

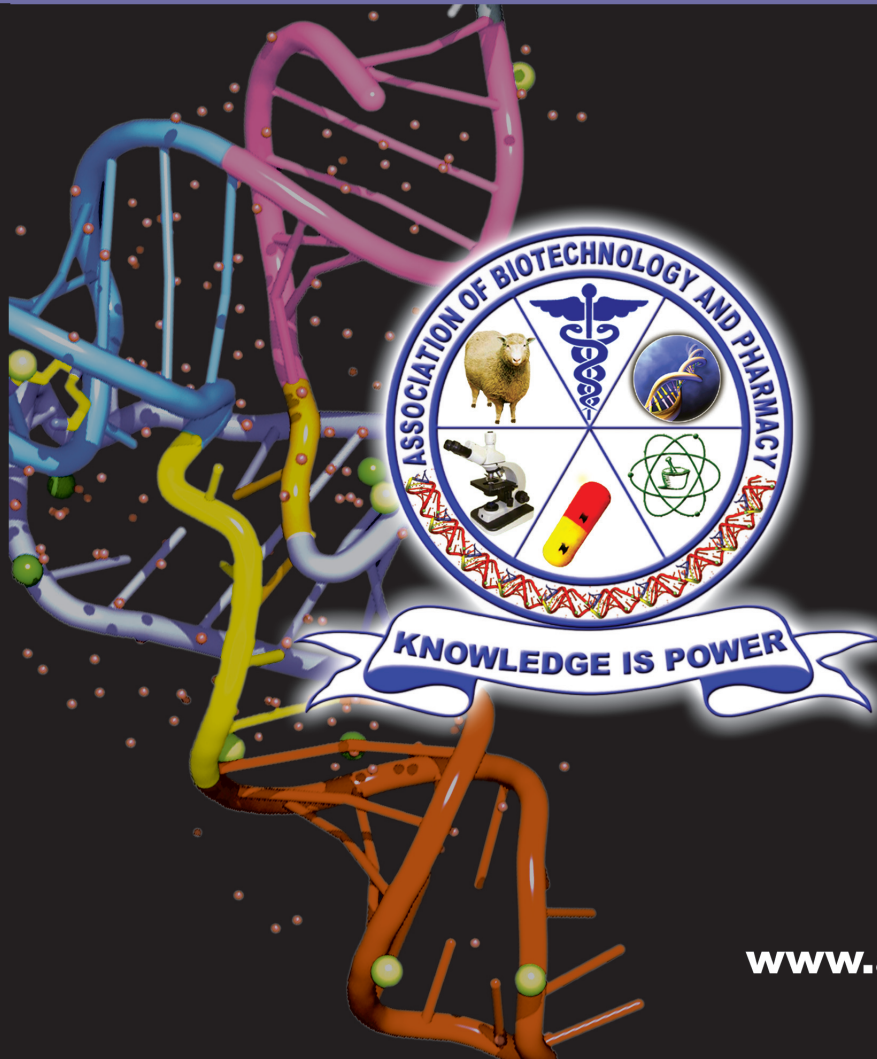
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Information to Authors

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Isolation of Endothelial cells from Goat Umbilical artery by an Optimized Technique and Characterisation by Cellular Morphology and Immunocytochemistry

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Abstract

The aim of the present study is to isolate endothelial cells from goat umbilical artery and to characterise them according to gross morphological and immunological criteria. A relatively simple, effective and less time consuming method was used for the first time to isolate and characterise goat umbilical artery endothelial cells (GUAEC). The isolated GUAECs were identified by cellular morphology and immunocytochemistry. Endothelial cells isolated were homogeneous in nature, oval shaped, with distinct cell borders and centrally located nucleus. It followed a striated array growth pattern. The growth curve of goat endothelial cells exhibited a typical 'S' shape. Von Willebrand factor VII and Ulexeuropaeus agglutinin-I were confirmed by immunocytochemistry. VEGF transcripts were positive from these endothelial cells by RT-PCR. In conclusion, the findings of the present study for GUAEC isolation may allow more detailed analysis of their biological properties and pharmacological characteristics thus promoting successful application of translational research in vascular biology.

Keywords: Endothelial cells, Goat umbilical artery, Ulexeuropaeus agglutinin-I, Von Willebrand factor VII.

Introduction

The vascular endothelium maintains a vascular tone and physiological homeostasis by establishing a balance between the release of endothelium-derived relaxing factors (EDRF) and endothelium derived contracting factors (EDCF) (1). Generally, blood vessels are made of several cell types, thereby making it difficult to elucidate the direct effect of pharmacological agents on the endothelium *in vivo*. *In vitro* culture of endothelial cell is an appropriate method to decipher the effect of such substances on blood vessels. However, endothelial cells from different species and different location differ in terms of their culture requirements (types of growth supplement and types of extracellular protein composition), morphology, cellular junctions, antigenic protein expression, and function (2,3,4). Hence, inspite of several studies on isolation of endothelial cells from bovine (5,6,7), ovine (8,9), porcine (10) and primate (11), very few studies are available on *in vitro* culture of goat endothelial cells (12). Moreover, umbilical artery as a source of endothelial cell in goat is not studied yet. Again, endothelial cell lines in umbilical vessels mediate vasodilation via an endothelium- and NO-sGC independent processes (13), involving the activation of Na⁺, K⁺-ATPase, and subsequent hyperpolarization via K⁺ efflux (14,15) which contradicts the obligatory role of endothelial cells

in mediating acetylcholine-induced vasorelaxation by release of nitric oxide as reported in several pharmacological preparations (16,17,18,19, 20, 21). In view of this heterogeneity of endothelial cells, it would appear logical to study endothelial cells derived from goat umbilical artery. Moreover, umbilical cord acts as a communicating bridge between foetus and mother in mammals and is frequently considered a discarded sample that can be used as a source of endothelial cells. Therefore, the present study aims to isolate culture and characterize the endothelial cells extracted from the umbilical artery of goat.

In previous studies, several investigators have tried to establish human endothelial cell line *in vitro*. Warren Lewis (1922) (22) was the first to publish a study of *in vitro* plasma clot culture of vascular endothelium. About 50 years later, the successful *in vitro* culture of umbilical vein derived endothelial cells was established by Jaffe et al. (1973) (23). These landmark studies laid the foundation for modern vascular research. Later on, several studies reported the importance of *in vitro* culture of endothelial cells for therapeutic investigation (24-32). Abnormality of the endothelial cell causes thrombosis, hepatic vascular defects and atherosclerosis in mammals (33,34). Similarly, endothelial dysfunction served as a key marker for atherosclerosis, preceding angiographic or ultrasonic evidence of atherosclerotic plaque (35). These findings of endothelial studies have profound prognostic implications and are an independent predictor of atherosclerosis disease progression and cardiovascular event rates.

Materials and Methods

Isolation and culture of goat umbilical artery endothelial cells : Isolation of goat umbilical artery endothelial cells was done as previously described by (36) with some modification. The experiment was conducted with prior approval from Institutional Animal Ethical Committee (IAEC), College of Veterinary Science and Animal Husbandry, Odisha, India.

A gravid uterus was collected from the pregnant goat from a nearby slaughter house, followed by sealing of the uterine orifices with sterile catgut and then placed in an autoclaved bag. After collection of the whole uterus, all further processing were done under a BSL-2 laminar hood. The whole uterus was then sprayed with 70% alcohol and washed with Phosphate-buffered saline (PBS) containing 1x penicillin and streptomycin (P/S) for 2 times. The umbilical artery was exposed by opening the umbilical cord transversely, it was ligated at both the ends before cutting and the isolated umbilical artery was collected in a pre-chilled PBS containing P/S.

Before isolation of the endothelial cells, the umbilical artery was again washed in 70% alcohol followed by 3 times washing in PBS containing P/S. End ligation of the artery was then removed and one end of the artery was then clamped with an artery forcep and the other end of the artery was fixed to the opening of a 5 mL syringe (Fig. 1A). Flushing of endothelial cells from lumen was done by infusing trypsin (0.05 %) into the artery lumen through a syringe, incubating at room temperature for 5 min and gently massaging the artery (Fig. 1B). The trypsin containing the cell suspension was collected by removing the clamp from another end by releasing the pressure in 50 ml falcon tube containing complete media to neutralize trypsin (Fig. 1C). The whole trypsinization process was repeated for 3 times. The cell suspension was then washed and centrifuged at 250 x g for 5 min, the cell pellet was resuspended in pre-warmed complete endothelial growth medium (Cat No. M-200, Invitrogen) supplemented with low serum growth supplement (Cat No. S003K, Invitrogen), 1x P/S (Cat No. 15140-122, Gibco) and 10 µg/ml Fungin (Cat No. ant-fn-1). Thereafter, the cells were seeded in 0.05% gelatin (Cat No. TCL109, Himedia) coated cell culture flask and allowed to grow on a humidified incubator of 5 % CO₂ / 95 % air at 37°C.

Histopathology: The trypsinized and untrypsinized umbilical arterial tissues were preserved in 10% formalin buffer solution (Cat

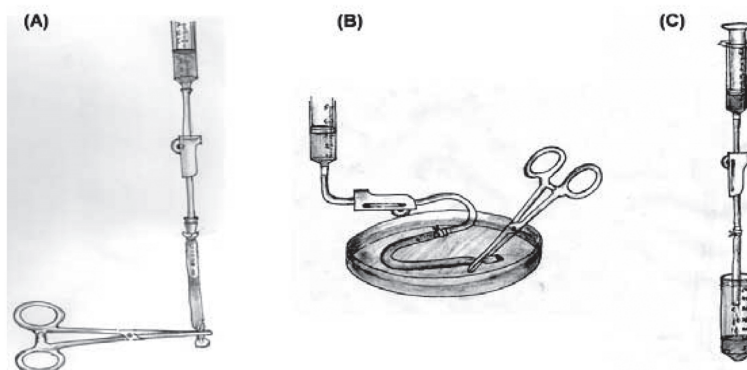


Fig. 1 A-C. Pictorial representation of technique used for endothelial cell isolation from an umbilical artery of *Capra hircus*. (A) One end of the artery was clamped with an artery forceps and the other end of the artery was fixed to the opening of a 5 mL syringe. (B) Trypsin (0.05 %) was infused into the artery lumen through a syringe, incubated at room temperature for 5 min and simultaneously massaged along the artery wall. (C) The trypsin containing the cell suspension was collected by removing the clamp from other end and releasing the pressure into 50 ml falcon tube containing complete media.

No. HT501128, Sigma). For histopathological assessment 5 micron tissue sections were prepared from formalin-fixed paraffin embedded tissue blocks. For haematoxylin and eosin staining, slides were deparaffinized and hydrated with deionized water followed by haematoxylin (Cat No. MHS1, Sigma) staining for three minutes and eosin for two minutes. Next, slides were thoroughly washed in H₂O and dehydrated through sequential alcohol grading then cleared in xylene and mounted with permanent mounting media (Cat No. H-5000, Vector Lab). All the stained slides were observed under Leica DM500 light microscope and representative photographs were taken at 40x magnification.

Immunofluorescence : To make sure that the isolated cells were a pure population of endothelial cells, P₂ passage cells were grown on poly L-lysine (Cat No. P4707, Sigma) coated glass coverslips in six-well plates, and immunostained with Anti-Von Willebrand Factor antibody (Cat No. ab6994, abcam) and Fluorescein labeled Ulexeuropaeus agglutinin-I (Cat No. FL1061, Vector Lab). For Anti-Von Willebrand Factor immunostaining, cells were

washed with PBS for 5 min followed by fixation with ice cold mixture of methanol and acetone (1: 1 ratio) for 10 min at -20 °C. Nonspecific binding was blocked with 2 % BSA in PBS for 30 min. The cells were then incubated with Anti-Von Willebrand Factor antibody (1: 100) for overnight in humidified chamber at 4°C, followed by washing and incubation with anti-rabbit IgG-ALEXA FLOUR 594 (Cat No. A-11080, Invitrogen) 1:500 dilution for 45 min at room temperature. Finally, cells were washed with PBS and mounted with mounting medium with DAPI (Cat No. S36938, life technologies) to stain the nuclei. For Fluorescein labeled Ulexeuropaeus agglutinin-I immunostaining, cells were washed with PBS for 5 min followed by fixation with 4% paraformaldehyde at room temperature. Nonspecific binding was blocked with 2% BSA for 30 min. The cells were then incubated with Ulexeuropaeus agglutinin-I (20 µg/ml) for overnight in humidified chamber at 4°C. Thereafter, cells were washed with PBS for 5 min and mounted with mounting medium with DAPI to stain the nuclei. Stained slides were checked under Leica DMIL LED microscope and images were captured at 20 x magnification.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) : Total RNA was isolated from goat umbilical artery and goat endothelial cells using Trizol reagent (Cat No. 4368814, Applied Biosystems) according to the manufacturer's instructions and dissolved in 50 μ l of RNAase free water. First-strand cDNA synthesis was performed from 2 μ g of total RNA using High Capacity cDNA synthesis kit according to the manufacturer's instructions. The semiquantitative analysis of transcripts encoding for VEGF was carried out using sense and antisense PCR primers, GAPDH served as an internal positive control. Gene specific primers were designed for Vascular Endothelial Growth Factor (VEGF) and Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) from the corresponding NCBI Reference Sequence (NM_001287034.1; XM_005680968.1) using online Primer 3 software. Primers for *VEGF* [Forward: GAAAGATAAAGCAAGGCAAG, Reverse: GTGTTTTTGCAGGAACATTT] and *GAPDH* [Forward: GAGATCAAGAA GGTGG TGAA; Reverse: CATACCAGGA AATGAGC TTG] were commercially procured from Eurofins Genomics, India. 2 μ l of each cDNA samples were used as a template for performing PCR reaction using Taq Polymerase (Invitrogen, US). The following cycling conditions were employed for all the genes: pre-incubation at 95 °C for 5 min, denaturation at 94 °C for 30 sec, annealing at 55 °C for 20 sec and extension at 72 °C for 30 sec. PCR products were resolved on 2 % agarose gel at 60 V, and the presence of amplicons were documented using gel documentation system. The data shown were obtained with 30 PCR cycles.

Results

Effect of trypsin digestion on umbilical artery histology: To check the effect of trypsin digestion on goat umbilical artery, tissue samples were collected on 10 % formalin buffer before and after trypsin digestion and processed for histological analysis. The inner lining of endothelium to the tunica intima was lost leaving intact elastic lamina compared to the umbilical artery without trypsin

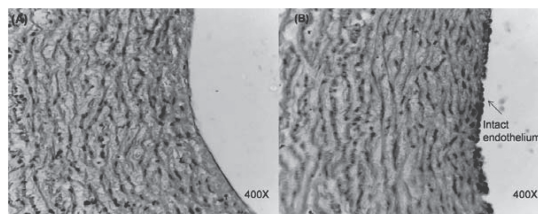


Fig. 2. Photomicrograph revealing the cross-sectional view of endothelium-denuded (A) and endothelium-intact (B) GUA ring under magnification of 400 X.

digestion (Fig. 2A-B).

Cell morphology studies : After 3 days of endothelial cells isolation, cells were growing in monolayer clumps and very tightly attached to each other. After the first round of trypsinization (P1 passage), endothelial cells were no more growing in clumps but in a whirling pattern. They were mostly homogeneous in nature, oval shaped with distinct cell borders and centrally located nucleus (Fig. 3A-D).

The endothelial cells of goat umbilical artery begin to adhere after 9 h of post seeding,

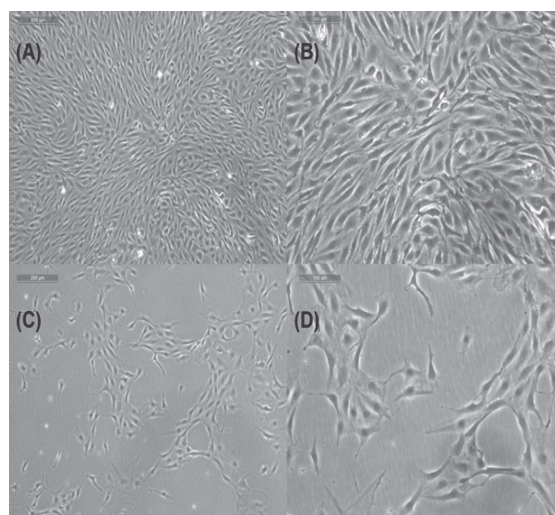


Fig. 3. Cell morphology of endothelial cell line isolated from Goat umbilical artery in a high confluent plate with 10x (A) and 20x (B) resolution and low confluent plate with 10x (C) and 20x (D) resolution.

first with small, rounded and non-uniform cell architecture followed by changing their morphology into spindle shape 72 h later (Fig. 3). Gradually, these cells grew non uniformly for 5 days and reached at 80% confluency after 7 days.

Growth curves and cell viability analysis : These cells entered to an exponential growth phase on the 4th day and reached a growth plateau on the 11th day of passage (Fig. 4). The growth curve of goat endothelial cells exhibited a typical 'S' shape. The latent phase of growth curve was about for 72 h, which was a result of trypsinization. The exponential phase got started on around 4th day with stationary phase on around 11th day. Finally, cell growth ceased as a result of contact inhibition from the day 12.

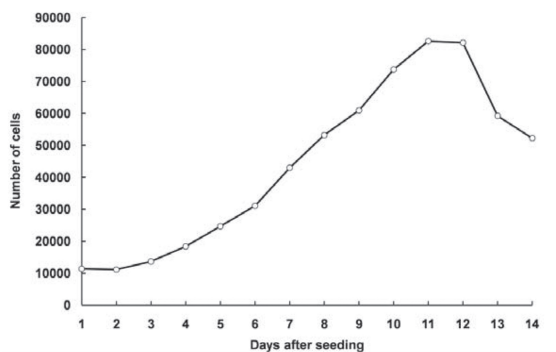


Fig. 4. Growth curve and cell viability analysis of endothelial cells from goat umbilical artery.

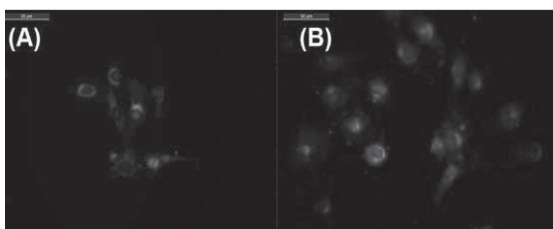


Fig. 5. Expression of Ulexeuropaeus agglutinin-I (A) and vWF (B) in endothelial cells isolated from goat umbilical artery as determined by immunofluorescence (40x).

Molecular characterization of goat umbilical artery endothelial cells : The detection of Von Willebrand factor VII and Ulexeuropaeus agglutinin-I confirmed that these cells have an endothelial origin (Fig. 5). Moreover, we were also able to detect VEGF transcripts from these endothelial cells by RT-PCR (Fig. 6).

