phospholipids under cytotoxic conditions.

Materials and methods

Reagents : The medicinal plant *L.acutangula* was purchased from Dr. Madhava Shetty, Department of Botany, Sri Venkateswara University,Tirupati, Andhra Pradesh, India. Thiobarbituric acid (TBA), 1,1,3,3, tetraethoxypropane (TEP), t-butyl

hydroperoxide(*t*-BHP), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), GSH, SOD, xanthine, xanthine oxidase, nitroblue tetrazolium, GSH-Px, reduced nicotinamide adenin dinucleotide phosphate (NADPH), bovine serum albumine and glutathione reductase (GR) were purchased from Sigma Aldrich Corp. (St. Louis, MO, USA.)

Extraction : Fruit hexane (FHE), fruit methanolic (FME) and fruit aqueous (FAE) extraction procedure was performed as described by Reddy e*t al.* (19). Briefly around 300g of fresh plant material (fruit) was washed with tap water, air dried and then chopped into small fragments which were shade dried and reduced to coarse powder with mortar and pestle. The powdered materials were extracted thrice times with hexane (2.5 l), then extracted three times with methanol (2.5 l) and followed by distilled and deionised water (1 l) at room temperature in cycle of 48 h each on orbital shaker. The combined methanolic extracts were then concentrated in a rotavapour at reduced pressure, below 40^{o}C and pooled water extracts were concentrated by lyophilization.

Partial purification : Since methanolic extract had shown significant antioxidant activity, this extract was further fractionated chromatographically as described by Reddy et al., 2009(20). Initially, 15 g of FME was loaded on a silica column (column height and diameter were 24 and 2 inches, respectively) and was eluted with stepwise gradient elution of ethyl acetate-methanol (4:1→0:1, v/v). Four fractions were collected at a regular interval and named as F1, F2, F3 and F4. Antioxidant activity of these fractions was evaluated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay and found that only fraction F2 was showing significant activity. Hence, this fraction was further fractionated with the solvent system methanol-water (4:1→0:1, v/v). Four fractions collected at a regular interval were named as F2-1, F2-2, F2-3 and F2-4.

HPTLC (High Performance Thin Layer Chromatography) fingerprinting : Fingerprinting of *Luffa acutangula* was performed on 5x10 cm HPTLC plates coated with 0.25 mm layer of silica gel 60F254. Before using, the plates were washed with methanol and activated at 110^{0}C for 5min. Samples were applied as 4mm wide bands and 6mm apart by using a Camag (Muttenz, Switzerland) Linomat IV sample applicator equipped with a 100 µl syringe. A constant application rate of 6 µl/sec was used. The chromatograms were monitored at 600nm using benzene : acetone (60:40) as mobile phase and vanillin H_2SO_4 as reducing agent.

DPPH free radical scavenging activity : The DPPH free radical scavenging activity was measured using a method described previously by Reddy *et al.* (19). Briefly, the stock test extracts of hexane, methanolic and aqueous fruit were dissolved in Dimethyl sulfoxide (DMSO) at a concentration of 1 mg/ml, the methanolic fractions, (F2-1, F2-2, F2-3 & F2-4) were dissolved in DMSO at concentration of 25 µg/ml and ascorbic acid was dissolved in DMSO at a concentration of 100 µg/ml. DPPH was prepared freshly in absolute alcohol at a concentration of 4.9 mg/25ml. The reaction mixture consisting of 125 µl of DPPH, 100µl of freshly prepared 0.5mM tris buffer (pH 7.2) and 25 µl test extracts or standard was added to 96 well plates. The plates were incubated at room temperature for 10 min and then absorbance was measured at 517 nm by a UV–visible spectrophotometer (SPECTRA max PLUS®, Molecular Devices, USA). The percentage of free radical scavenging activity was determined from the following formula:

$$\text{Radical scavenging } (\%) = \frac{(\text{CONTROL - SAMPLE})}{\text{CONTROL}} \times 100.$$

Erythrocytes preparation : Erythrocytes were prepared as described in Betul *et al.* (21) Human

blood was collected from healthy donors (we don't have any institutional review board protocol to draw the blood, it is done by our technician) and centrifuged at 600 g for 5 min. The clear plasma and buffy coat were discarded. Erythrocytes were washed thrice with phosphate- buffered saline (PBS; pH 7.4) by 600 g for 30 sec. The washed erythrocytes were used for subsequent antioxidant activity assay of *Luffa acutangula.*

Erythrocyte treatment : Stock solutions of fruit methanolic fraction (F2-3) were prepared in DMSO then diluted with distilled water to obtain 12.5, 25 and 50 µg/ml (DMSO concentration lower than 1%). Erythrocytes at 5% hematocrit in PBS which are treated by 1.5 mM *t*-BHP for 1 h were incubated for different intervals (30, 60 and 90 min) at 37^0 C (with continuous mixing) with fruit sub fraction (F2-3) at different doses from 12.5 –50 µg/ml. Samples of erythrocytes a without fruit sub fraction (F2-3) is used as controls. After incubation, the mixtures were haemolysed in -20^0 C. The mixtures were thawed the following day and then centrifuged at 3600 rpm for 15 min. All assays were performed in these supernatants. The concentration of haemoglobin (Hb) was determined using the method described by (22). All experiments described in these studies were reproduced with at least three separate isolates.

Lipid peroxidation : The concentration of the malondialdehyde (MDA) in blood was estimated as described by Stocks and Dormandy (23). The principle of this method is spectrophotometric measurement of pink color produced by the reaction of thiobarbituric acid with MDA. The results were expressed as nmol/ml. Briefly 1 ml of 28% trichloroacetic acid–sodium meta arsenite solution was added to 2 ml supernatan and centrifuged at 2000 rpm for 15 min. Then 0.5 ml of 1% thiobarbituric acid (TBA) was added to supernatants and placed in a boiling water bath for 15 min. After reaching room temperature, absorbance was measured using a spectrophotometer at 532 nm. MDA values in erythrocytes were determined from the standard curve using 1,1,3,3,tetraethoxypropane (TEP) as standard.

Total glutathione (GSH) : Determination of total gluathione content in incubation solutions was done according to the method of Betul (21). The principle of this method is that the oxidized glutathione (GSSG) is converted into GSH in the presence of NADPH and glutathione reductase, The chromophoric product 2-nitro-5-thiobenzoic acid, resulting from reaction of the 5, 5' dithiobis-(2-nitrobenzoic acid) (Ellman reagent) with GSH possesses a molar absorption at 412 nm. 25 µl of the supernatant was added to standard assay mixture containing 0.6 µmol DTNB, 10 µg glutathione reductase and 0.2 µmol NADPH. The reaction was initiated by the addition of NADPH and the color development at 412 nm was followed for 6 min. The concentrations of total glutathione were calculated from a standard curve prepared with GSSG and were expressed as µmol glutathione/g Hb.

Superoxide Dismutase (SOD) : Activity of the SOD enzyme in incubation solution was determined according to the method described by Sun *et al.* (24). Xanthine reacts with xanthine oxidase and generates superoxide radicals that react with nitrobluetetrazolium to form formazan dye. To analyze this, 400 µl of incubation solution was haemolysed by four times cold water and Hb was removed by adding mixture containing 0.6 ml chloroform ($CHCl_3$) and 1 ml ethanol (EtOH) to haemolysates, mixed vigorously and centrifuged. A 600 µl of reaction mixture containing 0.1 mmol of xanthine, 0.1 mmol of EDTA, 50 mg of bovine serum albumin, 25 µmol of nitrobluetetrazolium per liter was added to 125 µl supernatant or 125 µl SOD standard solutions,

then 25 µl of 9.9 nmol xanthine oxidase solutions was added to each tube at 30 s intervals. Each tube was incubated for 20 min at room temperature and the reaction was terminated by adding 0.5 ml of 0.8 mM $CuCl_2$ solution per tube every 30 s. The production of formazan was determined at 560 nm. Under these conditions, the absorbance at 560 nm of the blank tube was about 0.25. SOD enzyme concentrations were determined from the standard inhibition curve with the x-axis being the logarithmic SOD concentrations and the Y-axis represent percent inhibition and expressed as IU/g Hb.

Catalase (CAT) : CAT activity was measured according to the method described by Aebi (25). The principle of the assay is based on the determination of the rate constant of hydrogen peroxide decomposition by CAT enzyme. A mixture containing 50 mM phosphate buffer (pH 7.0), 20 mM H_2O_2 and 500 ìl of supernatant was incubated at room temperature for 2 min. The change in absorbance at 240 nm in 2 min was calculated and the decrease in H_2O_2 was measured spectrophotometrically at 240 nm, for 3 min at 25^0 C. The catalase activity was expressed as mmole H_2O_2 consumed /min/g Hb.

Statistical Methods : All the data were expressed as mean ± S.D. using Microsoft XL 2007 software.

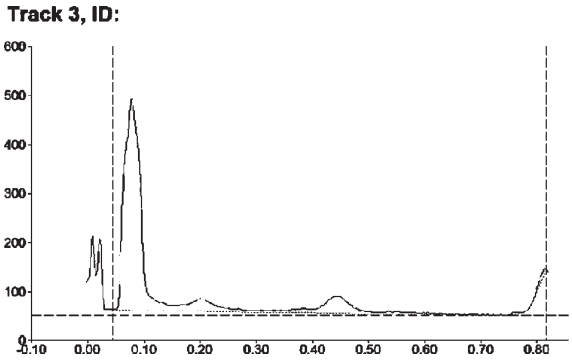


Fig. 1. HPTLC chromatogram of fruit methanolic extract (mobile phase of benzene : acetone at (60:40); chromatogram was monitored at 600 nm; Y-axis legend, Rf; X-axis legend, AU) (20).

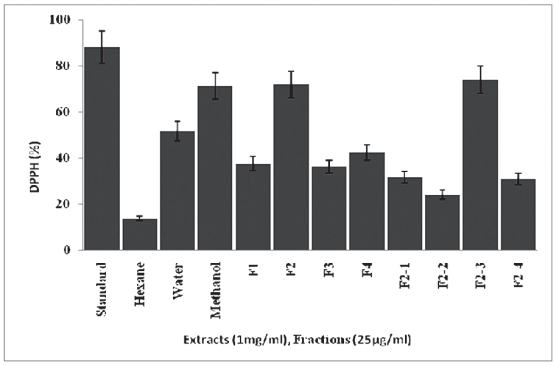


Fig. 2. Antioxidant activity of the extracts (hexane, methanolic, aqueous and methanloic fractions (F1, F2, F3, F4 and F2-1, F2-2, F2-3, F2-4) was estimated using the DPPH method. Results were shown as mean ± SD.

Results and Discussion

The percentage extraction yield of hexane, methanol and aqueous extract of *L. acutangula* fruit was 3.2 %, 15.02 % and 9.98 %, respectively. HPTLC fingerprinting of *L. acutangula* fruit methanolic extract is presented in Figure 1 (20)

In this study, all the three crude extracts, Fruit Hexane Extract (FHE), Fruit methanolic Extract (FME) and Fruit Aqueous Extract (FAE), were initially tested for their antioxidant activity, FME was showed higher antioxidant activity compared to FHE and FAE (Fig. 2). Hence this extract was selected for further purification using column chromatography. Fractions were collected at regular intervals and named as F1, F2, F3, and F4 and these fractions were also subjected to antioxidant activity. F2 demonstrated higher antioxidant activity (Fig. 2) than the other fractions (F1, F3 and F4). Fraction F2 was further fractionated chromatographically and named as F2-1, F2-2, F2-3 and F2-4. Out of these four fractions, F2-3 was found to possess antioxidant activity (Fig. 2). Hence, fraction F2-3 was selected to evaluate the oxidative stress inhibitory activity

in the human erythrocyte model by measuring different parameters such as lipid peroxidation, SOD, catalase and GSH levels.