Discussion

During the whole process of endothelial cells isolation and culture, contamination of samples might happen at multiple steps. Particularly, samples isolated from abattoir needs extreme precautions. We noticed that the collection of the whole uterus with a suture at the uterine neck and transportation of the whole uterus to the lab for further processing has substantially reduced contamination. Dipping the same into 70% alcohol before opening the uterine wall also helped to kill most of the microbes present on the uterine surface. Though a gravid uterus is believed to be sterile; however, the presence of some microbes at sub-clinical level can't be ruled out. Therefore, collection of the umbilical cord in antibiotic containing PBS

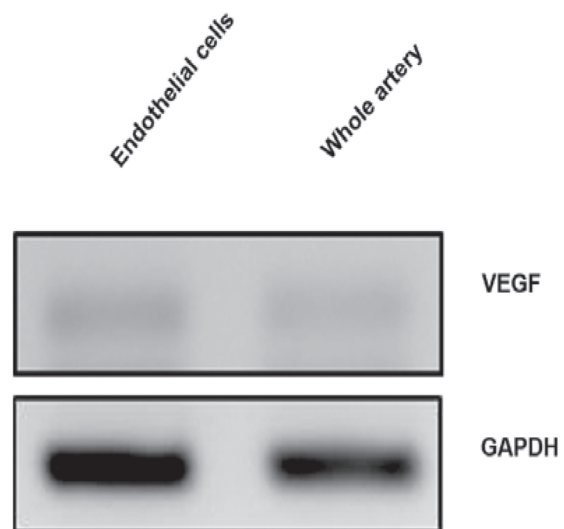


Fig 6. RT-PCR expression of VEGF transcripts from endothelial cells and whole artery.

and washing of the ligated umbilical cord with 70% ethanol significantly reduced the level of infection. We believe that before isolating endothelial cells, washing of the umbilical artery (ligated at both the ends) with alcohol followed by antibiotic containing PBS which not only helps in reducing the microbial load but also kills the umbilical matrix cells that are present on the outer surface of the artery, and this might have an additive advantage in reducing contamination of stromal cells in the isolated endothelial cells. During all the steps of alcohol washes, precautions were taken not to leave any residual alcohol that might come in contact with endothelial cells and compromise their viability.

Umbilical artery is opaque and more turgid in comparison to the vein, both encapsulated inside whartons jelly. Moreover, histology of the tissue also showed thicker tunica media, which is a characteristic of an artery. Similarly, the presence of endothelial cells in the inner luminal membrane of umbilical artery (without trypsinization) and absence of this lining in the photomicrograph of umbilical artery injected with trypsin clearly shows the effectiveness of cell dissociation and isolation process (Fig. 2A). These findings suggested that trypsin was only able to detach the endothelial cells, keeping smooth muscle cells unaffected (Fig. 2B). Selective removal of endothelial cells increases the chance of isolating pure cells.

Endothelial cells are flat, polygonal cells that are elongated in the direction of blood flow and, on cursory examination, appear identical in different parts of the body; but, in reality, there is significant heterogenic diversity. When grown in the culture medium, the isolated endothelial cells form a typical cobblestone pattern (37). The intensity of cobblestone pattern expression is also variable depending on the site of origin of the cells and the growth medium (38). Initially, for first few days these endothelial cells represented a cobblestone morphology, which gradually exhibited a more scattered and disarray pattern (data not shown). Gradually, these cells grew non-uniformly for 5 days and reached at 80%

confluency after 7 days. The growth pattern of endothelial cells is completely distinct from fibroblast. The later cells exhibit a striated array growth pattern whereas former cells displayed a disarray patterns. The morphology of endothelial cells in the present study is similar as to that of cells reported by previous investigators (23).

The growth curve of goat endothelial cells exhibited a typical 'S' shape, with exponential phase on 4th day, stationary phase on 11th day, and cessation of cell growth around 12th day. The latent phase of growth curve was about for 72 h, which might be a result of trypsinization. A similar kind of slow growth rate of endothelial cells has also been reported in infantile haemangioma (39) and human bone marrow (40). These results indicated that the endothelial cells from the umbilical artery of goat maintain normal characteristics throughout the culture. Though, much difference was not noticed in the morphology of early and late passage cells, but we observed a slower growth rate of cells above 10th passage of these cells (data not shown). The observed slower growth rate of late passage primary endothelial cells might be due to replicative senescence and/or due to an increase in apoptosis rate. von Willebrand factor (vWf) is one of the important endothelial cell restricted marker widely used by researchers in *in vitro* and *in vivo* experiments (41). It is a glycoprotein, synthesised and stored within endothelial cells and megakaryocytes (platelet precursors) in electron dense Weibel-Palade (WP) bodies (42-44). Similarly, lectin (*Ulexeuropaeus* agglutinin-I) a multifunctional globular protein is generally used as a suitable marker for endothelial cells (45). The detection of vWfVII and *Ulexeuropaeus* agglutinin-I confirmed that these cells have an endothelial origin.

Vascular Endothelial Growth Factor (VEGF) was initially called vascular permeability factor because its main effect appeared to increase the permeability of blood vessels (46). It is widely accepted that Vascular Endothelial Growth Factor Receptor (VEGFR2 or Flk 1) is the main receptor involved in endothelial proliferation,

survival, migration, permeability and vascular tube formation (47). Therefore, the presence of VEGF transcripts confirmed that these cells are genetically characterized endothelial cells.

In conclusion, we have successfully established for the first time a relatively simple, effective and less time consuming method for the isolation and characterisation of the endothelial cell from goat umbilical artery using tissue culture method. Studies of endothelial cell culture and their will form a startup for therapeutic strategies in the arena of vascular biology.

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Pharmacokinetics of Ivermectin (Ivermic Super®) following Single Dose Subcutaneous Administration in Cattle Calves

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Abstract

The present study was undertaken to evaluate the pharmacokinetics of Ivermectin (Ivermic super®) 0.2 mg/kg b.wt in cattle calves. The study was conducted in four cross-bred male cattle calves (1.0-1.5 yrs in age, weighing 110±5 kg). The plasma concentration of ivermectin was determined by HPLC. The decay in plasma concentration of drug was biexponential in cattle calves. The C_{max} value of 28.18 was obtained at T_{max} of 4.7 days in cattle calves, following SC administration of Ivermic super®. The elimination half life (Beta HL), volume of distribution (V_{1F}) and AUC were calculated as 19.18 days, 2.64 L.kg⁻¹, 370.15 day.ng/mL in cattle calves, following SC administration of Ivermic super®. A dosage regimen of 0.2 mg/kg at 14 days interval is recommended in cattle.

Key words: Cattle calf, Ivermectin, Pharmacokinetics, Subcutaneous

Introduction

Ivermectin was the world's first endectocide, forerunner of a completely new class of antiparasitic agents, potentially active against a wide range of internal and external nematodes and arthropods. It is a semisynthetic derivative of avermectin B1 and consists of an 80:20 mixture of the equipotent homologous 22, 23-dehydro B1a and B1b. This antiparasitic agent, developed by Merck & Co., is frequently used in veterinary medicine, due to its broad spectrum of activity, high efficacy and wide margin of safety

(1, 2). It is a highly lipophilic substance that dissolves in most Organic solvents, but is practically insoluble in water (0.0004% m/v). Ivermectin was first marketed in 1981 by Merck Sharp and Dohme as an antiparasitic agent (2), and it remains the leading worldwide antiparasitic agent for livestock.

At a dosage of 0.2 mg/kg, Ivermectin has been shown to be highly effective against at least seven species of gastrointestinal nematodes including the adult and larval stages of *Ostertagiaspp*, *Trichostrongylus spp.* (3) *Oesophagostomum spp.* (4), *Haemonchus spp.* (3), as well as the lung worm *Dictyocaulus viviparus* (5). Minimum effective concentration- In cattle, plasma concentrations of 0.5-1 ng/mL are required for optimal anthelmintic activity against most gastrointestinal and lung nematodes (6) plasma concentrations of 0.5 ng/mL also control *Hypoderma spp.* Flies (7). Maximum effective concentration: Single subcutaneous injection up to 6 mg/kg. Neurological symptoms at 8mg/kg body weight in cattle calves (8)

It has exceptional potency against endo- and ectoparasites at extremely low doses (doses recommended are expressed as µg/kg), this accounts for its large margin of safety. Toxicity to ivermectin is rare across animal species. The signs of toxicosis are mydriasis and depression, followed by ataxia, recumbency, and death. It has

no adverse effects on breeding performance. Many rumino-reticular delivery systems, as well as oral, topical, and injectable formulations of ivermectin, are currently available at the dosage recommended by manufacturers, namely, 200 µg/kg in ruminants (500 µg/kg for topical application) and equines, 300 µg/kg in pigs, and 6 µg/kg in dogs. Its use has revolutionized the treatment of nematode and arthropod parasites in animals and has provided hope for the control or even eradication of filariases in humans (9). All important gastrointestinal and lung nematodes are susceptible to the drug, including sensitive mites (10), ticks (11), biting flies, and parasitic dipteran larvae (12).

The pharmacokinetic parameters of Ivermectin vary extensively and in accordance with many factors that can all influence the drug's plasma concentration. These factors, which include the species, route of administration, vehicle used in the commercial formulation, bodyweight, body condition, physiological status, and amount and type of nutrition, create difficulties when extrapolating data from one species to another and should be considered in clinical practice in order to achieve effective levels that will last as long as possible. The present study was undertaken to elucidate disposition kinetics and dose regimen of Ivermectin in cattle calves. The purpose of the present study was to determine the pharmacokinetics and dosage regimen of Ivermectin following single dose subcutaneous (SC) administration.

Materials and Methods

Experimental animals: The present study was conducted in four cross-bred male cattle calves (1.0-1.5 yrs in age, weighing 110±5 kg). Cross-bred male cattle calves for this study were procured from Instructional Dairy Farm (IDF), of college of veterinary and animal sciences, Pantnagar. All these animals were housed in animal house of department of Veterinary Pharmacology and Toxicology and kept on pre-experimental period for one month before the commencement of experiment to acclimatize

them to new environment. Physical and clinical examination was done before the start of experiment. The animals were reared under uniform management and husbandry conditions, maintained on standard ration and water provided *ad libitum*. The animals were kept under constant observation before the commencement of the experiment.

Ethical approval: Institutional Animal ethics committee principles were followed strictly throughout the course of this study. Animals were handled gently and carefully. Deworming was done one month before the start of experimentation with the help of fenbendazole which was given at the rate 5mg/kg body weight.

Instruments used: HPLC system (Shimadzu Corporation, Kyoto, Japan, Model RF-10A_{XL}, LC10AT) comprised of double plunger pump, Rheodyne injector with 20 µl loop, Fluorescence detector, C₁₈ reverse phase column (Lichrospher 100 RP-18, 5µm (125 mm x 4 mm) with a guard column (Lichrospher 100 RP-18e (5µm), Merck Kga A, 64271 Darmstadt, Germany, Hamilton Syringe, Manufactured by Hamilton (Co., RE No. Nevada, USA) volume 20µl, to load the sample into the injector, Refrigeration Centrifuge machine

Drugs and Chemicals used: Pure technical standard Ivermectin (Sigma Aldrich Ltd), Methanol (HPLC grade), Acetonitrile (HPLC grade), Water (HPLC grade), Trifluoroacetic anhydride (Avra[®]), 1-methyl imidazole (HIMEDIA[®]), Heparin (LobaChemie[®]), Acetic acid (HPLC grade)

Estimation of Ivermectin: Injectable formulation of Ivermic super[®] (M/s Montajat Vet. Pharmaceuticals Ltd.,) was used in the study. Pharmacokinetic study of Ivermectin was conducted following a single dose (0.2 mg kg⁻¹) Subcutaneous (SC) injection in neck region of cattle. The blood samples were collected from jugular vein of calves in heparinized microcentrifuge tubes by disposable plastic syringes at time interval of 0 min, 15 min, 30 min,

1h, 3 h, 6 h, 12 h, 1 day, 3 day, 6 day, 9 day and up to 42 days. The blood samples collected in heparinized tubes following administration of Ivermectin were centrifuged at 5000 rpm (15 min) for separation of plasma. The plasma thus obtained was collected in micro centrifuge tubes and stored at -20°C till further analysis. An intervening wash out period of one month was given to all the animals before commencement of new experiment.

Extraction and Derivatization of Ivermectin from plasma samples:

Extraction of plasma samples was carried out as per the method described by Perez *et al.* (13) and Na-Bangchanget *al.* (14) with slight modifications. 1 ml of acetonitrile and 0.25 ml of deionised water was added to 1 ml of plasma sample, vortex mixed for 20-30 seconds and centrifuged at 12,000g for 12 minutes (4°C). The supernatant was transferred to a clean tube and evaporated to dryness under a stream of nitrogen at 30-40°C. The residue was subjected to derivatization according to the method of De Montigny *et al.* (15). The residue was dissolved in 100 µL of 1-methylimidazole solution in acetonitrile (1:2 v/v). To initiate the derivatization, 150 µL of Trifluoroacetic anhydride solution in acetonitrile (1:2 v/v) was added. After completion of the reaction (< 30 s), an aliquot (20 µL) of this solution was injected directly in to HPLC. The isocratic mobile phase consists of acetic acid (0.2% in water), methanol, and acetonitrile (4:32:64, v/v/v). The flow rate was kept at 0.7 ml.min⁻¹ at a temperature of 30°C with fluorescence detection at an excitation wavelength of 365 nm and an emission wavelength of 475 nm. Ivermectin was quantified from its respective retention time.

Preparation of Standard Curve: The standards for Ivermectin were made by dissolving 1 mg of pure Ivermectin in 1 ml of methanol from which concentrations of 100, 50, 25, 10, 5, 1 ng.ml⁻¹ were made in methanol. 20 µl of these concentrations was injected into HPLC system and quantified under the HPLC conditions mentioned above. The standard calibration curve

for Ivermectin was obtained by plotting concentrations *versus* mean of the peak areas obtained for their respective standards. The limit of quantification (LOQ) for Ivermectin was 1 ng.ml⁻¹. The method for Ivermectin was found to be linear and reproducible in the concentrations ranging 100 to 1 ng.ml⁻¹. A retention time of 24.1 min for Ivermectin was observed (Fig. 1).

The concentrations of the Ivermectin standard were made in drug free plasma as 100, 50, 25, 10, 5, 1 ng.ml⁻¹ applying serial ten times dilution (100 µl standard + 900 µl drug free plasma) of 1000, 500, 250, 100, 50, 10 ng.ml⁻¹ of standard in methanol, in equal volumes of drug free plasma, each time. The extraction from plasma was done by the same procedure as mentioned earlier. The areas obtained by chromatography were plotted against concentration in order to get a standard calibration curve. Recovery of the drug was done by deproteinizing the plasma having above mentioned drug concentration. Recovery percent of Ivermectin from plasma was 83.2. The method was linear in the range of 1-100 ng/ml with correlation of coefficient of 0.995. The intra and inter coefficient of variations were 6.5 and 7.2%, respectively.

Pharmacokinetic analysis of data: The plasma concentrations and pharmacokinetic variables of Ivermectin were expressed as mean ± S.E. The pharmacokinetic analysis of the plasma concentration obtained following SC administration of Ivermectin in this study was done by pharmacokinetic software "PhasightWinNonlin" version 5.3.

Results

The plasma concentration-time profile following single dose (0.2 mg.kg⁻¹) subcutaneous administration of Ivermectin (Ivermic super®) in male cattle calves is depicted in Table-1 and Fig. 2. The plasma samples were collected up to 42 days. The concentration of Ivermectin could be detected only up to 33 days. The mean peak plasma concentration was 28.188±0.5 ng.ml⁻¹

attained at 4.7 days post administration which decreased slowly to a minimum of 2.110 ± 0.03 ng.ml⁻¹ at 33rd day. The observed C_{max} value mentioned in table 1 is 41.05 ng/ml, whereas the value mentioned in text is 28.18 ng/ml which is the calculated value as indicated in table 2. The observed T_{max} value mentioned in table 1 is 6 th day, whereas the value mentioned in text is 4.77 th day which is the calculated value as indicated in table 2

The pharmacokinetic parameters describing the disposition kinetics of Ivermectin (Ivermicsuper®) following single dose (0.2 mg.kg⁻¹) subcutaneous administration are presented in Table-2. A two-compartment model adequately ($r = 0.89$) described the plasma concentration-time profile of Ivermectin in male cattle calves following single dose subcutaneous administration.

The mean values of zero-time intercept of distribution phase (A) and elimination phase (B) in the present study were calculated to be 3465.89 ± 509 ng.mL⁻¹ and 0.315 ± 0.04 ng.ml⁻¹, respectively. The elimination rate constant of first phase (K_{10}) and second phase (Beta) were 0.203 ± 0.001 and 0.037 ± 0.004 day⁻¹, respectively, with an elimination half-life of first phase (K_{10_HL}) and second phase (Beta_HL) calculated as 3.394 ± 0.01 and 19.180 ± 2.71 day, respectively. The transfer rate constant from central to peripheral compartment (K_{12}) and from peripheral to central compartment (K_{21}) were 0.002 ± 0.0006 and 0.038 ± 0.004 day⁻¹, respectively. The volume of distribution of central compartment (V_{1_F} ; when fraction of drug absorption is not known), and volume of distribution of peripheral compartment (V_{2_F} ; when fraction of drug absorption is not known) were 2648.6 ± 47.01 and 212.999 ± 77.9 ml.kg⁻¹ respectively. The clearance from central compartment (CL_F ; when fraction of drug absorption is not known) and clearance from peripheral compartment ($CLD2_F$; when fraction of drug absorption is not known) were estimated as 540.834 ± 9.7 and 7.188 ± 1.65 ml.kg⁻¹.day⁻¹ respectively. The rate constant of distribution phase (a) was 0.207 ± 0.007 day⁻¹ with

distribution half-life (Alpha_HL) of 3.34 ± 0.01 day. The rate constant of absorption phase (K_{01}) was 0.212 ± 0.001 day⁻¹ with absorption half-life (K_{01_HL}) of 3.262 ± 0.01 day. The mean area under curve (AUC) was 370.15 ± 6.6 ng.ml⁻¹ day. AUC values, the time interval was 0-”

Discussion

A two-compartment model adequately described the plasma concentration-time profile of Ivermic super® in cattle calves following single dose (0.2 mg.kg⁻¹) SC administration in the present study. The values of C_{max} in the present study were 28.18 ng.ml⁻¹ in cattle calves following SC administration of Ivermic super®. These findings could be well corroborated with C_{max} (33.1 ng/mL) in cattle (16), 32.7 ng/mL in cattle (propylene glycol: glycerol-formal vehicle 60:40 v/v) following SC route of administration (17, 18) have also reported C_{max} of 28.5 ng/mL in cattle by intraruminal route of administration. The C_{max} in the present study could also be compared with other species viz sheep (32.2 and 30 ng/ml (19) and (20), respectively) and pigs (28.4 ng/mL (21).