Lipid peroxidation is an autocatalytic process, which may lead to an oxidative stress, inflammation, cancer, tissue damage, DNA damage and aging (26). Since, MDA is an end product of the lipid peroxidation, we evaluated the effect of F2-3 on MDA as an index for the lipid peroxidation. When erythrocytes were challenged with *t*-BHP, all the oxidative stress parameters, GSH, SOD, and CAT were significantly reduced but MDA levels were increased drastically (Fig.4, Fig5, Fig6 and Fig.3, respectively) indicating *t*-BHP induces oxidative stress in the erythrocyte. In experiments, where erythrocytes were pre-incubated with fraction F2-3 for one hour and following exposure to *t*-BHP, MDA levels were significantly reduced in a dose and time dependant manner. MDA levels in 12.5 µg/ml treated group at the time intervals of 0, 30, 60, 90 min and *t*-BHP-control and control were 68.1, 51.8, 46.5, 40.4, 70.34 and 21.2 nmol/gHb, at the concentration of 25 µg/ml MDA levels were 70.6, 46.7, 42.6 and 30.8 and at the concentration of 50 µg/ml MDA levels were 75.5, 40.6, 29.9 and 27.9 nmol/g Hb, respectively (Fig.3). These data suggested that the fraction F2-3 of *L. acutangula* fruit possesses lipid peroxidation inhibition properties.

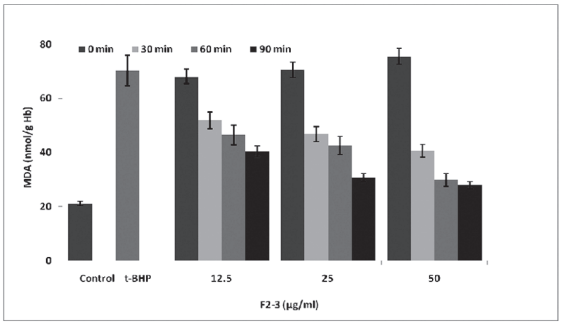


Fig. 3. Effect of methanolic fraction of *L.acutangula* fruit (F2-3) on *t*-BHP induced lipid peroxidation in erythrocyte was monitored by measuring the malonaldehyde (MDA). Values are mean ± SD.

GSH, one of the most potent biological molecules, it's can prevent occupation including free radicals reactions in the erythrocytes. The main defensive roles of glutathione against oxidative stress are: glutathione is a cofactor of several detoxifying enzymes against oxidative stress, e.g. glutathione peroxidase (GPx), glutathionetransferase etc. (27). GSH scavenges hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of glutathionperoxidase. In this study effects of F2-3 fraction on cellular GSH levels in *t*-BHP-treated cells were represented in Fig.4. GSH levels in 12.5 µg/ml treated group at the time intervals of 0, 30, 60, 90 min, *t*-BHP-control and control were 6.7, 9.0, 13.2, 13.6, 7.8 and 28.77 µmol/g Hb, at the concentration of 25 µg/ml GSH levels were 7.2, 12.3, 19.4 and 19.5 and at the concentration of 50 µg/ml GSH levels were 7.1, 13.2, 20.6 and 21.3 µmol/g Hb, respectively (Fig.4). The obtained results clearly indicating that fraction F2-3 has ability to replenish cellular redox buffer with GSH. Since GSH levels are increased, the co-factor for glutathione related enzymes, glutathione

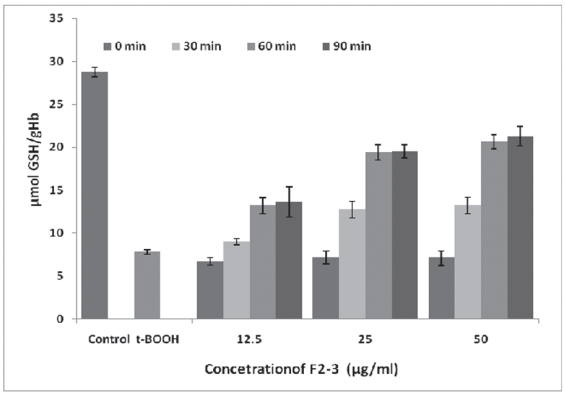


Fig. 4. Effect of methanloic fraction F2-3 on cellar redox buffer (GSH) was monitored in *t*-BHP treated erythrocytes. Values are mean ± SD.

peroxidase and glutathionetransferase, is readily available to neutralize the free radicals generated by *t*-BHP.

SOD catalyses the depletion of the superoxide radical and protects oxygen-metabolizing cells against harmful effects of superoxide free radicals. Some types of SOD like MnSOD which contains a manganese prosthetic group, resides in the mitochondria, perhaps because of the need to protect mitochondrial proteins, membranes, and DNA from $\cdot O_2$ generated as a result of the respiratory chain. Figure 5 showing total SOD levels in human erythrocytes with reference to sub fraction F2-3. SOD levels in 12.5 µg/ml treated group at the time intervals of 0, 30, 60, 90 min, *t*-BHP-control and control were 1.2, 1.9, 3, 3.23, 1.85 and 11.02 IU/g Hb protein at the concentration of 25 µg/ml MDA, SOD levels were 1.22, 2.5, 5.2 and 5.2 and at the concentration of 50 µg/ml SOD levels were 1.1, 5.3, 9.4 and 9.7 IU/g Hb, respectively (Fig.5).

CAT, a soluble protein in erythrocytes, plays a role in the decomposition of hydrogen peroxide to give H_2O. In humans, the highest levels of catalase are found in liver, kidney and erythrocytes, where it is believed to account for the majority of hydrogen peroxide decomposition. Figure 6 showing catalase activity in human erythrocytes with reference to FME sub fraction F2-3. CAT levels in 12.5 µg/ml treated group at the time intervals of 0, 30, 60, 90 min, *t*-BHP-control and control were 50.1, 55.4, 61.8, 62.4, 53.9 and 99.78 µmol H_2O_2/g Hb/min. at the concentration of 25 µg/ml MDA levels were 52.8, 60.9, 72.3 and 76.6 and at the concentration of 50 µg/ml MDA levels were 51.1, 62.5, 98.3 and 100.7 µmol H_2O_2/g Hb/min., respectively (Fig.6). Since both the enzymes SOD and CAT are directly involved in the neutralization of free radicals, these enzymes play a pivotal role in the oxidative stress. These enzyme levels were significantly reduced in *t*-BHP treated groups indicating that antioxidant ability of the erythrocytes are reduced in *t*-BHP treated group. Since these enzymes are replenished in response to F2-3 fraction of the fruit, erythrocytes again gained the antioxidant potentials to combat against the free radicals generated in the various metabolic reactions.

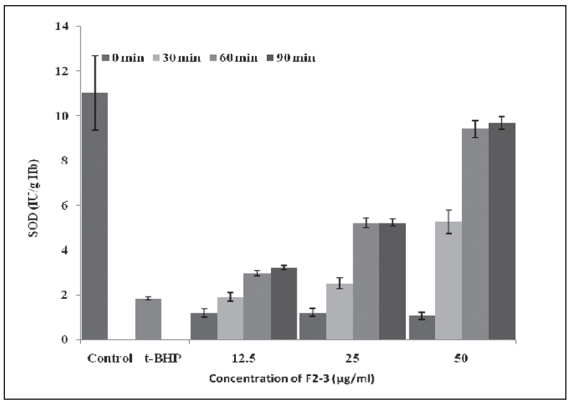


Fig. 5. Effect of fraction F2-3 on *t*-BHP induced oxidative stress in erythrocytes was monitored by measuring the superoxide dismutase activity. Results are mean ± SD.

The present findings show that *L. acutangula* fruit aqueous fraction F2-3 pretreatment attenuated *t*-BHP induced lipid peroxidation in human erythrocyes. Specifically, fraction F2-3 prevented *t*-BHP induced increases in MDA levels, and concomitantly restored GSH content, SOD and CAT activity in erythrocytes, though to a different degrees. These effects may reflect the ability of fraction F2-3 to enhance the scavenging and inactivation of H_2O_2 and hydroxyl radicals. In addition, fraction F2-3 may serve as a chealator and directly bind to Fe^{2+}, which catalyzes formation of free radicals via the Fenton reactions (28, 29). Fraction F2-3 may also terminate lipid peroxidation by induction of enzymatic and non-enzymatic antioxidants, such as GSH, SOD and CAT (30). Accordingly, the

protection afforded by fraction F2-3 against *t*-BHP induced ROS generation is likely attributable to its antioxidant effects.

In conclusion, *L. acutangula* methanolic extract fraction F2-3 showed significant antioxidant activity in human erythrocytes and further studies are required to elucidate the fraction components and their molecular mechanism. Systemically, superoxides could be produced in huge amounts by various metabolic and physiological processes (31, 32, 33). The formation of superoxide radical leads to a cascade formation of other ROS in the cell, whenever the antioxidant system fails to combat with ROS that can cause to lethal damage to the system (34). Hence, our data for the first time reports the oxidative stress inhibitory property of the *L. autangula* fruit. So that it may help to prevent diseases caused by the ROS. However, one thing we should consider that antioxidant activity may differ from organism to organism because of the genetic configurations to respective antioxidant mechanism (16). So we can expect less or more activity from different organism.

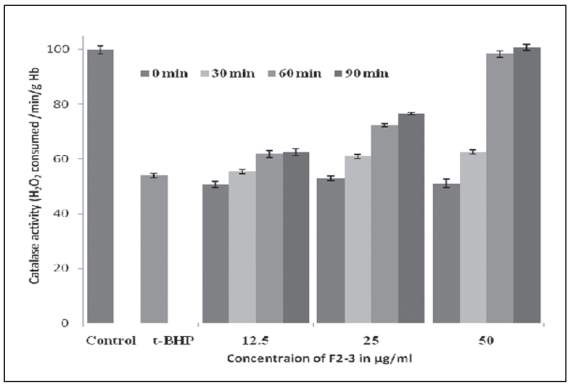


Fig. 6. Eeffect of fraction F2-3 on *t*-BHP induced oxidative stress in erythrocytes was monitored by measuring the activity levels of catalase. Results are Mean ± SD.

Acknowledgements

This study was supported by Financial support (Senior Research Fellowship) from CSIR, New Delhi, India.

Reference

1. Herbette Stephane, Roeckel-Drevet Patricia and Drevet, Joel R. (2007). Seleno-independent glutathione peroxidases: More than simple antioxidant scavengers. FEBS J., 274: 2163-2180.
2. Mc Cord, J.M. and Fridovich, I. (1969). Superoxide dismutase an enzymic function for erythrocuprein (hemocuprein). J Biol Chem., 244: 6049-55.
3. Marian, V. (2007). Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem, Cell Biol., 39: 44–84.
4. Aygul, R., Demircan B. Erdem .F, Ulvi, H., Yildirim, A. and Demirbas, F. (2005). Plasma Values of Oxidants and Antioxidants in Acute Brain Hemorrhage: Role of Free Radicals in the Development of Brain Injury. Biol Trace Elem Res., 108(1-3): 43-52.
5. Ji, L. (2007). Antioxidant signaling in skeletal muscle: A brief review. Exp Gerontol., 420: 582–593.
6. Bravo, L. (1998). Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. Nutr Rev, 56: 317-333.
7. Chung, K.T., Wong, T.Y., Wei, CI., Huang, Y.W. and Lin, Y. (1998). Tannins and human health: a review. Crit Rev Food Sci Nutr, 38: 421-464.
8. Crozier, A., Burns, J., Aziz, A.A., Stewart, A.J., Rabiasz, H.S., Jenkins, G.I., Edwards, C.A. and Lean Mej. (2000). Antioxidant

flavonols from fruits, vegetables and beverages: measurements and bioavailability. Biol Res., 33: 79-88.

9. Stefanos, T., Demosthenes, B. and Panagiotakos. (2010). The role of Mediterranean type of diet on the development of cancer and cardiovascular disease, in the elderly. A systematic review Maturitas, 65(2): 122-130.

10. Barry, H. (2002). Effect of diet on cancer development: is oxidative DNA damage a biomarker? Free Radic Biol Med, 32 (10): 968-974.

11. Greenwald, P., Clifford, C.K. and Milner, J.A. (2001). Diet and cancer prevention. Eur J Cancer. 37(8): 948-965.

12. Hubbard, R.W., Mejia, A. and Horning, M. (1994). The potential of diet to alter disease processes Nutr Res., 14(12): 1853-1896.