A lower peak plasma concentration (C_{max}) as compared to the present study has been observed by other workers in cattle using different formulations (22.6, 12.2 and 16 ng/mL (6, 22, 23), respectively). Sheep (24.1, 25.8 and 12.5 ng/mL (24, 25 and 26 respectively). Goats (21.8 and 9.3 ng/mL (27) and (28) respectively). However, higher peak plasma concentration (C_{max}) level compared to present study has been reported in cattle (42.8, 133.2, 40 and 39 ng/mL (29, 30, 31 and 18 respectively). Pigs (39.6 ng/mL (32), horses (51.3 ng/mL (33) and dogs (44.3 ng/mL (34). The higher peak plasma concentration (C_{max}) in the present study may be attributed to the formulation (propylene glycol: glycerol-formal 60:40 v/v) as a vehicle in the injectable product (Ivermic super®). Injectable product has the advantage that higher maximum plasma concentration are achieved and, thus presumably (by gradient diffusion) greater skin penetration and ectoparasiticidal activity, whereas the oral product is more easily

administered and may have greater activity against some intestinal nematodes.

The value of T_{max} in the present study was 4.77 days in cattle calves following SC administration of Ivermic super[®]. These findings could be well corroborated with T_{max} (4 days) in cattle (29). A lower value of T_{max} compared to the present study has been reported in cattle (2.25 and 2.32 days (6) and (16), respectively), sheep (1.7 and 1.24 days (35 and 26) respectively) and goats (3 and 2.85 days (27) and (36), respectively). However, higher level of T_{max} (15.1 day) compared to present study has been reported in cattle by sustained release bolus through intraruminal route (18).

The mean elimination half-lives in the present study were 19.18 days in cattle calves following SC route of administration of Ivermic super[®]. These findings could be well corroborated with mean elimination half-life of 17.2 days in cattle by subcutaneous route (29). However, lower mean elimination half-lives compared to present study has been reported in sheep (9.6 days (37), goats (7.4 days (27) and pigs (1.18 days) (38). The higher mean elimination half-life in the present study could be due to low water solubility of Ivermectin and its pre-cipitation in SC tissues favour slow absorption from the injection site, resulting in a prolonged presence in the bloodstream. Retention in the body is also increased due to slow absorption from the injection site.

Volume of distribution is a measure of extravascular distribution of a drug and higher values would always be advantageous for therapeutic purposes indicating excellent tissue penetration. In the present study, the volume of distribution (V_{1F}) was 2.64 L.kg⁻¹ in cattle calves following SC administration of Ivermic super[®]. These findings could be quite similar with volume of distribution (2.7 L.kg⁻¹) in cattle (39), goats (2.8 L.kg⁻¹) (27) and pigs (2.7, L.kg⁻¹) (38). Due to its high lipophilic nature, Ivermectin is extensively distributed with wide volume of distribution (V_d) in all species. Inter-individual variation can also

be attributed to differences in body condition, age, sex, and physiological status (35). A lower Volume of distribution (1.2 L.kg⁻¹) compared to present study has been reported in cattle (16). However, higher volume of distribution (3.4 L.kg⁻¹) compared to present study has been reported in cattle (29), sheep (5.3, 3 and 12.8 L.kg⁻¹) (40, 37 and (35) respectively).

The AUC is the parameter that integrates both time and intensity of drug concentration. The area under the concentration time curve characterizes the relative availability of drug in the body (41). The area under curve (AUC) in the present study was 370.15 ng.ml⁻¹day in cattle calves following SC administration of Ivermic super[®] respectively. These findings could be well corroborated with AUC (328.8 and 381.1 ng.ml⁻¹day (16) and (23) respectively) in cattle. However, higher AUC compared to present study has been reported in cattle (459 and 595.1 ng.ml⁻¹ day (29) and (23) respectively), sheep (440 ng.ml⁻¹ day (40), horse (550.4 ng.ml⁻¹ day (11). A lower area under curve (AUC) compared to present study has been reported in cattle (189 and 278 ng.ml⁻¹day; (6) and (31) respectively).

Plasma clearance of drug is the volume of the blood or plasma cleared of drug by metabolism and excretion per unit of time. It is a better index of efficiency of drug elimination than half-life as it gives the clearance of drug from blood per unit of time (10). The value of clearance in this study was 0.54 L.kg⁻¹ day⁻¹ in cattle calves following SC administration of Ivermic super[®]. These findings could be well corroborated with plasma clearance (0.48 L.kg⁻¹.day⁻¹) in cattle (29) and in sheep (0.56 L.kg⁻¹.day⁻¹) (40). However, higher plasma clearance compared to present study has been reported in sheep (1.11 and 3.24 L.kg⁻¹.day⁻¹) (37) and (35) respectively), goats (1.56 L.kg⁻¹.day⁻¹) (27) and pigs (4.15 L.kg⁻¹.day⁻¹) (38). A lower plasma clearance compared to present study has been reported in cattle (0.27 and 0.35 L.kg⁻¹.day⁻¹) (23) and (39) respectively).

Ivermectin persists in the body for a prolonged period, not only due to low plasma clearance but also due to the accumulation in fat tissue. Plasma clearance appears to be greater in pigs than in (goats > sheep > cattle) polygastric species(9).

Conclusion

A dosage regimen based on the pharmacokinetic data obtained following SC administration of Ivermectin (Ivermic super®) in adult male cattle was calculated with therapeutic concentration of 1 ng.ml⁻¹ at dosing intervals of 7, 14 and 21 days. Priming doses of 0.18928, 0.24523, and 0.31773 mg.kg⁻¹ and maintenance doses of 0.04318, 0.09915 and 0.1715 mg.kg⁻¹ was calculated (C_{min}^{ss}=46.30, 20.17 and 11.66 ng.ml⁻¹ respectively, C_{max}^{ss}=59.99, 33.86 and 25.34 ng.ml⁻¹, respectively) at 7, 14 and 21 days interval, respectively.

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Validation of Seedling Vigour QTLs in Rice (*Oryza sativa* L.)

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Abstract

Seedling vigor is an important trait for better crop establishment, wherein limited or excess moisture prevails under unfavorable soil conditions. Many high yielding indica cultivars of rice (*Oryza sativa* L.) performs well under transplanted conditions, but fail to establish under direct seeded conditions. Keeping in view, present study focused to validate reported QTLs for seedling vigor in a set of forty seven indica cultivars wherein, eight QTLs dispersed on chromosomes 1,3,5,6,7,10 and 11 respectively were selected, among them six QTLs (qGR-1, qGP-6, qFV-3-2, qFV5-2 and qFV-10) were present in genotypes screened, which showed high seedling vigour morphologically. The genotypes namely Dinesh, Pooja and Sabita showed the presence of multiple QTLs indicating that these genotypes have inherent capacity for early emergence with high seedling vigour and it may be due to the presence of these putative QTLs associated with different seedling vigour traits. It is evident that cultivars growing under aerobic conditions are having good seedling vigor than high yielding lowland cultivar. These cultivars can be utilized in breeding programme for development of genotypes which may be desirable trait for direct seeded conditions.

Key words: Rice, SSR markers, Seedling vigor, QTL.

Introduction

Better seedling vigor in direct seeded upland and lowland aerobic rice system is an

important trait for better yield under limited water conditions, which is directly related with better crop establishment. Seed vigor is an important characteristic related to seed quality and determines the rate of early embryo growth, germination, emergence and photosynthetically efficient seedlings. Uniform germination, faster canopy development, broader leaves at the surface level are the desirable traits for rice genotypes in aerobic rice system. Seeds with strong vigour may significantly improve the speed and uniformity of seed germination and the final percentage of germination and lead to perfect field emergence, good crop performance and even high yield under suboptimal conditions (4).

Earlier studies reported that rapid crop establishment directly related to weed suppression ability; the major trait needs to be improved under direct seeded system. It is reported that weeds are the major constraints for yield losses worldwide with an average of 16% yield reduction in tropics and subtropics (13). Rapid root and shoot growth, i.e., measured as plant height are the closely related with seedling vigor (16). Screening of germplasm for long coleoptile length and mesocotyls are directly related to seedling vigor (11). Measurement of seedling vigor at early stage is a better option for later selection of genotypes with agronomic traits and yield. However, high yielding cultivars lacks this trait when grown under unfavourable environments and these traits can be incorporated in the genetic background of elite high yielding varieties.

Redona and Mackill (14, 15) surveyed rice germplasm for its genetic variation in seedling vigour by measuring shoot weight, shoot length and coleoptile length across three environments and found significant genetic differences exists for all seedling vigour traits. They also found that seedlings of temperate *japonica* and *indica* varieties were more vigorous than tropical *japonica* types.

DNA markers and genome mapping techniques have become powerful tools for genetic analysis of quantitative trait loci (QTLs) controlling complex traits (18) and have been extensively used for genetic dissection of agriculturally important traits in rice (8). A large number of QTLs are reported for coleoptile length, germination index, germination rate, shoot length, fresh shoot weight, dry shoot weight, tiller number/plant, mesocotyl length, root fresh weight and root dry weight etc which directly or indirectly contributed to seedling vigour. Determination of the number, location and magnitude of effects through SSR linkages of QTLs underlying seedling vigour could elucidate the genetic basis of the trait leading to improved breeding and selection efficiency. However, the QTL mapping for entirely new population is more time consuming and resource intensive. Dang *et al.* (3) reported seedling vigour QTL through association mapping in 540 rice cultivars utilizing SSR markers. Hence, major QTLs reported for seedling vigour contributing traits and corresponding markers present in the interval mapping of putative QTLs were validated. Finally the selected markers had been evaluated for their association with the high seedling vigour trait. With this back drop, the author reports major QTLs associated with different seedling vigour traits based on the presence of alleles in different genotypes and identification of SSR markers showing highest phenotypic and genotypic correlation.

Material and Methods

Forty seven rice genotypes of diverse ecologies were utilized in screening for seedling vigour traits along with standard checks of high

and low seedling vigour genotypes, viz., IR 28, Teqing, Lemont and Labelle and employed to map the loci underlying seedling vigour traits (Table- 1).

Estimation of seedling vigour: Germination test was conducted on pure seed fraction using 100 seeds in four replicates following between paper (BP) method at 25°C temperature and 93 ± 2 per cent relative humidity (7). The germination percent was calculated based on the number of seedling germinated to total number of seedlings kept for germination. Ten normal seedlings selected at random from each of the replication from germination test were carefully removed on the 14th day and used for measuring seedling length and it is measured from the root tip to shoot tip and the mean seedling length was expressed in centimeters. These seedlings after removing cotyledons they were put in butter paper bags and kept in hot air oven at 80°C for 24 h. The dry weight of the seedlings was recorded and expressed in mg 10 seedlings⁻¹. The seedling vigour indices were calculated using the following formula suggested by Baki and Anderson (1): Vigour index-I: Seed germination per cent x seedling length (cm) and Vigour Index-II: Seed germination per cent x seedling dry weight (g).

QTL analysis: Based on the literature studies, major QTL's for various seedling vigour traits, viz., germination percentage, germination rate, germination index, coleoptile length at optimum temperature 25°C, coleoptile emergence in flooded soil, seedling height in drained soil, mesocotyl length at temperature at 25°C and proportion of phenotypic variation, the QTL's contributing phenotypic variation more than 20 per cent were selected for the present study to identify the role of these QTLs in 47 different varieties (Table-3). Between the marker interval of these QTL's, based on their physical map location, 30 SSR markers were selected. Genomic DNA from the leaf samples was isolated using the CTAB method following the procedure of Zheng *et al.* (20) with slight modifications. The isolated genomic DNA was diluted with TE buffer

Table 1. Details of the experimental material included for study

No.	Genotype	Parentage	Released for
1.	Dinesh	Jaladhi 2/ Pankaj	Deep water
2.	CSR 23	IR 64//IR 4630-22-2-5-1-3	Irrigated saline and alkaline
3.	NDR 359	BG 90-2-4/ OB 677	Irrigated Medium
4.	PR 106	IR 8/ Peta 5/ Bella Patna	Irrigated Medium
5.	Sabita	Pureline selection from Boyan	Semi deep water
6.	IR 36	IR 2071-625-1-252	Irrigated Mid-early
7.	MTU 1010	Krishnaveni/ IR 64	Irrigated Mid-early
8.	Salivahana	RP 5-32/Pankaj	Rainfed shallow lowland
9.	Vivekadhan 62	China 4/BG 367-4	Irrigated hills
10.	Jalmagna	Selection from Badhon	Irrigated lowland conditions
11.	PR 113	IR 8/ RP 2151-173-1-8// IR 8	Irrigated Mid-early
12.	Purnendu	Pantai 23/ Jaladhi 2	Deep water
13.	Vandana	C 22/ Kalakeri	Rainfed Upland
14.	Jaya	TN 1/T 141	Irrigated Medium
15.	Kalanamak	Pureline selection from KN 3-27-3-3	Aromatic short grain
16.	Savitri	Pankaj/ Jagannath	Rainfed shallow lowland
17.	Govind	IR 20/IR 24	Rainfed upland
18.	Kasturi	Basmati 370/ CRR 88-17-1-5	Basmati
19.	NDR 8002	IR 60290- CPA 5-1-1-1-1/ IR 52533-52-2-1-2-1-B-2-3	Rainfed upland
20.	NDR 97	N 22/ Ratna	Rainfed Upland
21.	BPT 5204	GEB 24/ TN 1// Mahsuri	Rainfed shallow lowland
22.	IR 64	IR 1561-228-1-2/IR1737//CR94-13	Irrigated mid-early
23.	IR 50	IR 2153-14-6-6-2/ IR 28/IR 2070-625-1-25	Irrigated Early
24.	Vasumati	PR 109/ Pak. Basumati	Basmati
25.	Lalat	OBS 677/ IR 2071// Vikram/ W 1263	Irrigated Mid-early
26.	Pooja	Vijaya/ T 141	Rainfed shallow lowland
27.	Bhadshabog	Land race	Rainfed upland
28.	Varalu	Erramallelu/ CR 544-1-2	Rainfed upland
29.	RP BIO 226	BPT 5204*4/SS 1113	Rainfed shallow lowland
30.	Krishna Hamsa	Rasi/Fine Gora	Boro
31.	Triguna	Swarnadhan/ RP 1579-38	Irrigated medium
32.	Mandya vijaya	Sona/ Mahsuri	Rainfed shallow lowland
33.	Ajaya	IET 4141/ CR 98-7216	Irrigated medium
34.	DRR Dhan 38	BPT 5204/ KMR-3	Irrigated Medium
35.	Prasanna	IRAT 8/ N 22	Rainfed upland
36.	Swarna	Vasistha/ Mahsuri	Rainfed shallow lowland
37.	Tulasi	Rasi/ Fine Gora	Rainfed upland
38.	CSR 36	CSR 13/ Panvel 2// IR 36	Salinity
39.	Suraksha	Sayasree/ MR 1523	Irrigated medium
40.	Varadhan	Swarna/ IET 9314// BR 327-36	Irrigated Medium
41.	DRR Dhan 39	CSR 23/Kasturi	Irrigated saline and alkaline
42.	Akshaydhan	BR 827-35/SC 5109-2	Irrigated medium
43.	Sugandhamati	Pusa Basmati 1/ IET 12603	Basmati
44.	Swarnadhan	Sona/ RPW 6-13	Rainfed shallow lowland
45.	Rasi	TN 1/CO 29	Irrigated Early
46.	Sampada	Vijaya/ C 14-8	Irrigated medium
47.	Vikas	IR 8/ TKM 6	Irrigated saline and alkaline

Table 2. ANOVA for seedling vigour related traits among rice genotypes

Source of variance	df	Seedling g vigor index I	Seedling g vigor Index II	germination percent	Shoot Length (cm)	Root Length (cm)	Seedling g Length (cm)	Root: Shoot ratio	Root fresh weight (mg)	Shoot fresh weight (mg)	Seedling fresh weight (mg)	Seedling dry weight (mg)
Treatments	46	631678.0**	330136.0**	0.37**	6.26**	15.48**	7.25**	0.16**	200.00**	23541.9**	32160.0**	3258.00**
Error	94	23162.0	1760.0	0.010	0.963	0.354	0.715	0.010	3.10	9.219	8.11	58.90
CV (%)		3.01	5.03	2.15	6.10	4.16	3.80	7.4	4.20	0.43	0.42	4.38

to get required concentration of DNA (approximately 30 ng/ml) in each sample. The PCR plate was labeled with respect to sample number and 2 ml (*i.e.*, 50-100 ng) of template DNA was added to the respective wells. The master mix consisted of 0.5 l forward primer, 0.5 ml reverse primer, 0.5 ml dNTP's, 0.6 ml *Taq* DNA polymerase (M/s Bangalore Genei Pvt. Ltd.), 1.0 ml of 10 x PCR buffer (Tris with 1.5 mM Mg Cl₂) and 4.9 ml of sterile distilled water was added to make up the volume to 10 ml. Then the master mix (8.0 ml) was dispensed to the PCR plate with template DNA. The PCR plate was covered with a sealing mat. It was then placed in a programmable thermal cycle (M/s Applied Biosystem, USA) for DNA amplification.

The above steps are repeated for 35 cycles for amplification of DNA. After completion of the PCR the samples were collected and stored at -20°C. Later the products were resolved on agarose gel. PCR amplified products were resolved in 3 per cent agarose (3 g of agarose dissolved in 0.5 X TBE buffer, M/S USB, USA) gels in 0.5 X TBE buffer at 100V for 3.5 h in Hoefer super submarine electrophoresis unit (Pharmacia biotech) containing 0.5 X TBE buffer solution. Prior to loading, 1/6th volume of gel loading dye (40% sucrose; 0.25% bromophenol blue) was added to the PCR amplified products and ensured proper mixing. The sizes of amplified fragments were determined by comparing with 100 bp ladder (MBI Fermentas). The gels were stained in ethidium bromide (10 mg ml⁻¹) and placed over the UV-transilluminator and documented using ALPHA IMAGER gel documentation system (M/s Alpha innotech) for documentation.

Genomic SSR data scoring: Quantitative multistate traits depicting an array of characters were converted into binary characters (17) based on the variations present. Markers were scored for the presence and absence of the corresponding bands of the genotypes. The core 1 and 0 indicates the presence and absence of bands, respectively. In case of binary coding, a data matrix comprising 1 and 0 formed depending on the character and this data matrix was subjected to further analysis.

SSR marker trait correlation: The correlation of the marker with the trait was determined by the

presence of number of marker alleles corresponding to positive and negative control in morphologically characterized low and high vigorous genotypes (6).

Results

The analysis of variance of rice genotypes showed highly significant variation for seedling vigour related parameters (Table-2). The qGR-1, qGP-6, qGI-11 for seedling germination with phenotypic variance 68.5, 5.24, 54.9 respectively, whereas qRZ395 for mesocotyl length and qRZ448 for coleoptile emergence accounts 55.1 phenotypic variance. qFV3-2 for coleoptile seedling emergence accounts 7.8, qFV5-2 and qFV-10 for seedling height accounts 7.8 and 9.8 phenotypic variance respectively.

In the present study, a total of 30 SSR markers which were dispersed throughout the 7 chromosomes were used to assess the association of markers with seed vigour traits in 47 different rice genotypes by taking high and

low vigour parents as positive and negative control respectively. Among them, 19 markers were amplified, out of them a total of 11 markers showed different marker alleles (polymorphic) and remaining 8 markers showed same marker allele (monomorphic) across the varieties tested.