13. Goksel, G and Mehmet Zeki, H. (2008). Evaluation of antidiabetic, antioxidant and vasoprotective effects of Posidonia oceanica extract. J. Ethnopharmacol, 1150 (1, 4): 122-130.

14. Phil, S.O., Se,i L. and Kye, T.L. (2007). Inhibitory effect of glycoprotein isolated from Ulmus davidiana Nakai on caspase 3 activity in 12-O-tetradecanoylphorbol 13-acetate–treated liver cells through the reduction of intracellular reactive oxygen species. Nutr Res, 27: 432–439.

15. Wang, H. and Ng, T.B. (2002). Luffangulin, a novel ribosome inactivating peptide from ridge gourd (Luffa acutangula) seeds. J Life Sci, 11: 899-906.

16. Kettelin, A. Arbos, Ligia, M. Claro., Lucielly Borges., Cid, A.M. Santos., Almeriane, M. and Weffort-Santos. (2008). Human erythrocytes as a system for evaluating the antioxidant capacity of vegetable extracts. Nutr Res, (28): 457–463.

17. Cawthon, D., McNew, R., Beers, K.W. and Bottje, W.G. (1999). Effect of t-butyl hydroperoxide on hepatic mitochondrial function, glutathione, and related thiols. Poultry Science. 78: 114–124.

18. Kaur, P., Kaur, G. and Bansal, M.P. (2008). Upregulation of AP1 by tertiary butylhydroperoxide induced oxidative stress and subsequent effect on spermatogenesis in mice testis. Molecular and Cellular Biochemistry, 308: 177–181.

19. Reddy, B.S., Reddy, B.P., Raghavulu, S.V., Ramakrishna, S., Venkateswarlu, Y. and Diwan, P.V. (2008). Evaluation of antioxidant and antimicrobial properties of Soymida febrifuga leaf extracts. Phytother Res., 22: 943-7.

20. Reddy, B.P., Goud, R.K., Mohan, S.V. and Sarma, P.N. (2009). Antiproliferative and antiangiogenic effects of partially purified Luffa acutangula fruit extracts on human lung adenocarcinoma epithelial cell line (A-549). Curr Trends Biotechnol Pharm., 3(4): 396 – 404.

21. Betul, K.C., Sibel, O. and Buket, A. (2007). Effects of trichlorfon on malondialdehyde and antioxidant system in human erythrocytes. Toxicol *In Vitro,* 21: 1538–1544.

22. Eugene, F., Roth, J.R., Donna, S.B., Jerome, P.V. and Seymour Schulman. (1986). Malarial pigment-dependent error in the estimation of hemoglobin content in plasmodium falciparum-infected red cells: implications for metabolic and biochemical studies of the erythrocytic phases of

malaria am. I Trop Med Hyg., 35(5): 906-911.

23. Stocks, J. and Dormandy, T.L. (1971). The autoxidation of human red cell.Lipid induced by hydrogen peroxide. Br J Haematol., 20.: 95-111.

24. Sun, Y., Oberley, L.W. and Li, Y. (1988). A simple method for clinical assay of superoxide dismutase. Clin Chem., 34: 497–500.

25. Aebi, H. (1984). Catalase *in vitro* Methods Enzymol., 105: 121–126.

26. Srinivasan, R., Chandrasekar, M.J.N., Nanjan, M.J. amd Suresh, B. (2007). Antioxidant activity of Caesalpinia digyna root. J Ethnopharmaco1., 13: 284–291.

27. Masella, R., Benedetto, R.D., Vari, R., Filesi, C. and Giovannini, C. (2005). Novel mechanisms of natural antioxidant compounds in biological system: involvement of glutathione and glutathione-related enzymes. J Clin Biochem Nut, 16: 577–586.

28. Jiao, Y., Wilkinson, 4th J, Christine Pietsch, E., Buss, L., Wang, W, Planalp R, Torti F.M. and Torti, S.V. (2006). Iron chelation in the biological activity of curcumin. Free Radic. Biol. Med. 40, 1152–1160.

29. Jiao, Y., Wilkinson 4th J., Di, X., Wang, W., Hatcher, H., Kock, N.D., D,'Agostino Jr R., Knovich, M.A., Torti, F.M. and Torti, S.V. (2009). Curcumin, a cancer chemopreventive and chemotherapeutic agent, is a biologically active iron chelator. Blood, 113: 462–469.

30. Miquel, J., Bernd, A., Sempere, J.M., Díaz-Alperi, J. and Ramírez, A. (2002). The curcuma antioxidants: pharmacological effects and prospects for future clinical use. A review Arch Gerontol Geriatr, 34: 37–46.

31. Gqlc¸in I., Beydemir, S., Alici, H.A., Elmasta, Y.M. and Bqyqkokuro lu, M.E. (2004). *In vitro* antioxidant properties of morphine. Pharmacol Res, 49: 59- 66.

32. Blaszczyk, J., Kedziora, J., Luciak, M., Sibinska, E., Trznadel, K. and Pawlicki, L. (1994). Effect of morphine and naloxone on oxidative metabolism during experimental renal ischemia and reperfusion. Exp Nephrol, 2: 364 -70.

33. Bedard, L., Young, M.J., Hall, D., Paul, T. and Ingold, K.U. (2001). Quantitative study on the peroxidation of human low-density lipoprotein initiated by superoxide and by charged and neutral alkylperoxyl radicals. J. Am. Chem Soc., 123: 12439-48.

34. Halliwell, B. and Gutteridge, J.M.C. (1985). Free radicals ageing and disease, free radicals in biology and medicine. 2nd ed. Oxford, UK: Clarendron Press. p. 279-315.

Current Trends in Biotechnology and Pharmacy
Vol. 5 (1) I-VII January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

NEWS ITEM

Indian Scientists need to think Big – PM

Prime Minister of India, Manmohan Singh urged Indian scientists to "think big" and "out of the box" for scientific advancement and innovations in the country. Manmohan Singh said in his inaugural address at the 98th Indian Science Congress organized at SRM University, Chennai The time has come to produce Ramans and Ramanujans of the 21st Century as we usher in the decade of innovation". The prime minister stressed on the need for better higher education in the country and claimed that the government was already moving forward in this direction. He said that the government has paid special attention to the growth of our university system. In the past six years, our government has established eight new Indian Institutes of Technology and five institutes" of higher education and research. "The growth of our economy, defense of our people and security of our people depends on scientific and technological competence," and he declared the congress open. "Come back home, the country needs you", that was the message for thousands of young non-resident Indian scholars from Prime Minister Manmohan Singh while inaugurating the 98th session of the Indian Science Congress. Singh also asked universities to open their doors for young scientists studying abroad so that they can chart out a new research career here. Hoping that "The Year of Science in India" would unleash energies of our young scientists, Singh said: "Science is ageless, but our scientists must be younger" The prime minister-a former university teacher in economics-suggested universities should be "more hospitable to creativity and genius, and less captive to bureaucracy and procedure." The biggest conclave of Indian science has 7,500 scientists, including six Nobel laureates, from across the world attended the meeting.

India is a world power, can work together for global peace: Obama

US President Barack Obama said India was now a world power and the two countries can work together on issues like counter-terrorism to promote peace, stability and prosperity for the whole world. The US President said he has undertaken the trip to India to strengthen what is already an incredible friendship that would be one of the defining partnerships of the 21st Century, to build on commercial ties and strengthen cooperation in bilateral relations and international economy. He said that the two countries would be able to focus on issues like counter-terrorism in order to ensure that both the US and India are secure well into the future". Obama said that US and India as two largest democracies share "extraordinary" people-to-people contact and more importantly share the same set of core values. He said during the visit he would be able to continue to build on the commercial ties that the two countries already have and to strengthen cooperation in bilateral relations and international economy. He also referred to the contributions made by the millions of Indian Americans and said they have helped the Americans appreciate the Indian life. "Given that India is not simply an emerging power but now it is a world power, US

Vol. 5 (1) I-VII January 2011. ISSN 0973-8916 (Print), 2230-7303 (Online)

and India can work together to promote international principles, rules, and relations between nations they can promote peace, stability, prosperity not only for just two nations but for the whole world," he said.

India welcomes US decision to lift export control on dual-use technology

Prime Minister Manmohan Singh said that India welcomes US decision to lift export control on dual-use technology. "We have agreed on steps to deepen cooperation on nuclear, defence and other high-end spectrum," he said. He also said that India and the US will start a new Homeland Security dialogue. The prime minister also said that Indian investments helped increase competitiveness of US economy

Give Quality Education to the Youth: President Pratibha Patil

India President Pratibha Patil at the Golden Jubilee ceremony of Kohinoor Institute said that the Indian Government has made plans and is working hard to educate and train over half a billion people by the year 2022. This will be done with a view to build a skillful and self-reliant workforce in the nation with the goal of making India a superpower. With an intention of achieving this goal, she said that India's youth needs quality education which is value-based. As the youth population of India is very high, an educated youth will surely form a strong backbone for the Indian Government and the Indian economy. On the occasion, Patil said, "The Union Government has started National Skill Development Mission to train 50 crore people. With this, the quality of education will improve and in turn the knowledgeable and skilled workforce will help in the development of the nation." Patil hailed the progress made by India in the healthcare sector after independence. She said, "The country has done a good job in the health sector but more work is required. We have to achieve the goal of 'health for everyone' and focus more on rural area, women, children and other weaker sections of the society by providing them easy and cheap health facility." She added that we have over 54 crore young population and by giving them quality education and teaching them values we can use this youth power towards the development of the country.

India Aims to raise Enrolment in Higher Education - President

President Pratibha Patil at the sixth convocation of the Mizoram University said India intended to increase enrolment in higher education from the present 14 million to about 40 million. Patil said "Higher education has been accorded priority in our country. It is our aim to increase gross enrolment ratio in higher education to 30 percent by the year 2020, which means almost tripling the enrolment from the present 14 million to about 40 million". She said "Universities of the country, existing and the new ones, will be responsible for achieving this target". The president said that "Periodic short-term courses for the local community on issues relevant to them like floriculture, mushroom cultivation and medicinal plants can be organized where students also would participate." Bamboo flowering and "Jhum" ('slash and burn' method) cultivation, which had caused setbacks to agriculture in the state, need to be studied in-depth, with the aim to deal with them on a scientific basis. Underlining

the need for undertaking a programme for proper documentation of the state's rich biodiversity, the president said: "It is also important that the university creates certain awareness of the adverse impact of climate change and encourages people to adopt energy efficient and eco-friendly measures."

Meira Kumar proposes University for artisans

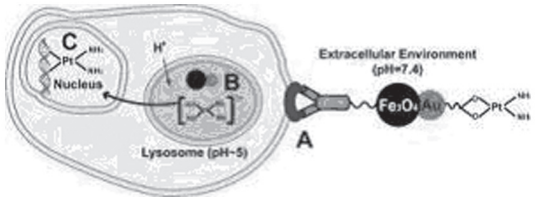
Lok Sabha Speaker of India, Meira Kumar has proposed the UPA government to come up with a University that would cater to the "working class" and offer courses for artisans and craftsmen so that they can obtain formal degrees. Meira Kumar is pitching for the establishment of the University as it would be able to offer formal degrees ranging from Bachelors to Doctorates to artisans and craftsmen that would show for their skills. According to Kumar, such a "Labor University" would also help in saving traditional knowledge and skills which might soon be extinct as India undergoes development. The university would be catering to the section of the society that have a vast variety of skills but cannot obtain regular university degrees.