For the QTL qGR-1, six markers viz., RM10936, RM10066, RM11099, RM11449, RM11111 and RM11114 were analyzed using IR 28 as positive control. The negative control was not included due to unavailability of reported cultivar, *i.e.*, Daguandao. Among these markers, four markers got amplified (RM10936, RM10066, RM11099 and RM11114), out of which RM11114 showed marker allele 250 bp in IR 28. Remaining three markers found viz., RM10936, RM10066 and RM11099, although amplified there was no size difference in the marker alleles (monomorphic banding pattern) observed among the varieties. Whereas for qGP-6, among the five markers, viz., RM20536, RM20608, RM20583, RM20615 and RM20623 analyzed using IR 28

Table 3. List of QTL's validated in the present study

S.No.	Parents	QTL's	Chromosome No.	Marker interval	Trait	PV%	Reference
1.	IR 28/Daguandao	qGR-1	Chrm-1	RM9-RM7075	Germination rate	68.5	Wang <i>et.al</i> -2010
2.	Labelle/Blackgora	RZ448	Chrm-3	RM15500-RM15573	25 ⁰ C- coleoptile length	25.1	E. D. Redona-1996b
3.	Lemont/Teqing	qFV-3-2	Chrm-3	OSR31-RM168	Flooded soil-coleoptile emergence	7.8	Liang Zhou <i>et.al</i> - 2006
4.	Lemont/Teqing	qFV-5-2	Chrm-5	RM161-CDSR49	Drained soil treatment – seedling height	14.0	Liang Zhou <i>et.al</i> - 2006
5.	IR 28/Daguandao	qGP-6	Chrm-6	RM528-RM340	Germination percentage	24.0	Wang <i>et.al</i> -2010
6.	Labelle/Blackgora	RZ395	Chrm-7	RM6326-RM21960	Temperature - 18 ⁰ C	28.5	E. D. Redona-1996b
7.	Lemont/Teqing	qFV-10	Chrm-10	C223-RM228	Seedling height	9.8	Liang Zhou <i>et.al</i> - 2006
8.	IR 28/Daguandao	qGI-11	Chrm-11	RM3428-RM6091	Germination index	54.9	Wang <i>et.al</i> -2010

as positive parent, three markers got amplified, viz., RM20536, RM20608 and RM20623 showed band at 60, 90 and 80 bp approximately. Out of them, two were found to be polymorphic (RM20608 and RM20623). Similarly for the qGI-11, three markers were analyzed viz., RM6091, RM26580 and RM3428 using IR 28 as positive control all showed monomorphic banding pattern.

For the QTLs RZ448 and RZ395, a total of seven markers were analyzed using Labelle as negative control, viz., RM15337 for RZ448 and RM6326, RM21918, RM21925, RM21936, RM21956 and RM21960 for the QTL RZ395. Among these markers, RM15337 for RZ448 and RM6326, RM21918, RM21925 and RM21936 for RZ395 were amplified. RM15337 found to be monomorphic and formed band at 400 bp approximately. RM6326 and RM21925 were found to be polymorphic for the QTL RZ395, which formed band approximately at 100 and 200 bp respectively and remaining two markers RM21918 and RM21936 were monomorphic showing bands at 175 and 90 bp respectively.

Similarly for the QTLs in stress conditions qFV-3-2, qFV-5-2 and qFV-10, nine markers were analyzed using Teqing as positive and Lemont as negative control. For the qFV-3-2, out of two markers used, two markers got amplified and found to be polymorphic viz., RM15626 and RM15630. The marker RM15626 showed allele of positive control at 150 bp and negative control allele of 500 bp and RM15630 showed positive control allele at 240 bp and negative control allele at 250 bp. Among three markers analyzed for qFV-5-2 viz., RM18799, RM18704 and RM18770, two markers were amplified and showed polymorphism (RM18799 and RM18704). The marker RM18799 showed positive control allele as 250 bp and RM18704 showed positive control allele as 200 bp and negative control allele as 250 bp. In the same way for the qFV-10, from the four markers analyzed (RM25905, RM25919, RM25925 and RM25940) two markers were amplified, viz., RM25919 and RM25940. The marker RM25919 found to be monomorphic by showing positive

control allele as 70 bp and negative control allele as 50 bp and the marker RM25940 was polymorphic showing bands of positive control allele as 250 bp and negative control allele as 200 bp.

SSR marker trait correlation: In this study, more diversity was observed both in morphological analysis and SSR analysis. In the present study, 8 high vigour genotypes, 29 medium vigour genotypes and 10 low vigour genotypes were selected using morphological analysis. The correlation of SSR markers were presented in the Table-4.

The marker RM11114 showed the presence of alleles amplicons corresponding to low vigorous control in all morphologically categorised genotypes and the alleles corresponding to high vigorous control were present in five genotypes among eight morphologically categorised. Hence, it expressed correlation of 81.25 per cent. Whereas RM15630 expressed 45 per cent correlation as it showed the presence of alleles corresponding to negative control in four genotypes and alleles corresponding to positive control in four genotypes. Similarly, RM15626 accounts the presence of alleles corresponding to low vigorous parent as negative control in two genotypes out

Table 4. Genotypic and phenotypic correlation for seedling vigour traits among rice genotypes

S.No.	Polymorphic Primers	Correlation (%)
1.	RM 11114	81.25
2.	RM 15630	45.00
3.	RM 15626	53.75
4.	RM 18704	23.75
5.	RM 18799	71.25
6.	RM 20608	81.25
7.	RM 6326	60.00
8.	RM 25940	50.00
9.	RM 21936	50.00
10.	RM 21925	50.00
11.	RM 20623	51.25

of ten morphologically categorized low vigorous genotypes and the alleles corresponding to high vigorous parent as positive are present in seven genotypes exhibiting the correlation of 53.75 per cent.

The marker RM18704 shows correlation of 23.75 per cent and accounts the presence of alleles corresponding to negative control in only one morphologically categorized low vigorous genotype and high vigour alleles were present in three genotypes. Whereas RM18799 expresses correlation of 71.25 per cent since the bands corresponding to low vigour parents were present in eight genotypes and to high vigour parents were present in five genotypes. The marker RM20608 exhibited 81.25 per cent correlation as it shows the presence of high vigour alleles in five genotypes and low vigour alleles in all low vigorous genotypes which were morphologically characterized.

The marker RM6326 exhibited the correspondence of positive (high vigour) control band in all high vigorous genotypes and negative control (low vigour) bands in only two genotypes among ten low vigorous genotypes used for the present study. Therefore it shows 60 per cent correlation. The marker RM21925 does not show any correlation between genotypic and

phenotypic analysis of high vigorous genotypes and showed the complete correlation for low vigorous genotypes and expresses the correlation of 50 per cent. Similarly, the marker RM21936 showed the correlation of 50 per cent as there was no corresponding band of positive control were present in high vigour genotypes and showed complete correlation among low vigour genotypes. The marker RM20623 showed correlation of 51.25 per cent as the bands corresponding to positive control were present in one genotype and to negative control were present in nine genotypes (Fig. 1). Correlation of 50 per cent were observed by the marker RM25940 which shows the presence of alleles corresponding to positive control in four genotypes which were morphologically categorized and bands corresponding to negative control in five genotypes. Among the 11 markers which were found to be polymorphic, RM11114 and RM20608 were identified as best marker which showed highest correlation between genotypic and phenotypic data. From the study, it was concluded that these markers were useful for determining the vigour traits in the selected 47 different rice genotypes.

QTLs for seedling vigour traits: A total of 8 QTLs were detected for the seven seedling vigour

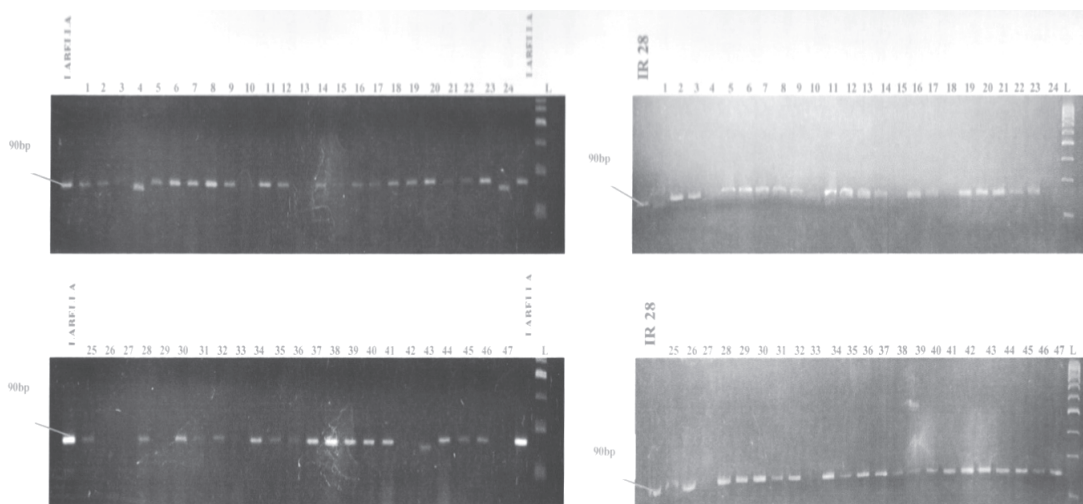


Fig 1. Banding pattern of marker RM 21936 and RM 20623 in 47 rice genotypes

traits and under different stress conditions. Based on the SSRs selected from the interval of these QTLs and marker analysis, QTLs associated with different seedling vigour traits were found to be present in some genotypes selected for the analysis.

The putative qGR-1 associated with germination rate was found to be present in 12 genotypes viz., Dinesh, CSR 23, NDR 359, PR 106, Sabita, MTU 1010, Salivahana, Vivekadhan 62, Vandana, DRR Dhan 39 and Swaradhan. In three genotypes, viz., Dinesh, Pooja and Tulasi, qFV-3-2 associated with coleoptiles emergence under flooded condition was found to be present. Similarly, qFV-5-2 associated with seedling height under drained soil condition was present in Vivekadhan 62, PR 113 and Improved Samba Mahsuri. The presence of qGP-6 accounting for germination percentage was found in the genotypes Dinesh and Lalat. RZ395, a putative QTL controlling mesocotyl length at temperature regime of 18°C was found in NDR 359, PR 106 and Vasumati. qFV-10 present on chromosome 10 expressing seedling height under stress condition was present in nine genotypes viz., Dinesh, Sabita, Vivekadhan 62, PR 113, Pooja, Triguna, Mandya vijaya and Ajaya (Table-5).

Some genotypes showed the presence of multiple QTLs, from the eight QTLs analyzed, Dinesh has four QTLs viz., qGR-1, qFV-3-2, qGP-6 and qFV-10 and Pooja has two qFV-3-2 and qFV-10. Similarly, Sabita has two QTLs viz., qGR-1 and qFV-10 and Vivekadhan 62 has two QTLs qGR-1 and qFV-10.

Discussion

Seed vigour is an important trait, wherein seed properties which determine the potential for rapid, uniform emergence and development of normal seedlings under a wide range of field conditions (9). The seedling vigor is directly related to better crop establishment measured in terms of plant height. In the present study, the traits of seed vigour during the germination stage, including germination rate, germination percentage, and germination index, were determined under laboratory conditions. McKenzie *et al.* (10) reported that seedling traits measured under controlled laboratory conditions were correlated with seedling vigour measured under field conditions. The results of germination percentage determined by standard germination test under optimum conditions usually overestimate field emergence under suboptimal field conditions. Cui *et al.* (2) indicated that

Table 5. Seedling vigour QTLs associated with selected genotypes

S.No.	QTL	Trait	Genotypes
1.	qGR-1	Germination rate	Dinesh, CSR 23, NDR 359, PR 106, Sabita, MTU1010, Salivahana, Vivekadhan 62, Vandana, DRR Dhan 39 and Swaradhan
2.	qFV-3-2	Coleoptiles emergence under flooded condition	Dinesh, Pooja and Tulasi
3.	qFV-5-2	Seedling height under drained soil treatment	Vivekadhan 62, PR 113 and RP Bio 226
4.	qGP-6	Germination percentage	Dinesh and Lalat
5.	RZ395	Mesocotyl length at temperature 18°C	NDR 359, PR 106 and Vasumati
6.	qFV-10	Seedling height under flooded condition	Dinesh, Sabita, Vivekadhan 62, PR 113, Pooja, Triguna, Mandya vijaya and Ajaya

germination rate (speed) and early seedling growth were interrelated in rice.

There are several reports of QTLs for rice seed vigour, mainly focused on seedling growth, seed longevity and tolerance to adverse conditions. For example, Cui *et al.* (2) mapped 31 QTLs for five traits of seedling vigour using a set of 241 F₁₀ recombinant inbred lines (RILs). Zhang *et al.* (21) identified a total of 34 QTLs for four traits of seedling vigour using 282 F₁₃ RILs. Miura *et al.* (12) found three putative QTLs for seed longevity, *qLG-2*, *qLG-4*, and *qLG-9*, using 98 backcross inbred lines (BILs). Fujino *et al.* (2008) detected three putative QTLs (*qLTG-3-1*, *qLTG-3-2*, and *qLTG-4*) associated with low-temperature germination using 122 BILs, and *qLTG-3-1* was map-based cloned. Six QTLs genotypes viz., *qGR-1*, *qGP-6*, *qFV-3-2*, *qFV5-2* and *qFV-10* were found to be present in some genotypes which showed high seedling vigour morphologically and genotypes viz., Dinesh, Pooja and Sabita showed the presence of multiple QTLs concluding that the genotypes which showed high vigour were due to the presence of these putative QTLs associated with different seedling vigour traits. Similarly, Wang *et al.* (19) reported the *qGR-1*, *qGR-2*, *qGR-6*, *qGP-4*, *qGP-6*, *qGP-8*, *qGP-11*, *qGI-1*, *qGI-7* and *qGI-11* were responsible for germination in recombinant inbred lines (RIL) derived from the cross between *japonica* Daguandao and *indica* IR 28.

The genotypes having multiple QTLs are having the trait acquired from one of its donor parent or cultivated and adapted for years under unfavourable ecosystems. Sabita, Dinesh and Pooja are all well adopted for deep water ecosystem and has better seedling vigor trait. Similarly, traditional and upland cultivars are having in build vigour, good emergence and fast canopy cover at early crop stage and these are all needed traits for suppression of weeds and drought escape mechanism. Hence, faster canopy cover suppresses the weed growth and also conserves moisture level. However the present day high yielding varieties, viz., Jaya, IR

64, IR36, NDR 359, Swarna, MTU 1010, BPT 5204 etc are having poor seedling vigour under direct seeded and submerged conditions. Seedling vigour in most of the cases negatively correlated with yield and selection for higher yield eliminates genotypes having better seedling vigour and by evolution these traits are lack in majority of lowland irrigated varieties. It is extremely difficult to improve rice seedling vigour by conventional strategies, due to undesirable traits associated with seedling vigour, such as large grain size (2, 10). Cui *et al.* (2) found two QTLs for seedling vigour and α -amylase, not linked to seed weight, which will be useful for MAS to improve seedling vigour. However, with the number of QTLs increasing, the utility of MAS becomes less obvious (4) and the complexity of other physiological process involved in different genetic background. Therefore, marker-assisted introgression of the six major QTLs detected here is expected to significantly improve seed vigour in rice.

Conclusion

Present study clearly elucidates six QTLs presence in genotypes individually or in combination and its mechanism in expression of high seedling vigour morphologically. The interaction mechanism between QTLs where genotypes having more than one QTLs are present needs to be investigated. The QTLs detected here may be used for developing new varieties with a high level of seed vigour by marker assisted back cross breeding.

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Pharmacological Evaluation of Leaf Ethanol Extract of *Crotalaria hebecarpa* (DC) Rudd.

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Abstract

Crotalaria hebecarpa is a prostrate herb with slender branches belonging to the family Fabaceae. Though the identity and distribution of the plant is known, but its medicinal uses, phytochemicals and their mechanisms are not known. In the present paper, we are reporting the pharmacological evaluation of ethanol extracts of leaf using standard pharmacological methods. Our results indicated that the plant leaf contains bioactive molecules needed for eliciting potent activity with low IC₅₀ values for the activities of 5-Lipoxygenase, Tyrosinase and Acetyl cholinesterase. Our results also indicated that the plant leaf extract is not having potency, a reflection of lack of required bio active molecules for inducing effective activity against biological activities, like cytotoxicity and α -glycosidase inhibition activity. Nevertheless, the results reveal the very potent activity of extract against acetyl cholinesterase, 5- lipoxygenase and tyrosinase activities. However, more detailed investigations need to be carried out for isolation and characterization of bioactive molecules responsible for observed activities.

Key words: *Crotalaria hebecarpa*, leaf ethanol extract, in vitro Pharmacological Screening.

Introduction

The medicinal plants form an important component of human health care system since ancient days. Owing to this, extensive

phytochemical and pharmaceutical screening has been carried out identifying a range of biologically active molecules and their therapeutic uses. Though much work has been done, but is not sufficient to meet the demand due to emergence of new diseases and their causative agents. Hence, a large scale screening has to be carried out on a range of unexplored potential medicinal plants. In the present work, we report the pharmacological evaluation of an important unexplored medicinal plant, *Crotalaria hebecarpa* (DC) Rudd.

Crotalaria hebecarpa (DC) Rudd is a prostrate annual herb with slender, hairy branches, belonging to the family Fabaceae. It is commonly known as Fuzzy Fruited Rattle pod and is observed in open forests and as a weed in cultivated fields. The plants prefer a sunny situation on fresh moist soil. The substrate should be sandy-loamy, gritty-loamy or sandy clay soil. The whole part of this plant is mixed with curd and orally given once in a day for two days to cure gastric dysentery (1). Barring this report, there is no work on pharmacological evaluation of this plant.

Hence, in the present paper, we are reporting various pharmacological activities using leaf ethanol extract. The leaf ethanol extract is a good source of secondary metabolites and screening that may lead to discovery of new drugs or new mode of treatment of a disease.

Indeed, our investigation revealed that it contains potent acetyl cholinesterase, tyrosinase inhibition, and lipoxygenase inhibition activities. Thus, our work confirms that *Crotalaria hebecarpa* is a good source of biologically active molecules.

Materials and Methods

Preparation of plant material: For the investigation, the leaves of *Crotalaria hebecarpa* were collected from A.N.U Campus, Guntur District, and were identified by the Botanical Survey of India, Deccan Regional Centre, Hyderabad. One voucher specimen was deposited there containing accession number BSI/DRC/2015-16/662. The leaves of *Crotalaria hebecarpa* was collected and dried, then the dried samples subjected to course grinding. The powdered material was extracted by ethanol using the soxhlet apparatus for 10h. Then the extract was filtered through a cotton plug followed by what men filter paper no 1. The extract was then concentrated by evaporating solvent.