Sibal discuss educational ties with French Education minister

Human Resource Development Minister Kapil Sibal meets Higher Education and Research Minister Valerie Pecresse in order to discuss the development of educational ties between the two nations as they both go through similar educational reforms and face similar challenges. The French education and research minister was accompanied by French president Nicholas Sarkozy during his three-day visit to India. There was no discussion of government-to-government deals on the agenda. Instead, the meeting between the two ministers was of importance because of the similarities between the higher educational reforms that are being prioritized by the governments of the both India and France. A French official has said that France is a country that despite being developed, is still undergoing challenges to the administration of higher education in their country which is similar to the ones being faced by India. "It is our belief that India and France have much to learn from each other regarding strategies and experiences," he said. The higher educational institutions in France, similar to Indian higher educational institutions, lack autonomy and are mostly governed by the state. They are almost completely funded by the government and require sanctioning before changing the fee structure, introducing or changing courses or hiking salaries of teachers. Higher educational reforms have been made a core component of Nicholas Sarkozy's political agenda, similar to how it has been done in India by Prime Minister Manmohan Singh.

SCIENTIFIC NEWS

Twin Nanoparticle Shown Effective at Targeting, Killing Breast Cancer Cells



Breast cancer patients face many horrors, including those that arise when fighting the cancer itself. Medications given during chemotherapy can have wicked side effects, including vomiting, dizziness, anemia and hair loss. These side effects occur because medications released into the body target healthy cells as well as tumor cells. The trick becomes how to deliver cancer-fighting drugs directly to the tumor cells. Brown University

chemists created a twin nanoparticle that specifically targets the Her-2-positive tumor cell, a type of malignant cell that affects up to 30 percent of breast cancer patients. The researchers created the twin nanoparticle by binding one gold (Au) nanoparticle with an iron-oxide (Fe3O4) nanoparticle. On one end, they attached a synthetic protein antibody to the iron-oxide nanoparticle. On the other end, they attached cisplatin to the gold nanoparticle. Visually, the whole contraption looks like an elongated dumbbell, but it may be better to think of it as a vehicle, equipped with a very good GPS system, that is ferrying a very important passenger. In this case, the GPS comes from the iron-oxide nanoparticle, which homes in on a Her-2 breast-cancer cell like a guided missile. The nanoparticle vehicle "docks" on the tumor cell when the antibody and the antigen become connected. Once docked, the vehicle unloads its "passenger," the cisplatin, into the malignant cell. The combination nanoparticle binds to the Her-2 tumor cell and unloads the cancer-fighting drug cisplatin directly into the infected cell. The result is greater success at killing the cancer while minimizing the anti-cancer drug's side effects. In laboratory tests, the gold-iron oxide nanoparticle combination successfully targeted the cancer cells and released the anti-cancer drugs into the malignant cells, killing the cells in up to 80 percent of cases.

M. Haritha

New Markers for Allergic Disorders Derived from Analysis of Medical Databases

Researchers at the University of Gothenburg, Sweden developed new methods for analysing medical databases that can be used to identify diagnostic markers more quickly and to personalise medication for allergic disorders. They could also reduce the need for animal trials in clinical studies. The study builds on data analyses of freely available medical databases representing studies of countless numbers of patients in the PubMed database, and microarray data in another major database. The use of microarrays is a method that allows scientists to study all 20,000 human genes at the same time for various disorders. Groups of researchers in Gothenburg, Oslo and Rome have developed computational methods to simulate how a change in the interaction between several different genes in the lymphocytes (a kind of white blood cell) controls the immune system. They identified the genes by reviewing abstracts of all 18 million articles included in PubMed, and then constructed a network model of how these genes interact. The researchers then carried out data simulations of how the network model reacted to repeated exposure to particles, which resulted in four reaction patterns, one of which was to suppress the immune system, while the other three were to trigger it in various ways. These methods become increasingly important in the future, as the huge amount of information in medical databases is growing all the time. This information could serve as an important resource for researchers in their endeavours to investigate and verify medical hypotheses. They could also mean quicker and better-designed experiments and their results could generate new knowledge about diagnostic markers or new medicines.

N.Vijaya Sree

New Method for Combating Prostate Cancer

Prostate cancer is the second leading cause of cancer-related death for men. Present treatments for metastatic prostate cancer (cancer cells that spread to other parts of the body) include hormonal therapy, chemotherapy and radiotherapy, which frequently have serious side effects. The well known drug, paclitaxel, exhibits a wide spectrum of anti-tumor activity. However, its therapeutic application in cancer therapy is limited, in part, due to its low water solubility,

making it difficult to effectively deliver the drug to the points needed. It is also known to induce hypersensitivity reactions. Therefore, A novel method of drug delivery to inhibit the growth of prostate cancer cells has been developed that would allow for delivery of effective concentrations of paclitaxel over extended time intervals while minimizing toxicity. It has been shown that the HER2 receptor is over-expressed in prostate cancer cells. It was also known that trastuzumab (an antibody) binds specifically to HER2. But there had been no clinical data indicating that this antibody would provide any relief for prostate cancer patients. Danny Goldstein, a student of Prof. Simon Benita, showed that attaching trastuzumab molecules to the surface of oil droplets in nanoemulsions made possible the targeting of such droplets to cells over-expressing the HER2 receptor. He coupled trastuzumab with emulsions containing the toxic agent paclitaxel-palmitate and evaluated the efficiency of these emulsions in laboratory tests on cancerous prostate cells and on mice with induced prostate cancer. He found that this emulsion compound did not cause a hypersensitive reaction upon injection and even yielded better results than known drug treatments while inhibiting tumor growth substantially.

S.Shankar

EDUCATION

PhD Programs

PhD Opening 2011, Faculty of Life Sciences, University of Copenhagen, Faculty of Life Sciences, Universitetsparken 2, 2100 Copenhagen, Denmark. Applications are invited in the area of Bioinformatics. All applicants interested in doing a Ph.D must hold a Master's degree in Bioinformatics, computational biology, biology or similar and have basic knowledge of biological sequences, experience with algorithm development, Perl or Python (or similar), experience with one (or more) of C, C++ or Java and have general knowledge about RNA structure and folding, RNA gene search algorithms and algorithms for structural alignment or RNAs. Interested students can send their applications by email no later than the 1st February, 2010 together with a brief cover letter, Curriculum Vitae, Basic Data Sheet and publications. More details about ILS PhD Program could be gathered from the website www.life.ku.dk. Applications without Curriculum Vitae and necessary documents will not be processed.