In vitro methods

DPPH free radical scavenging activity: DPPH(1,1diphenyl-2picryl-hydrazyl) free radical scavenging of test compounds was determined by the method of Lamaison *et al.*, (2) which depends on scavenging of colored free radical (DPPH) in ethanol solution by the test drugs. The reaction mixture was prepared using DPPH and test drugs in final concentrations of 3 ml. Absorption of DPPH at its adsorption maximum 516 nm is inversely proportional to the concentrations of the scavenger (Test drug). The activity was expressed as inhibitory concentrations 50 (IC_{50}) i.e., the concentration of the test solution which showed 50% reduction in absorbance of the test solution as compared to that of blank solution.

$IC_{50} = [(OD \text{ of control} - (OD \text{ of test} - OD \text{ test blank}) / OD \text{ of control}]$

DPPH Free radical scavenging activity inhibition was calculated using following formula.

$\% \text{ inhibition} = (\text{Control O.D} - \text{Test O.D}) / \text{Control O.D} \times 100$

Brine Shrimp Lethality Assay: Brine shrimp lethality assay was carried out according to the method of Meyer *et al.* (3). Brine Shrimp (*Artemia salina*) nauplii were hatched in sterile brine solution (sea salt 38g/l pH 8.5) under constant aeration for 38h. After hatching, 10 nauplii were placed in each vial and added various concentrations of leaf extract in a final volume of 5 ml, maintained at 37° C for 24 h under the light of incandescent lamps and surviving larvae were counted. Each experiment was conducted along with control at various concentrations of the test substances. Percentage lethality was determined by comparing the mean surviving larvae of test and control tubes. The result of the test compound was compared with the positive control, Podophyllotoxin.

Alpha glycosidase inhibition assay: Alpha glycosidase inhibitory activity was determined according to the standard methods. In a micro plate well, 50 ml of enzyme (0.4 U/ml) was taken, to this, 90 ml of 100mM phosphate buffer pH 7.0 and 10 μ l test substances were added and mixed well. The reaction mixtures was incubated at room temperature for 5 min and 50 ml of p-Nitro phenyl alpha-D- Glycosidase (20 mM) as substrate was added, mixed well, and incubated for 15 min at room temperature. The reaction was stopped by the addition of 30 ml of sodium carbonate solution (200 mM). The absorbance was measured at 405 nm using micro plate reader. Control and test blank OD's were obtained by replacing enzyme with buffer. Alpha glycosidase inhibition was calculated using following formula.

$\% \text{ Inhibition} = (\text{Control O.D} - \text{Test O.D}) / \text{Control O.D} \times 100$

Estimation of acetyl cholinesterase assay:

The acetyl cholinesterase activity was determined using photometric method as described by Ellman *et al.*, (4) Acetylthiocholine substrate is hydrolyzed by AchE in the sample and forms thiocholine, which will react rapidly and irreversibly with 5,6-thio-bis nitro benzoic acid (DTNB) producing a yellow anion of 5-thio-2 nitro

benzoic acid. The increase in color intensity was measured spectrophotometrically at 412 nm. Twenty microliters of 0.075 M acetylcholine iodide was added to the reaction mixture in the cuvette and mixed well and the absorbance for 5 min at an interval of 15 seconds each, the change in absorbance per minute (ΔA) was calculated (5).

Calculation of AchE activity = $5.74 \times 10^{-4} \times (\Delta A) / \text{Protein content of the sample}$

5-Lipoxygenase inhibition assay: The lipoxygenase inhibition activity was assayed using photometric method as described by Reddanna *et al.*, (6). The assay mixture contained 2.97 ml of 50 mM phosphate buffer (pH 6.3), 5 μ l of 80 mM Linoleic acid and sufficient amount of potato 5-Lipoxygenase enzyme. The enzyme solution was stored in ice and controls were measured at intervals throughout the experimental period to ensure that enzyme activity was constant. The reaction was started by the addition of substrate (Linoleic acid) and the increase in UV absorption at 234 nm was followed at 25 $^{\circ}$ C. The activities were measured at every two minutes. The reaction was linear during this time period. In the inhibition studies, the activities were measured in the presence of various concentrations of extract. All the assays were performed in duplicate or triplicate. The percent inhibition of 5-Lipoxygenase activity was calculated as follows;

$\% \text{ Inhibition} = (\text{Control O.D} - \text{Test O.D}) / \text{Control O.D} \times 100$

Tyrosinase inhibition assay: Tyrosinase inhibition assay was carried out according to the method of Ohguchi *et al.*, (7). The assay mixture contained 250 μ l of enzyme, 50 μ l of drug carrier, and 1250 μ l of 8 mM M L-Dopa. The reaction was started by addition of substrate, and then incubated for 1 min; the activity was measured at 475 nm. In the inhibition studies, the activities were measured in the presence of various concentrations of test substances. All the assays were performed in duplicate or triplicate.

Tyrosinase inhibition was calculated using following formula.

$\% \text{ Inhibition} = (\text{Control O.D} - \text{Test O.D}) / \text{Control O.D} \times 100$

Results

DPPH – inhibition assay: For determining the antioxidant potential (DPPH radical scavenging assays) of *Crotalaria hebecarpa*, the stock solution (100 mg/ml) of ethanol leaf extract was prepared. From this stock solution, different dilutions (50,100,150, 200 and 250 μ g/ml) of extracts were prepared in ethanol and water and were taken in 4 different test tubes, compared with standard vitamin – C (0.5, 10, 15, and 20 μ g/ml). Experiments on antioxidant potential of *Crotalaria hebecarpa* revealed that the percentage of DPPH inhibition increased with increase in the concentration, the highest percentage of inhibition was observed at extract concentration of 200 μ g/ml with 67.45% inhibition (Fig.1). IC_{50} value of *Crotalaria hebecarpa* extract is 177.63, whereas the IC_{50} value of standard is 30.41 (Fig.2), indicating the plant extract is having very low antioxidant activity when compared with standard drug vitamin-C (Fig.2).

Brine Shrimp Lethality Assay: The ethanolic extracts of *C. hebecarpa* were assessed for cytotoxicity using brine shrimp lethality assay on *Astemia salina*. The *A.salina* was subjected with extracts for 24 hours and in place of extracts podophyllotoxin was used as control. The aqueous extracts were prepared by dissolving in water with concentrations ranging from 50-250 μ g/ml. The IC_{50} values were obtained with Fenny probed analysis software. The results indicated that the *C. hebecarpa* leaf extract is having low cytotoxic potential with IC_{50} value of 96.40 (Fig.3), whereas standard drug is potent with IC_{50} value of 6.09 (Fig.4). The results also confirm that the leaf of *C. hebecarpa* lack active principles needed for inducing optimal cytotoxic response.

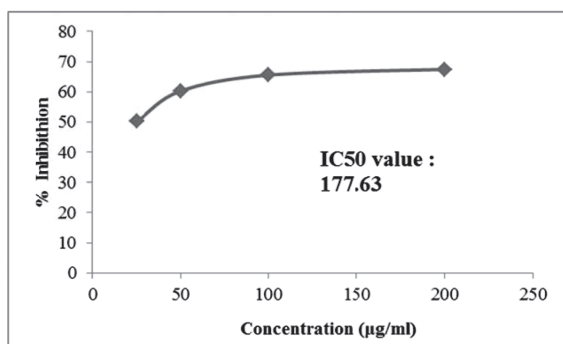


Fig. 1. DPPH scavenging activity of *Crotalaria hebecarpa*

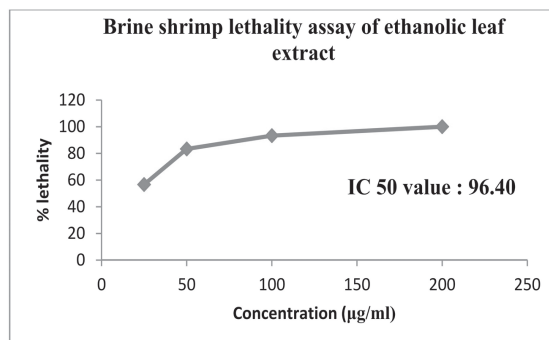


Fig. 3. Brine shrimp lethality assay of ethanol leaf extract of *Crotalaria hebecarpa*

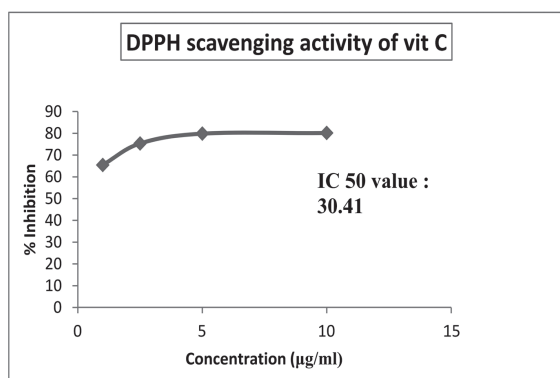


Fig. 2. DPPH scavenging activity by Vitamin - C (standard)

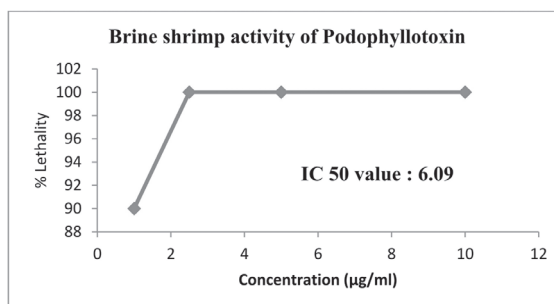


Fig. 4. Standard drug of Podophyllotoxin

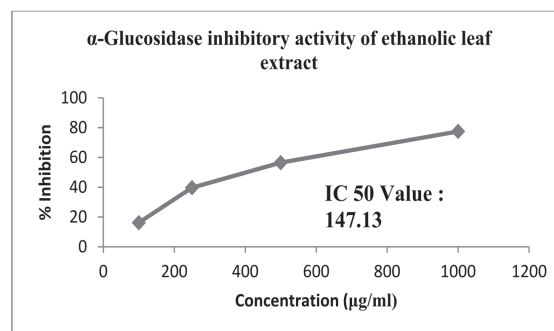


Fig.5. Alpha Glucosidase inhibition activity of *Crotalaria hebecarpa* ethanol leaf extract.

Alpha Glycosidase inhibition assay: To do alpha glycosidase inhibition assay, the ethanolic leaf extracts were prepared in different concentrations ranging from 200 to 1200 µg/ml. As a standard, Acarbose is used with concentrations ranging from 2 to 12µg/ml. The results revealed that the ethanolic extract of leaf is low in α - glucosidase inhibition with IC_{50} value of 147.13 (Fig.5). Whereas the standard drug acarbose displayed good inhibition activity with IC_{50} value of 38.81(Fig.6). Basing on the results, it can be concluded that the potency of α -glucosidase inhibition is low in leaf extracts.

Estimation of acetyl cholinesterase activity: The acetyl cholinesterase activity was carried out according to the method of Ellman *et al.*, (4). For

assessing the activity, the leaf ethanol extracts were prepared in different concentrations ranging from 20-120 µg/ml and the standard drug in the concentration ranging from 2 to 12µg/ml. The assay revealed that the leaf ethanol extract is very potent inhibitor of acetyl cholinesterase activity (Fig.7) with IC_{50} value 208.60. Whereas

standard drug displayed IC₅₀ value of 8943.42 (Fig.8). The enormous differences (3 fold) in the IC₅₀ value of extract and drug indicates that the extract contains active biomolecules needed for potent inhibitory activity against acetyl cholinesterase whose identity need to be confirmed (Fig.7-8).

5- Lipoxygenase inhibition assay: The 5-Lipoxygenase inhibition assay of leaf ethnolic extracts were carried by preparing extract in different dilutions(5,10,15,20,25 and 30µg/ml) and compared against standard drug Zileuton prepared in the concentrations of 2,4,6,8,10 and 12 µg/ml. The experimental results (Fig.9) indicated that the ethanolic extract of leaf contain very potent inhibitory activity as evidenced by low IC₅₀ value (16.65 µg/ml). But the standard drug contains high activity with IC₅₀ value of 311.70 (Fig.10). It is very exciting to see high potent activity of leaf ethanol extract with very low IC₅₀ value (16 µg/ml) as against to high IC₅₀ value (311 µg/ml) of standard (Fig.9-10). Further work need to be carried out to isolate and characterize the active principle responsible for the observed inhibitory activity.

Tyrosinase inhibition assay: The ethanolic leaf extracts of *C.hebecarpa* were screened for tyrosinase inhibition assay. For performing the assay, the extracts were prepared in different concentrations 20 to 120 µg/ml. The drug resveratrol is used as standard and is prepared in different concentrations ranging from 2 to 12µg/ml. The experimental results indicated that the *C.hebecarpa* extract is very potent in inhibiting tyrosinase activity with IC₅₀ value of 40.15 (Fig.11). In against to this, the drug displayed poor activity with high IC₅₀ value 1456.16 (Fig.12). It is very interesting to note that, very low IC₅₀ value of extract in comparison with pure drug. Our results are interesting and promising in confirming that the *C.hebecarpa* extract contain compounds that can elicit high inhibition activity against tyrosinase. However, the compounds responsible for observed activity need to be isolated and characterized *in vivo* and *in vitro*.

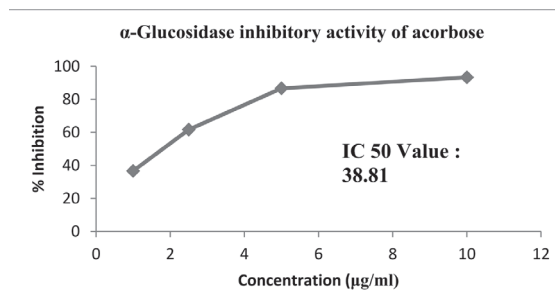


Fig.6. Alpha Glucosidase inhibition by acarbose standard

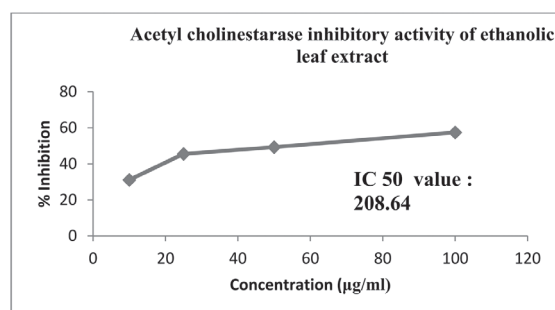


Fig.7. Acetyl cholinesterase inhibition activity of ethanol leaf extract

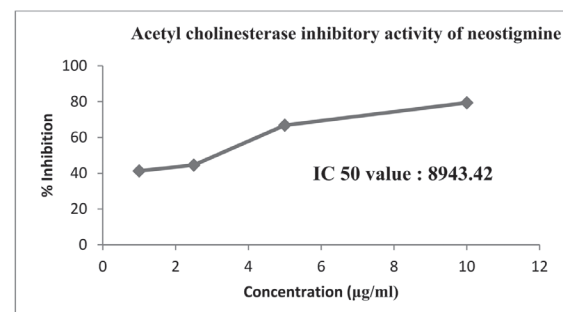


Fig.8. Acetyl cholinesterase inhibition of Neostigmine (Standard drug)

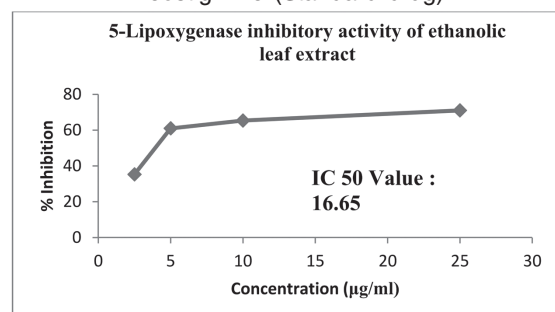


Fig.9. 5-Lipoxygenase inhibition of *Crotalaria hebecarpa* ethanol leaf extract

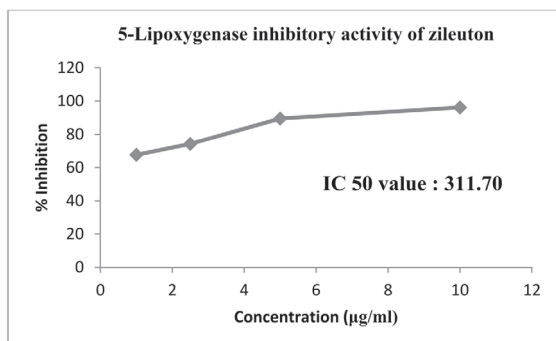


Fig.10. Lipoxygenase inhibition of Zileuton (Standard drug)

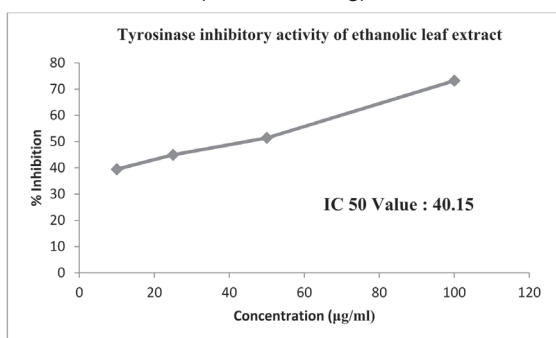


Fig.11. Tyrosinase inhibitory activity of *Crotalaria hebecarpa* leaf ethanol extract.

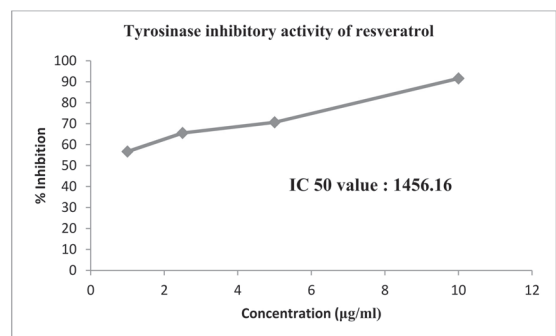


Fig.12. Tyrosinase inhibition of resveratrol (Standard drug)

Discussion

The nutritional values and antioxidant activity of wild plants are regarded worldwide as an important area of the phytotherapeutic research (8). Many antioxidants can be obtained from food sources such as grains, fruits and

vegetables. Consumption of high antioxidants may provide positive effects on human health. Most of the diseases are caused by the oxidative stress. Antioxidants are playing an important role in delaying, intercepting or preventing oxidative reactions catalyzed by free radicals (9). Plant products including phenolics, flavonoids, tannins, proanthocyanidins and various plant or herbal extracts have been reported to be scavengers and inhibitors of lipid peroxidation. Therefore, in view of the importance of these substances to health, phenolic have been proposed as health-promoting products in prophylactic medicine (10).

Plant derived cytotoxic constituents have played an important role in the development of clinically useful anticancer agents. Various medicinal plants are being used in cancer therapy due to their cytotoxicity property (11). Brine shrimp assay was considered as useful tool for preliminary assessment of toxicity and it has been suggested for screening the pharmacological activities of plant extracts. The brine shrimp lethality test is considered to be useful in determining various biological activities such as cytotoxicity, phototoxic, pesticidal, trypanocidal and enzyme inhibition activities (12, 13 and 14). *E. indica* produced physical sign of toxicity in albino Wister rats depending on the dose given ranging from writhing, decreasing respiration and death 24 h after administration of the leaf ethanolic extract (15). The best extraction method for the whole plant sample of *E.indica* is through mixture of ethanol and water as it is demonstrated maximum mortalities (100%) and high LC₅₀ values against the brine shrimp. Contrasting to this, aqueous leaf extract of *Crotalaria hebecarpa* is having low cytotoxic potential with IC₅₀ value of 96.40 (Fig.3), whereas standard drug is potent with IC₅₀ value of 6.09(Fig.4). It indicated the lack of bio active molecules against cytotoxicity.

Ethano botanical information indicted that more than 800 plants are used for the treatment of diabetes throughout the world (16). Many therapeutic approaches are available in managing diabetes and among them is to

decrease the post-prandial hyperglycemia (17). It can be overcome by the inhibition of carbohydrate hydrolyzing enzymes like alpha amylase and alpha glucosidase (18). Several alpha glucosidase inhibitors have been isolated from medicinal plants to develop as an alternative drug with increased potency and lesser adverse effects than the existing drugs (19). However, the methanolic extract of *Citrullus lantus* is a potent inhibitor of alpha amylase and alpha glucosidase enzymes based on the breakdown of starch to glucose. The LC 50 values were 76.68 and 54.44 for alpha amylase and alpha glucosidase respectively (20). But our results revealed that the *Crotalaria hebecarpa* ethanolic extract of leaf is low in α -glucosidase inhibition with IC_{50} value of 147.13 when compared to standard drug.

Acetyl choline esterase (AChE) is an enzyme involved in the breakdown of the neurotransmitter acetylcholine into acetic acid and choline. Inhibition of AChE is needed for deceleration of neurodegeneration in Alzheimers, Parkinsons and other neurodegenerative diseases. Plant secondary metabolites have been used as inhibitors of various classes of enzymes. Several thousands of plant extracts have been screened against AChE from different parts of the world. More than 35 alkaloids have been purified from plants so far with AChE inhibitory activity (21). The extracts of *Rosmarinus officinalis*, *Mentha spicata*, *Majorana syrica* and *Nigella sativa* were proved to have a great potential and should be considered for further studies to identify the constituents responsible for the AChE inhibitory activity (22). However, our results indicated that *Crotalaria hebecarpa* has very potent activity against acetylcholinesterase when compared to standard drug with IC 50 value 8943.42.

Lipoxygenases (LOXs) are non-heme iron-containing dioxygenase and widely distributed in plants and animals. LOX catalyzes deoxygenating on poly saturated fatty acids to yield cis, trans-conjugated diene hydroperoxides. These are the key enzymes in the biosynthesis of leukotrienes that play an important role in

several inflammatory diseases such as arthritis, asthma, cancer and allergic diseases. All *Caralluma arabica* extracts exhibited strong LOX inhibitory activity with LC50 values ranging from 11.2 to 30.77 μ g/ml (23). *Camellia sinensis*, *Rhodiola rosea* and *Koelreuteria henryi* had notable significant inhibitory activities against lipoxygenase (24). Whereas *Crotalaria hebecarpa* ethanolic extract of leaf contain very potent inhibitory activity as evidenced by low IC_{50} value (16.65 μ g/ml). Antioxidants and polyphenols are widely distributed in nature and some studies have revealed that poly phenols constitute rich inhibitors of LOX product synthesis (25). High polyphenols and antioxidants may provide the LOX inhibiting properties in *Crotalaria hebecarpa*.

Tyrosine is an enzyme present in plant and animal tissues which is responsible for the synthesis of melanin pigments from tyrosine by oxidation (26). In humans, increased activity of this enzyme leads to over production of melanin in epidermal layers causing hyper pigmentation of the skin (27). Hyper pigmentation can also be caused by excessive exposure to UV light, adverse drug reaction, melanoma and ageing (28). Hence, tyrosinase inhibitors can be clinically very useful for the treatment of some skin disorders associated with melanin hyper pigmentation and depigmentation after sunburns. The isolated methanolic extracts of *Piper betel* and *Cinnamomum zeylanicum* can serve as therapeutic or cosmetic agents and can be used as potential sources of novel tyrosine inhibitors for treating variety of skin related disorders. Generally, phenylethanoid glycosides showed moderate tyrosinase inhibitory activity with the exception of diglycosides. The inhibitory activity of phenylethanoid glycosides could be attributed to the presence of o-hydroxyls on the phenolic rings, which give them the property to chelate with metals. Whereas, in the case of *Crotalaria hebecarpa*, ethanolic extract shows high inhibition activity against tyrosinase. The high terpenoid compounds in *Crotalaria hebecarpa* plant may be contributing to the tyrosine inhibition activity.

Conclusion

The leaf ethanol extracts of *Crotalaria hebecarpa* showed different pharmacological activities such as cytotoxicity, DPPH-inhibition assay, alpha-glucosidase inhibitory, acetyl choline esterase inhibitory, lipoxygenase inhibitory and tyrosinase inhibitory activity. Of which, very potent activities were observed with acetyl cholinesterase, lipoxigenase and tyrosine activities and remaining showed very poor activities. Further studies are required to identify the specific compounds which are responsible for potent activities.

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Immunological and Antioxidant Response of *Litopenaeus vannamei* fed With *Lactobacillus* species under WSSV challenge

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Abstract

Two-phased experiment was conducted to investigate the effects of dietary supplementation of probiotic bacterium on shrimp physiology. In first phase shrimp fed with probiotic supplemented feed for 21 days, in second phase challenged with White spot syndrome virus (WSSV) and their physiological responses were investigated for 2 days post-challenged. The probiotic bacteria *Lactobacillus sps* was added to the formulated basal diet at 5%, 10% and 15% concentration. Growth, immune response, antioxidant status of shrimp were evaluated. The results showed that dietary supplementation of probiotics in shrimp had significant ($P < 0.01$) impact on growth. The treated shrimp groups showed significant increase in THC, percentage of phagocytosis and phenoloxidase enzyme activity. IgG, IgA, and IgM like substances in the haemolymph of treated shrimp were significantly increased when compared to control. Higher levels of these substances were observed in 10% treated shrimp than other two groups. The antioxidant enzymes like catalase and superoxide dismutase enzymes also significantly increased in probiotic treated shrimp when compared to control. In addition, dietary supplementation of all the three concentrations of *Lactobacillus sps* probiotic bacterium was effective in improving the resistance of shrimp against WSSV as they had higher THC, higher percentage of phagocytosis, phenoloxidase enzyme and immunoglobulin like substances level.

Key words: Antioxidant response, immune response, Probiotics, WSSV

Introduction

Shrimp farming is very important in aquaculture because consumer demand is increasing day by day. The most important farming shrimp is *Litopenaeus vannamei*. Though all the conditions are given for successful growth of the shrimp the diseases are becoming a risk factor for the shrimp farming, which affect shrimp production severely (1). In particular white spot syndrome virus (WSSV) causing white spot disease is considered as a severe threat to aquaculture industry because it can cause cumulative mortalities up to 100% within 7-10 days in shrimp (2).

Antibiotics and vaccination are used as traditional disease control strategy, however long term uses of antibiotics leads to negative impacts like drug resistance and drug residues (3). In the last few years biological control has been beneficial technique which consist use of probiotic bacteria capable of improving health and growth (4). The mechanism of probiotic to host is reported by many people, products of substances that inhibit growth of the pathogen (5); competition exclusion (6), stimulation of immune response, production of enzymes involved in digestive function, antiviral effects and improved water quality. A wide range of yeasts, microalgae, gram-positive bacteria such as *bacillus*, *lactobacillus*, *streptococcus*,

enterococcus and gram negative bacteria such as *Aeromonass*, *alteromonas*, *pseudomonas* are used as probiotics (7,8)

Lactobacillus is an important species used in aquaculture because of its properties like low virulence, production of antimicrobial substances which can act against certain pathogens (9). But there are only few reports about their use in shrimp culture are present (10, 11, 12). In the shrimp innate immune system is present consists of cellular and humoral reactions which are very important defence mechanism. The aim of this work was to study the growth, change in immunological parameters like total haemocytes, phagocytic activity, antibody like substances, phenoloxidase activity and antioxidant enzymes like catalase, superoxide dismutase in the haemolymph in the probiotic diet fed shrimp and in virus challenged shrimp

Materials and Methods

Animals: Healthy shrimp (wt of approximately 6 gms) was collected from a commercial farm and bring to lab immediately. The collected shrimp were acclimatized to culture conditions for one week in tanks containing filtered sea water and freshwater which is UV treated. Salinity was maintained at 20ppm and continuous aeration was given.

Preparation of probiotic formulation: The strain *Lactobacillus*, which was isolated from shrimp cultured ponds in Nellore (13) was used in this study. Bacteria were grown in sterile conditions using MRS broth until final density reaches to 1×10^6 Cells per ml. These cells were mixed with a commercial gel (Him C, Himalaya chemicals) to attain different concentrations (5%, 10%, and 15%) for using as feed supplement to study its effect on biochemical and immunological parameters.

Experimental design

Stage-1: Feeding the shrimp with different probiotic concentration supplemented: For each parameter to study a group of 24 animals were taken and acclimatized to laboratory

conditions for a period of one week. These animals were divided into 4 groups each consists of 6 animals all groups were run in triplicate. Three diets containing different doses (5%, 10%, 15%) of probiotic mixture were prepared. Keeping one group as control and the remaining three groups were fed probiotic supplemented feed (1×10^6 @ 10gm gel /kg feed, 50gms, 100gms, 150gms of gel was added per Kg feed to make 5%, 10% and 15%). The feed was given to each group for a period of three weeks. For control group the feed was supplied only by mixing with commercial gel without probiotic. The feeding experiment was done up to three weeks; in this feeding trial all shrimps were fed diets four times a day at 4% of the body weight.

During the experiments 30% of seawater was exchanged daily to maintain the water quality and the water temperature was maintained at $28 \pm 1^\circ\text{C}$ the pH at 7.5-8.4 and the salinity was maintained at 20ppm. At the end of this experiment shrimp weight was measured to find weight gain in treated shrimp.

Total weight gain = weight at the end of experiment - weight at start of experiment

Specific growth rate was calculated by the following formula:

$$\text{SGR (\% g/day)} = 100 \times \frac{\ln W_t - \ln W_0}{t}$$

Where SGR is the specific growth rate in weight (%g/day), and W_t and W_0 are the weight of shrimp at current time (t) and at the commencement of the experiment (0), respectively.

Stage-2: Challenging test with WSSV: Shrimp were challenged at PL40 by feeding with macerated shrimp (mincemeat) that had been prepared from a severely WSSV-infected shrimp. The shrimp were fed with probiotic for 3 weeks. In the 4th week of study all the shrimp groups were challenged at PL40 WSSV. 1gm of abdominal tissue was macerated in 10mL of phosphate buffer saline (PBS) 1:10 (w: v) and centrifuged at 3000X g for 20min at 4°C. The supernatant was centrifuged again under the

same conditions (14), and the final supernatant was injected (0.2mL) into the second abdominal segment of probiotic fed shrimp. Control shrimp were injected with PBS. All shrimp injected with infected supernatant were moribund and were dead by 72 h post challenge (hpc). The haemolymph (0.8µl) was extracted and maintained at -80° C for further haemolymph analysis and enzyme assays.

Haemolymph collection: Haemolymph (0.8µl) of individual shrimp (probiotic treated and virus challenged) was drawn from the ventral sinus in the first abdominal segment with a sterile 1-ml syringe (26-gauge hypodermic needle). The syringe was loaded with 200µL of pre cooled anti coagulant solution (10mM Tris Hcl, 250mM sucrose, 100mM sodium citrate, at pH 7.6). More anticoagulant was added to make equal volume ratio of haemolymph to anticoagulant. Six haemolymph samples from each treatment were analyzed individually. 50µl of the anticoagulant-haemolymph mixture was diluted with equal volume of neutral buffered formalin (10%) for 30min to measure the total haemocytes.

The remaining anti coagulated haemolymph was centrifuged at 3000 g for 10 min at 4°C to separate plasma and haemocytes. Then these were immediately frozen in liquid nitrogen and thawed once for assays.

Total haemocytes: Fixed haemolymph was diluted with ice cold phosphate buffer saline (PBS, 20mM, pH 7.2). Total haemocytes and phagocytes were counted using a hemocytometer (Boeco, Germany) and light microscope at 100X.

Percentage of phagocytosis: Phagocytic activity was measured by mixing 200 µl of the diluted haemolymph sample was mixed with 0.2 ml 0.1% paraformaldehyde for 30 min at 4°C to fix the haemocytes then centrifuged at 800g (Model 5403, Eppendorf, Hamburg, Germany) at 4°C. Two hundred haemocytes were counted (15)

Percentage phagocytosis was calculated as follows

Percentage phagocytosis = (no of phagocytic cells / no. of cells observed) X100

Phenoloxidase activity in plasma:

Phenoloxidase activity was measured by recording the formation of dopachrome produced from 3,4-dihydroxyphenyl-L alanine (L-DOPA). Plasma (50µl) was taken and 100µl L-DOPA (3,4-Dihydroxyphenyl- L alanine, 1.6 mg/ml in HBSS) was added. After 10 minuits, OD was measured at 490 nm using spectrophotometer (Hitachi U-2000) (16).

Superoxide dismutase activity. The enzyme activity was measured by the inhibition of photo reduction of Nitro blue tetrazolium (NBT) by the superoxide dismutase. 100µl of serum from each replicate was used. The result was reflected through its optical density at 550nm with UV spectrophotometer. The activity of the enzyme was expressed as units/mg protein. One unit defined as the amount of enzyme causing 50% decrease in the photo reduction of Nitro blue tetrazolium (17).

Catalase enzyme assay: The reaction mixture contained 2 ml of phosphate buffer (pH 7.0), 0.45 ml of hydrogen peroxide (30 mM H₂O₂) in 0.25 ml of enzyme source. The absorbance was read at 240 nm against a reagent blank. The enzyme activity was expressed as µ moles of H₂O₂ metabolized / mg protein / min (18).

Measurement of immunoglobulin like substances:

50ul of serum was diluted using 1ml of saline water then it was used for nephelometry. The concentrations of the immune factors in the serum such as immunoglobulin like substances were measured by the method of (19). 0.5ml of sheep anti-human serum was added to diluted haemolymph, these reagents were mixed thoroughly in the glass tubes and kept in the water bath (37°C) for 15 minutes. All the samples were measured by means of RA-50 Biochemical analyser.

Statistical analysis: The data were expressed as the arithmetic mean \pm standard deviation and were analyzed by one-way –ANOVA. Least significant difference test used to determine the differences among the data with SPSS statistical software (SPSS Inc., USA). Levels of $p < 0.01$ and $p < 0.05$ were considered significant.

Results

Growth of shrimp: After phase -1 experiment all the treated shrimp showed significant increase in weight gain. In 10% probiotic treated shrimp highest weight gain ($3.02 \pm 0.44g$) and specific growth rate (7.16 ± 0.02) was observed when compared to control group (Fig-1, Table-I)

Total haemocyte count: After three weeks of feeding the total haemocyte count in haemolymph of shrimp was significantly ($p < 0.01$) higher in all the probiotic (5%, 10%, 15%) treated groups and in virus challenged shrimp (Fig-2). After virus challenge the total haemocytes increased in the shrimp when compared to control and probiotic treated shrimp. But no significant difference found in 5%, 10% and 15% probiotic treated shrimp.

Percentage of Phagocytosis levels: Phagocytic levels of shrimp fed with probiotic bacterium diets and virus challenged shrimp were significantly ($p < 0.01$) higher than those of shrimp fed control diet after three weeks (Fig-3). Among the shrimp fed with 5%, 10%, 15% probiotic supplemented diets, 10% group shrimp showed high phagocytic levels $4.8 \pm 1.7\%$ and $6.35 \pm 0.26\%$ in shrimp before and after virus challenge respectively and the control group showed $3.9 \pm 0.55\%$.

Immunoglobulin like substances: IgG like substances: IgG like substances in haemolymph are increased in all probiotic treated groups significantly ($p < 0.01$) than that of control. High IgG levels were observed in virus challenged shrimp and the 10% diet fed shrimp showed high IgG levels than 5% and 15% fed shrimp (Fig- 4).

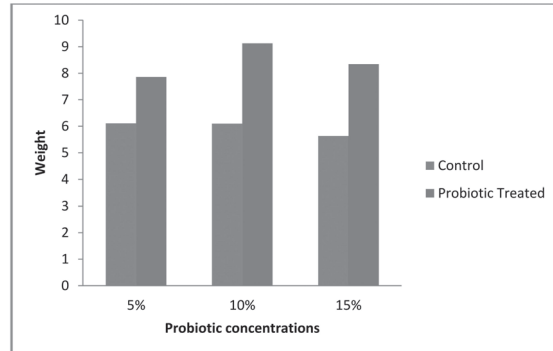


Fig. 1. Growth in weight of prawn *Litopenaeus vanameii* by using different probiotic concentrations

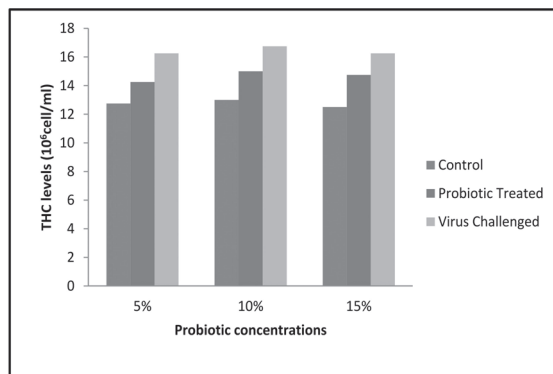


Fig. 2. Total Hemocyte Count levels (10^6 cell/ml) by using different probiotic concentrations

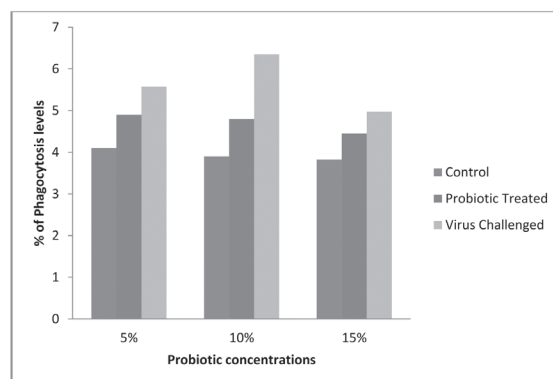


Fig. 3. Effect of different probiotic concentrations % of phagocytosis levels in haemolymph before and after challenge with WSSV

IgA like substances: The IgA like substances levels in probiotic treated groups and virus challenged shrimps was significantly higher ($p < 0.01$) than that of control shrimp groups (Fig-5). The virus challenged groups results higher variation when compared to treated and controls. In all the three groups 10% treated groups showed high IgA levels $3.487 \pm 0.27 \text{ mg/dl}$, the other groups 5% and 15% showed $3.05 \pm 0.29 \text{ mg/dl}$ and $3.09 \pm 0.13 \text{ mg/dl}$ respectively.