PhD Openings 2011, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, Andhra Pradesh, India. Applications are invited in the areas of various life sciences branches like Botany. Biotechnology, Zoology, Microbiology, Biochemistry etc. All applicants interested in doing a Ph.D must hold a Master's degree in any Life Science branch. Sale of Applications from: 17-01-2011, Cost of application : Rs 500/- Last date for submission of the completed Applications to the Director, Directorate of admissions - Without late fee : 14-02-2011 and With late fee of Rs200/- : 25-02-2011. For more details visit the website at www.anu.ac.in.

OPPORTUNITIES

Central Marine Fisheries Research Institute (CMFRI), Behind High court of Kerala, P.B No. 1603, Cochin-682018. Applications are invited from Indian Nationals to work as Junior Research Fellow (JRF) for one position in a research project funded by MoEF. Title of the project is "Bioinventorisation of coral fishes of South India with special reference to threats and conservation measures". Essential Qualifications are Post Graduation in Mariculture/Marine Biology/ Fishery Science/Zoology/Industrial Fisheries with a minimum of 65% marks required, Training in SCUBA DIVING. Resumes can be mailed to moefcmfri@gmail.com on or before 21st January 201. Date & Time of Walk-in-Interview: 25.1.2011 at 10.00 hrs. Venue: Central

Marine Fisheries Research Institute, Behind High Court of Kerala, P.B. No. 1603, Cochin-682 018. (ATIC Hall). Emoluments: Rs. 12,000 p.m. plus HRA 7.5%. Age limit :35 years for men & 40 years for women (age relaxation of 5 years for SC/ST and women and 3 years for OBC. The candidates are requested to bring their original certificates (or provisional postgraduate certificate) along with a set of attested copies.

Indian Institute of Science Education and Research (IISER), Department Of Biology, Bhopal. Applications are invited from Indian Nationals to work as Project Assistant (PA) for two positions in a research project funded by Department of Biotechnology (DBT) for a period of one year. Essential Qualifications are First class M.Sc. degree in any discipline of Life Science or Organic Chemistry from a recognized University/Institute. Preference will be given to those who have cleared NET (CSIR/UGC)/ NET(LS)/ICMR. Last date for applications is 31 January 2011. Emoluments: Rs. 12,000 p.m. plus HRA 7.5%. Age limit: Applicant should not have completed 28 years of age as on Dec 31, 2010. The application should contain a detailed résumé, photograph, and should follow the format provided with the advertisement. Applicants should send the application by email addressed to Dr. R. Mahalakshmi, Ph. D., Assistant Professor and Ramalingaswami Fellow, Department of Biology, Indian Institute of Science Education and Research Bhopal, ITI (Gas Rahat) Building, Govindpura, Bhopal – 462023. Email: maha@iiserbhopal.ac.in. Webpage: http:// home.iiserbhopal.ac.in/~maha/.

Global Hunt, E-45/6, Okhla Phase-II, New Delhi, 110020. Calls for Sr. Scientist/Associate Scientific Manager, Location: Bangalore. Candidate with 3-7 years experience in downstream processing, Pilot scale or production level experience in purification of biologicals especially monoclonal antibodies, Handling of large scale columns and involved in technology transfer and troubleshooting, Exposure to continuous centrifuge, TFF etc and have completed M.Tech – Chemical engineering and Biotech or Bioprocess Engineering are eligible. For More Details Please go through - Website http://www,globalhunt.in or Contact: Mamta Singh, Global Hunt, E-45/6, Okhla Phase-II New Delhi, NEW DELHI, Delhi, India 110020. Telephone 91-11-46547712.

SEMINARS/CONFERENCES

World Congress on Biotechnology: An international conference on World Congress on Biotechnology was going to held on March 21-23, 2011 at Hyderabad International Convention Centre (HICC), Hyderabad, India organized by OMICS Publishing Group, 5716 Corsa Ave., Suite 110, Westlake, Los Angeles, CA 91362-7354, USA. Abstract can be submitted Online at http:/ /omicsonline.org/biotechnology2011/abstract.php (or) E-mail: biotechnology2011@omicsonline.org, biotechnology2011@omicsonline.biz For further information contact: Organizing Committee, BIOTECHNOLOGY-2011, OMICS Publishing Group 5716 Corsa Ave., Suite 110, Westlake, Los Angeles, CA 91362-7354, USA, Ph: +1-650-268-9744; Fax: +1-650-618-1414; Toll free: +1-800-216-6499. E-mail: avens.solutions@gmail.com, trade.biotechnology2011@omicsonline.org,

International Symposium on Innovations in Free Radical Research and Experimental Therapeutics
&
5th Annual Convention of Association of Biotechnology and Pharmacy

(December 7-9, 2011)

Venue: Karunya University, Karunya Nagar,
Coimbatore – 641 114, Tamilnadu, India

Broad Areas of Focus

Oxidative stress, Free radicals and Antioxidants
Free Radical and Cancer
Life style diseases
Herbal Drugs, Nutraceuticals in experimental therapy
Immunomodulators and Radioprotectors
Immunopharmacology
Toxicology
Translation Research
Pharmaceutical Biology
Drug Metabolism and Drug Interactions
Complementary and Alternative Medicines

Contact for further details

Dr. Guruvayoorappan Chandrasekaran
Organizing Secretary
Department of Biotechnology, Karunya University
Coimbatore – 641 114, Tamilnadu, India
Email - gurukarunya@gmail.com

Registered with Registrar of News Papers for India
Regn. No. APENG/2008/28877

Association of Biotechnology and Pharmacy

(Regn. No. 28OF 2007)

Executive Council

Advisory Board

Executive Members

Printed, Published and owned by Association of Bio-Technology and Pharmacy # 6-69-64 : 6/19, Brodipet, Guntur - 522 002, Andhra pradesh, India. **Printed at :** Don Bosco Tech. School Press, Ring Road, Guntur - 522 007. A.P., India **Published at :** Association of Bio-Technology and Pharmacy # 6-69-64 : 6/19, Brodipet, Guntur - 522 002, Andhra pradesh, India.
Editors : Prof. K.R.S. Sambasiva Rao, Prof. Karnam S. Murthy