IgM like substances: The IgM like substances increased significantly ($p < 0.01$) in all probiotic treated groups as well as virus challenged shrimp. But high IgM was observed in virus challenged shrimp. Among the three treated groups 10% group showed high IgM levels $2.467 \pm 0.44 \text{ mg/dl}$ (Fig-6).

Phenoloxidase activity: Significant ($p < 0.01$) increase in phenoloxidase activity was observed in probiotic treated and virus challenged shrimp and high activity was observed in virus challenged shrimp. Among the three diets 10% diet fed shrimp showed high activity $0.25 \pm 0.03 \text{ min/mg}$ and after virus challenge $0.322 \pm 0.05 \text{ min/mg}$ (Fig-7)

Super oxide dismutase activity: SOD activity increased significantly ($P < 0.01$) in all the shrimp fed with 5%, 10% and 15% diets as well as after virus challenge (Fig-8). But after virus challenge SOD levels increased more when compared to control and probiotic treated shrimp. Among the three diets 10% fed shrimp group showed highest activity $19.585 \pm 0.95 \text{ U/mg protein}$.

Catalase activity: After three weeks of feeding the catalase activity was increased significantly ($p < 0.01$) in all treated shrimp (Fig-9). Among the three diet fed shrimp 10% diet fed shrimp showed high enzyme activity $35.187 \pm 1.48 \text{ } \mu\text{mol/mg/min}$ in probiotic treated shrimp and after virus challenge also high enzyme activity $39.09 \pm 0.91 \text{ } \mu\text{mol/mg/min}$. All the immune parameters of shrimp after feeding of different concentrations of probiotics and after challenge with WSSV were given in tables I & II respectively.

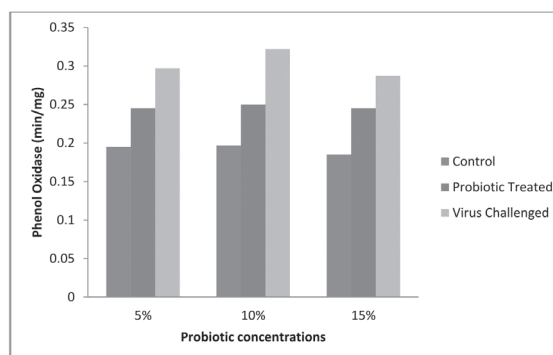


Fig. 4. Effect of different probiotic concentrations on shrimp IgG levels in serum before and after challenge with WSSV

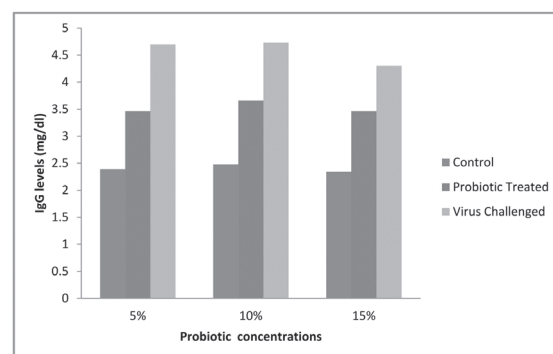


Fig. 5. Effect of different probiotic concentrations on shrimp IgA levels in serum before and after challenge with WSSV

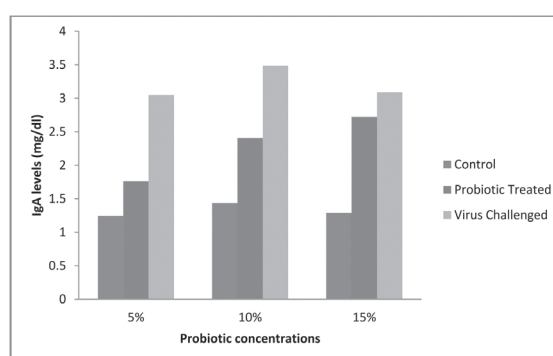


Fig. 6. Effect of different probiotic concentrations on shrimp IgM levels in serum before and after challenge with WSSV

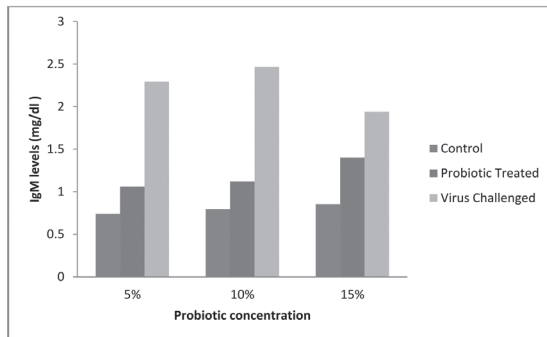


Fig. 7. Effect of different probiotic concentrations on shrimp phenoloxidase enzyme levels in serum before and after challenge with WSSV

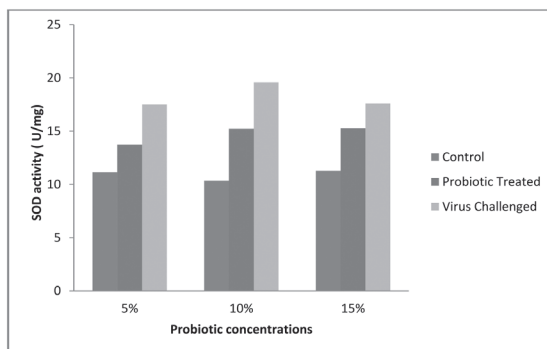


Fig. 8. Effect of different probiotic concentrations on superoxide dismutase levels in serum before and after challenge with WSSV

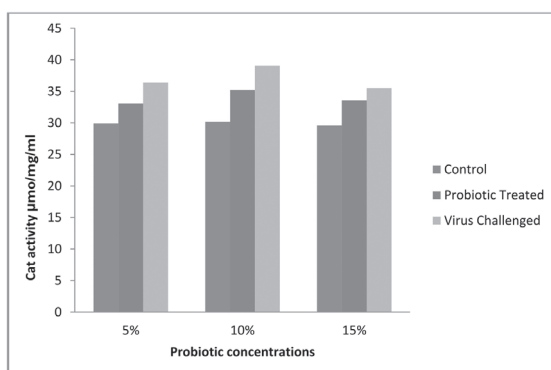


Fig 9: Effect of different probiotic concentrations on shrimp catalase levels in serum before and after challenge with WSSV

Discussion

Shrimp like other crustaceans lack adaptive and specific immune system so they depend on innate immune system (20). In the immune defence system circulating haemocytes play a central role (21). In the present study the mean values of haemocytes were increased significantly before and after virus challenge in all probiotic diet fed shrimp which was consistent with the previous results carried out with the yeast and *Lactobacilli* at the concentration of 1×10^6 CFU gm⁻¹ of feed in shrimp (10). In shrimp like other invertebrates total count of haemocytes are used as a health indicator because they are important non specific immunological parameter, higher THC numbers may provide improved immunity during the periods of increased pathogen loads. In our study we have demonstrated that the phagocytic activity significantly increased in probiotic fed shrimp especially in 10% probiotic fed shrimp. Our results agree with (22), who gave *B. subtilis* E.20 in the diet at a concentration of 10^8 CFU/kg to *L. vannamei* juvenile increases the disease resistance to the pathogenic bacterium, *V. alginolyticus* and immune responses including phagocytic activity. In the present study enhanced phagocytosis was observed in the probiotic fed shrimp after virus challenge. This result was agreed with the study in *L. vannamei* by (23) who observed the enhanced phagocytosis against shrimp pathogen after challenge with the *Vibrio harveyi*. Phenoloxidase cascade plays a key role in the shrimp humoral response (24). In the present study significant increase in phenoloxidase (PO) was observed in both probiotic diet fed shrimp and in virus challenged shrimp. Phenoloxidase activity was higher in shrimp when *Bacillus cereus* was fed along with diet than control diet shrimp (25). Another study also reported that PO activity tended to be high in the group fed with probiotic *L. plantarum* supplemented diet and challenged with *V. harveyi* in the shrimp *L. vannamei* (26), the present work results are coinciding with this. A significant alteration in PO activity was observed in probiotic treated shrimp when challenged with virus WSSV.

Table 1. Growth in weight of Prawn *L.vannamei* after feeding with different concentrations of probiotics

Concentration of the Probiotic	Control (gms)	Probiotic treated (gms)	Weight gain (gms)	DWG ^a (g/d)	RGR ^b (%)	SGR ^c (%/day)
Control	5.74±0.04	7.24±0.07	1.50±0.03	0.07±0.01	26.1±0.06	5.90±0.25
5%	5.86±1.54	7.61±0.61	1.75±0.87	0.083±0.05	29.86±0.12	5.95±0.01
10%	6.10±0.58	9.12±0.14	3.02±0.44	0.143±0.36	49.5±0.83	7.16±0.02
15%	5.63±0.32	8.34±0.11	2.71±0.21	0.129±0.1	48.13±0.03	7.01±0.30

Table 2. Immune parameters of shrimp after feeding with different probiotic concentration

Immune parameter	Control	5%	10%	15%
Total hemocytes(10 ⁶ /ml)	12.75±0.96	14.25±0.96	15±1.15	14.75±0.96
% of phagocytosis	4.1±0.47	4.9±0.48	4.8±0.71	4.45±0.34
IgG(mg/dl)	2.39±0.25	3.465±0.11	3.665±0.16	3.46±0.19
IgA(mg/dl)	1.242	1.76±0.17	2.407±0.37	2.72±0.66
IgM(mg/dl)	0.74±0.08	1.06±0.14	1.22±0.12	1.4±0.28
PO(U/min/mg)	0.195±0.03	0.245±0.02	0.25±0.03	0.24±0.02
SOD ((U/mg) a)	11.157±0.85	13.745±1.20	15.237±1.53	15.26±1.26
Catalase(μmol/mg)	29.887±1.54	33.057±1.52	35.187±1.48	39.08±0.91

Table 3. Immune parameters of shrimp after virus challenge

Immune parameter	Control	5%	10%	15%
Total hemocytes(10 ⁶ /ml)	12.75±0.96	16.25±0.5	16.75±0.96	16.25±1.5
% of phagocytosis	4.1±0.47	5.575±0.43	6.35±0.26	4.975±0.09
IgG(mg/dl)	2.39±0.25	4.73±0.19	4.3±0.19	
IgA(mg/dl)	1.245±0.18	3.487±0.27	3.09±0.13	
IgM(mg/dl)	0.74±0.08	2.467±0.44	1.94±0.17	
Phenol oxidase(min/mg)	0.195±0.03	0.297±0.01	0.322±0.05	0.287±0.03
SOD ((U/mg) ^d)	11.157±0.85	17.505±0.59	19.585±0.95	17.67±0.60
Catalase(μmol/mg)	29.887±1.54	36.40±1.97	39.08±0.91	35.51±1.48

a – Daily Weight gain

b-Relative Gain Rate

c-Specific Growth Rate

d-Super Oxide Dismutase

In the present study some proteins which are like IgA, IgM and IgG were measured in haemolymph of the probiotic diet fed and control group shrimp, we found that there is a significant increase of these immunoglobulin like substances in all group shrimp and after virus challenge. The high IgG, IgM, IgA were observed in the 10% probiotic diet fed shrimp than 5% and 15% diet fed shrimp. According to (27) eleven pattern recognition particles which include immunoglobulin like proteins are present in shrimp haemolymph play a role in the immunity of shrimp against infection. A higher immunoglobulin like substances in probiotic treated and virus challenged shrimp may be indicates possible immune reactive effect of lactobacillus against WSSV.

Shrimp fed with probiotic bacteria showed significant increase in weight gain and specific growth rate. Similar results were reported by in Indian white shrimp (28)

Fenneropenaeus indicus. As a result of pathogen pressure and environmental changes, aquatic animals were peculiarly susceptible to oxidative stress (29). Shrimp have integrated antioxidant system includes enzymatic antioxidants (30). In the present study increased SOD activity and catalase activity was observed both in probiotic fed shrimp and in virus challenged shrimp. High SOD activity was observed in the shrimp *L.stylosstris* which were fed with probiotic bacteria and infected with *V. nigripulchritudo* (29). This indicates that antioxidant system was enhanced by giving the diet containing probiotic bacteria. Administration of *L.Plantarum* in diet at 10^{10} CFU (Kg diet)⁻¹ induced increased SOD activity significantly and enhanced the immune ability of *L.Vannamei* and increased its resistance to *V.alginolyticus* infection (12). In our present study higher SOD activity levels were observed in the treated shrimp feed and virus challenged than control group, high or SOD activity was observed in 10% probiotic fed. This may suggest that superoxide radicals overly induced by immune responses

when virus challenged to counterbalance this radical SOD appeared better activity.

Conclusion

In conclusion, using the probiotic bacterium *Lactobacillus sps* results from stage-1 and stage- 2 showed that 10% concentration can achieved the highest score, followed by 15% and 5%. Furthermore, 10% probiotic concentration was the most effective in the shrimp diets. In the present study, shrimp fed probiotic supplemented diets produced beneficial outcomes in terms of immune parameters (THC, percentage of phagocytosis, immunoglobulin like substances levels) and antioxidant response (catalase and superoxide dismutase enzyme) when challenged with WSSV compared to control diet.

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Screening and Profiling of Sandal Seeds collected from Provenances of Karnataka

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Abstract

Sandalwood trees are the only source of precious natural sandal oil obtained from the heartwood. The tree develops heartwood over a period of twenty years. However, flowering and seed set are observed within two years of planting. Seeds serve as a potential source of an important therapeutic compound and therefore ensures steady income to the growers. Indian Sandalwood and Australian sandalwood are the source of a unique fatty acid called Ximenynic or Santalbic acid. Sandal grows in abundance in many states of South India. Santalum album or East Indian Sandalwood cultivation is on the rise in Karnataka. As the Indian Sandalwood (S.album) is grown in several parts of Karnataka state, a study was taken up to find out the relative content of Ximenynic acid from different provenances, besides comparing with the Australian Sandalwood. Seeds were collected from different provenances of Karnataka. Using hexane as a solvent extraction of seeds were carried. Seed were evaluated for their physico-chemical and fatty acid profiles. Since, Santalum spicatum, the Australian sandalwood is generally used as a source of Ximenynic acid. The present work has established the superiority of S.album seeds over S. spicatum with respect to Ximenynic acid content. Gas-Chromatography-Mass Spectrometry analysis of the fatty acid profiles of six samples of Santalum album L. recorded higher percent of Ximenynic acid as compared to Santalum spicatum. Therefore, Santalum album L. seed oil can be used as a potential source of intermedi-

ate income for farmers besides being a natural source of an anti-ageing ingredient.

Key words: Santalum album, Ximenynic acid, Gas chromatography, Provenances.

Sandalwood is one of the most valuable and commercially important tree belonging to the family Santalaceae. Among the eighteen known species of sandalwood belonging to the genus Santalum, the East Indian Sandalwood Santalum album L. is renowned for its oil which has multiple use in perfume, pharmaceutical and cosmetic sector [1]. Santalum album L. is native of south India mainly Karnataka, Kerala, Andhra Pradesh and Tamil Nadu., generally growing at altitudes of 2000-3000 feet, thriving in soils preferably on slopes of hills exposed to sunlight [2]. The heartwood is highly rated for its oil and valuable timber after twenty five years. However, the seeds can become a potential source of income, as intermediary because sandalwood tree produce seeds from 3 years of age and can yield 1-2 kg per tree on an average. Each seed weighs 2.2 g; large seeds are easy to collect and the hard shell keeps the seed intact intact [3]. In Australia, where S. spicatum sp. is found seed kernels are a valuable part of the traditional aboriginal diet and consumed as a treatment for Rheumatoid Arthritis. Seeds are valued as commercially viable nuts. Nuts can be developed as a food crop and this would provide continuing cash benefits for growers until the trees become ready for harvesting wood. These seeds are very rich in fixed oil use-

ful in making soaps and other preparations. The seed oil is characterized by the presence of unusual acetylenic fatty acid, known as Ximenynic acid/Santalbic acid which occurs only in Santalaceae and oleaceae families. Various works on Ximenynic acid have proved that it is one of the best cosmetic ingredient. It is shown to increase cellular detoxification and anti-oxidation capacity, in strengthening of cellular matrix, improving skin elasticity, dermal strength and very effective against cellulite and hair loss (Glenn, Evonik Industries; TEGO® Xymenynic). The global anti-ageing market is driven primarily by the rise in the ageing population in the world, especially the nations that hold the majority of the baby boomer population whose major concern is physical health and beauty. A Research report (Transparency Market Research, 2015) has forecast the global anti-ageing market to grow at a CAGR of 7.8 % between 2013 and 2019. With the market value being \$122.3 bn in 2013, it is expected to reach \$191.7-bn by the end of 2019. Also, as the area under cultivation is increasing due to farmer friendly policies of trade transactions, many farmers have taken up Sandalwood cultivation. Hence, seeds so produced shall not be wasted. Thus, the present research is aimed at commercialising the use of seeds by screening and profiling for active principle, the Ximenynic acid content to exploit its pharmacological application. Profiling of *Santalum album* seeds from different provenances of Karnataka were taken up to find out any inherent difference among the provenances.

Materials and Methods

The lab studies were carried out at Medicinal & Aromatic Crop section, Department of Horticulture, UAS, GKVK, Bangalore-65

Collection of Sandalwood Seeds: *S. album* seeds were collected from six regions of Karnataka viz. Magadi, Shimoga, Heggedevana Kote (Begur Tiger Reserve), Lingarajpuram (Bangalore), GKVK, UAS(B) campus and Meriyur-Kerala (Table 1).

Extraction of oil from Sandalwood Seeds:

The outer hard shell (Endocarp) of the seed was

removed manually, the inner, oil rich kernel was used for extraction of the fatty acid. A known weight of the seeds was taken and ground to a fine paste using electric blender (Havells). Hexane, the extracting solvent was used to seed oil was extracted oil from the seed paste using Soxhlet Apparatus. The seed oil extracted was concentrated using Rotary Vacuum Evaporator [4,5]. The extractable yield (%) of the oil was recorded and refrigerated in glass bottles until further use (Table 2).

Quality Analysis of Sandalwood seed oil:

The extracted oil was evaluated for its organoleptic (color and odour). Physical (Refractive Index) and chemical (Acid value, Saponification value, Ester value) for assessing the quality of the fatty acid (5) (Table 3).

Coverision of Fatty Acid to Fatty Acid Methyl Esters:

Esterification is the reaction of carboxylic acid with an alcohol to produce an ester and water in the presence of an acid catalyst such as H_2SO_4 . The decrease in the free fatty acid level of oil/fat is an indication that FFA are consumed in the esterification reaction. In the reaction, water and glycerol are the by-products.

The raw material used in the present esterification tests was the sandalwood seed oil using acid esterification method for converting free fatty acids in acid oil or acid fat into fatty acid methyl esters. The method involved addition of excess methanol and an acid catalyst to the oil/fat and subjecting the mixture to conditions that allow the formation of Fatty Acid Methyl Esters (FAMES). The excess methanol used is for driving the reaction to completion (6,7,8,9). In the present investigation about 0.5g – 1.0g of sandalwood seed fatty acid was weighed and treated with 8% sulphuric acid with methanol as a catalyst, to improve the reaction rate and yield. The mixture was esterified at 60°C for 2 hours on a hot plate (FIG 2) with the condenser attached. The optimal conditions used were consistent with the industrial practice. The

reaction mixture was cooled and about 100ml of distilled water was added to separating funnel followed by the addition of 100ml diethyl ether. The process was repeated 4 times. At the end of the reaction, the reaction mix can be separated into top lipid phase containing FAMES triglycerides and unreacted FFA and a bottom phase containing excess methanol, acid catalyst, water and glycerol along with impurities. The top lipid phase is filtered through sodium sulphate and evaporated to obtain FAME [shorter reaction time has to be followed because there is little gain after 5-10 hr of reflux]. Methyl esters offer excellent stability, high volatility, provide quick and quantitative samples for GC analysis (10).

Gas Chromatography-Mass Spectrometry:

Fatty acid profiling of the Fatty Acid Methyl Esters (FAME) was analysed through Gas Chromatography -Mass spectrometry in Shimadzu-QP-2010 S instrument, equipped with DB-23, 30m*0.25mm*0.25 μ m. Helium was used as a carrier gas (1ml/min), inlet and detector temperatures are 220°C and 225°C respectively (3). The fatty acid profile of sandalwood seed oil extracted from seeds procured from six different provenances of Karnataka are tabulated (Table 4)

Results and Discussion

Traditionally the seeds have been used in Ayurvedic treatments to make skin smoother, tauter and more velvet. Various pharmacological studies of the sandalwood seed oil have also proved neither toxicity nor pathological damaging. The seeds are very rich in fixed oil and is a rich source of phytochemicals exhibiting antioxidant property. Sandalwood seed oils are widely used in cosmetics and nutraceuticals, with high concentrations of Ximenynic acid and other poly unsaturated fatty acids [11].

The present research work was aimed at the Indian sandalwood (*Santalum album*) seed oil as a rich source of Ximenynic acid compared to *Santalum spicatum*. The seed oil was extracted from seeds collected from six provenances of Karnataka (Table 2 & 3) using hexane as an

extracting solvent in Soxhlet Apparatus. The study revealed that sandalwood seeds have been shown to have a promising oil yield ranging from 15.27% -56.24% (Table 5). The highest seed oil yield was recorded in seed sample collected from Shimogaprovenance (56.24%) followed by Lingarajpuram (40.20%) and least was recorded in GKVK, UAS(B) campus (15.27%). Hettiarach chii et al. (11), reported that heavier kernels produce lesser oil yield(%), this could be due to variation in carbohydrate and protein content compared with lipids. Geographic, nutritional, climatic factors and other conditions diminish lipid synthesis while other constituents relatively increase producing heavier kernels with less oil. The physico-chemical parameters of sandalwood seed oil investigated for the samples collected from six provenances of Karnataka are presented in Table 4.

Fatty acid Profile: Fatty acid composition of the Sandalwood seed oil was analysed by Gas Chromatography-Mass Spectrometry for their methyl esters. In addition to the presence of oleic acids, a distinctive feature of sandal wood seed oil is the presence of unusual and rare acetylenic fatty acid, Ximenynic acid which was significant in all the six samples. The present work highlights the superiority of *S. album* seeds (69.32-85.25%) over *S. spicatum* (32-39%) [12] which is generally used as a source of Ximenynic acid.

In the present study, the fatty acid profile of the respective methyl esters of the respective samples recorded Oleic and Ximenynic acid together made up to 93.80 % of the total fatty acids recorded (Fig 1, 2). The sandalwood seeds collected from six different provenance showed variation in the Ximenynic acid content (%) in the range of 69.32-85.32%. The fatty acid extracted from seeds of GKVK, Bangalore campus location was found to be richest in Ximenynic acid (85.32%) followed by Meriyur (85.25%), Lingarajpuram (84.33%) and least was recorded in the fatty acid extracted from Magadi region sample (69.32%).

Fig 1: Fatty acid profiling of Standard fatty acid mixture

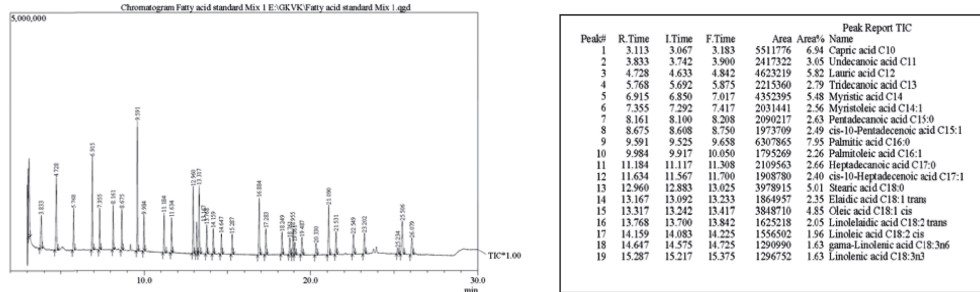


Fig 2. Fatty acid profiling of Sandal seed oil

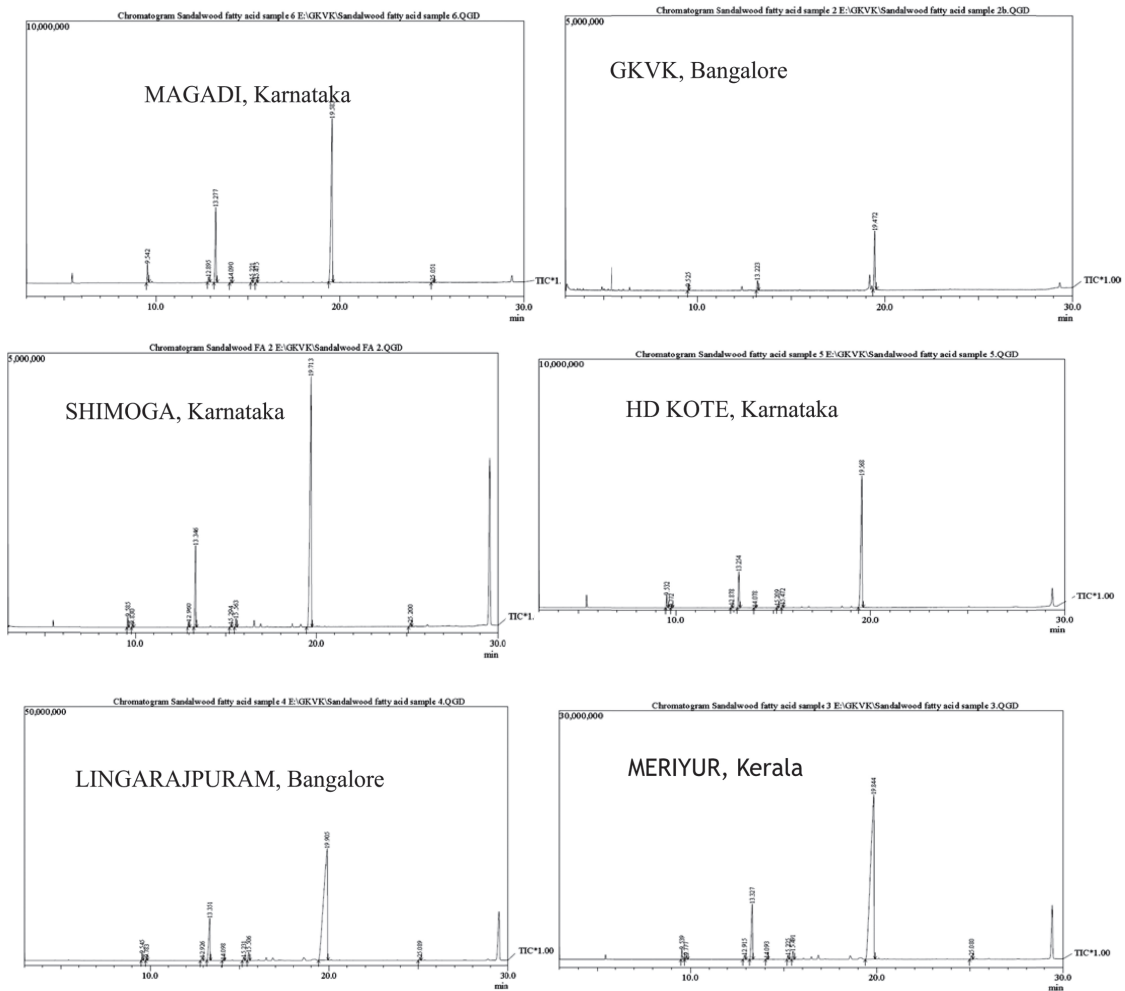


Table 1 : Provenances details of Sandal trees

Sl.No.	Sample	Date of Harvest	Age of tree (yrs)
1	Magadi, Karnataka	1-10-2014	3
2	GKVK, Bangalore	11-08-2015	10
3	Shimoga, Karnataka	19-09-2015	20
4	H.D.Kote, Karnataka	05-09-2015	16
5	Lingarajpuram, Bangalore	17-09-2015	40
6	Meriyur, Kerala	-	-

Table 2: Morphometric parameters of seeds

Sample	Mean Diameter (mm)	Seed : Seed coat
1 Magadi, Karnataka	8.37	76.3:23.6
2 GKVK, Bangalore	9.62	59.8:40.1
3 Shimoga, Karnataka	8.00	*
4 H.D.Kote, Karnataka	7.87	60.6:38.4
5 Lingarajpuram, Bangalore	7.75	*
6 Meriyur, Kerala	7.50	*

Note: * seeds without seed coat

Table 3: Physical and Chemical parameters of the Sandal seed oil

Sl.No	Sample	Color	Appearance	RI@26.5°C	AV	EV	SV
1	Magadi, Karnataka	YELLOW	VISCOUS	1.488	00.75	157.11	157.86
2	GKVK, Bangalore			1.491	03.70	327.30	331.00
3	Shimoga, Karnataka			1.479	11.22	402.12	413.34
4	H.D.Kote, Karnataka			1.482	04.11	329.18	333.29
5	Lingarajpuram, Bangalore			1.490	10.20	459.08	469.30
6	Meriyur, Kerala			1.481	10.20	510.09	520.29

[RI: Refractive Index; AV: Acid Value; EV: Ester Value; SV: Saponification Value]

Table 4: Fatty acid profile of Sandal Seed oil -Gas Chromatography-Mass Spectrometry

Sl.No. Sample	Seed oil (%)	Oleic acid (%)	Ximenyic acid (%)
1 Magadi, Karnataka	20.80	21.47	69.32
2 GKVK, Bangalore	15.27	11.22	85.32
3 Shimoga, Karnataka	56.24	14.44	79.89
4 H.D.Kote, Karnataka	36.60	13.44	79.28
5 Lingarajpuram, Bangalore	40.20	10.35	84.33
6 Meriyur, Kerala	20.96	8.5	85.25
<i>Santalum spicatum</i> *	50-55%	27.9-37.3	27.9-37.3

Note: * Butand *et al.*, 2008

The oleic acid content recorded was in the range 10.35-21.47%. Of all the six samples analysed, seeds collected from Magadi recorded highest content of oleic acid(21.47%) as compared to the seeds collected from other regions, the least being recorded in Meriyur sample(9.87%).

The variation of Ximenynic and oleic acid in the Santalum album L. seed oil observed might be due to differences in geography and climate, age of the tree and season of harvest. It is observed that oleic and Ximenynic acid concentration increase as the seed matures while the other saturated and unsaturated fatty acids decrease. It is reported that Tropical varieties are rich in Ximenynic acid when compared to temperate and arid regions. A geographical study conducted reported that in a single season Ximenynic content varied from 27-38% within different geographical and climatic zones of Western Australia [3]. Oleic acid is a precursor for sterolic acid which then gives rise to Ximenynic acid. Production of Ximenynic acid in seed oils of Santalaceae family is also considered to be a defense mechanism against predators or pathogens. And these acetylenic moieties have been shown to possess medicinal properties such as antimicrobial, anti-fungal, insecticidal and anticancerous.

Conclusion

Santalwood seeds [*Santalum album*] from provenances of Karnataka and Kerala have been found to contain high percentage of anti-ageing compound as compared to *Santalum spicatum*. Observed variability of Ximenynic acid could provide better utilization of the natural resource. *Santalum album* seeds can thus be utilized as the best natural and sustainable source of Ximenynic acid in high end cosmetics.

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Detection and Production of Gallic acid from Novel Fungal strain- *Penicillium crustosum* AN₃ KJ820682

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Abstract

Phenolic compound 3,4,5-trihydroxy benzoic acid commonly known as gallic acid, an important multipurpose ingredient harboured from an ascomycetes fungus which was previously isolated from an apple orchard soil and identified as *Penicillium crustosum* AN₃ (Gene bank accession no. KJ820682). The product gallic acid was obtained during fungal fermentation studies. To achieve maximum yield of the product, optimization studies were performed and maximum yield of 9.29 mgg⁻¹ was obtained at 30°C with fermentation medium pH 5.5, 0.5% tannic acid as inducer and 30% substrate moisture ratio. The obtained gallic acid was detected using TLC and Rf value of 0.57 was obtained. The structural compounds containing OH, COOH, C-C bonds were deduced by ATR-FTIR spectroscopy. HPLC was carried out to calculate the peak and retention time (tR = 5.749 min) of product formed by the fungal isolate.

Keywords: Gallic acid, *Penicillium crustosum* AN₃, Production optimization, Analytical techniques

Introduction

3,4,5-trihydroxy benzoic acid, a phenolic compound commonly known as gallic acid found in gallnuts, grapes, tea, hops and oak bark. From the available literature it was found that a Swedish chemist Carl Wilhelm Scheele was the first one who studied about gallic acid in the year 1786. It is formed during shikmic acid pathway in the

plants (5). Due to its wide applications in several aspects it became an important product for which studies to be taken. Literature cited that it is an important precursor for trimethoxybenzaldehyde which in turn is a precursor for the production of trimethoprim, an antibacterial agent used in combination with sulfonamide (7). Propyl gallate (gallic acid ester) used for pyrogallol preparation whose application in staining fur, leather and hair (7). In writing inks and dyes, gallic acid used as photographic developer (11, 12). In the findings of Rajendran *et al.* (19) gallic acid is one of the important ingredients in horse gram decoction or kulatha kasaya, which further act as a purifying agent during the preparation of *Lauha bhasma*. Gallic acid-lecithin complex (combined by a non-covalent bond) is an effective scavenger of DPPH radicals (10). Besides this various literature available which shows the gallic acid and its derivatives possess a large number of biological activities like antimicrobial, anticancer, antiviral, anti-inflammatory, analgesic and anti-HIV activities (1, 4, 9, 17, 20, 23, 24).

Microorganisms - the alternative source of gallic acid:

Due to complex structures and stereo specificity of plant derived compounds there chemical synthesis become uneconomical (15). Further, due to interference of unknown impurities, extraction of plant secondary metabolite is not easy. Chemical hydrolysis of tannic acid results in poor and less yield of gallic acid (2). This results in the release of the huge amount of toxic effluent which act as pollutant

and affecting the ecosystem (3). Ultimately an economical, non hazardous and eco friendly process for gallic acid production is the use of microorganisms in fermentation processes through cheap agrobased residues. Present work was therefore envisaged towards gallic production through SSF of pine needles using novel fungal strain (*P. crustosum* AN₃ KJ820682) and its detection through analytical techniques viz., TLC, FTIR and HPLC studies.

Material and Methods

Substrate: Mature fallen pine needles were collected from fields of Silviculture, Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni (Solan), washed thrice with running water, dried at 50°C in oven for 72 h and then ground in electric grinder to a fine powder. These were packed in polythene bags for further studies.

Microorganism: *Penicillium crustosum* AN₃ KJ820682 used throughout this work was previously isolated from apple orchard soil (Distt Kinnaur, H.P) identified by 18SrRNA sequencing, following nucleotide blast and deposited in the National Centre for Biotechnology Information (NCBI). Spore suspension (containing about 1×10^7 spores ml⁻¹) was freshly prepared from preserved cultures of *P. crustosum* AN₃ grown on malt extract agar (MEA) slants at 30°C and mixed with 10 ml of 0.1% sterile Tween -20 prepared in normal saline.

Gallic acid production by solid state fermentation: Fungal strain was cultivated in 300 ml glass jars containing 3g of pine needles as support material for solid state fermentation with mineral salt solution (9 ml) having the composition of (g/l): Potassium dihydrogen phosphate (KH₂PO₄) -0.5, dipotassium hydrogen phosphate (K₂HPO₄) - 0.5, magnesium sulphate (MgSO₄) -2.0, ammonium chloride (NH₄Cl) - 3, calcium chloride (CaCl₂) -1.0, agar -20 having pH 5.5.

Optimizing parameters for gallic acid production: Tannase biosynthesis was performed by optimizing various physicochemical

process parameters where optimum conditions found for each parameter was settled for the subsequent experiments. Process parameters viz., effect of incubation temperature (25 to 45°C) for different incubation periods (24 to 120 h) was explored. Initial pH value of the fermentation medium (4.5 to 6.5) was adjusted by 0.1 M HCl or NaOH. Effect of moisture content (10% to 50%) was estimated. The effect of various initial tannic acid concentrations (0.05 to 1.5 g %, (w/v) was also optimized.

Estimation of gallic acid: Gallic acid was estimated by spectrophotometric method using methanolic rhodanine at 520 nm as per the procedure of Sharma *et al.* (2000). Gallic acid is expressed as mgg⁻¹.

Detection of gallic acid by thin layer chromatography (TLC): TLC on silica gel plates G-60 F254 with solvent system of ethyl acetate, chloroform and formic acid (4:4:1) was performed for gallic acid detection in fermented filtrate. Spotting of standard gallic acid and sample (fermented filtrate) was carried out on the same plate with the help of capillary. Plates were kept in TLC chamber for two hours and a solution of FeCl₃ was used as spraying reagent for plates development (13). Retention factor (Rf) value for standard and sample was calculated from the chromatogram.

Detection of gallic acid by Fourier-transform Infra-red spectroscopy (FTIR spectroscopy): Instrument for FTIR analysis was first calibrated for background signal- scanning without any sample (air blank), and then the experimental samples were scanned. Crude fermented filtrate was lyophilized in the lypholyser (CHRIST, Delta 2-24 LSC plus Lypholizer) and then detected by ATR FTIR (Attenuated Total Reflectance) spectroscopic analysis against commercially available gallic acid powder used as standard at Center of Innovative and Applied Bioprocessing (CIAB), Mohali.

Detection of gallic acid by high performance liquid chromatography (HPLC): The separation and identification of gallic acid was performed

by HPLC (Agilent Technologies, 1260 infinity, DAD) at CIAB, Mohali. Mobile phase was a mixture of methanol :water (1:9 both containing 0.02%TFA) delivered at a flow rate of 0.5ml min⁻¹. Standard gallic acid (1mg/ml) and fermented filtrate were injected at a volume of 50 μ l to eclipse plus C18 column (4.6 x100mm, 3.5 μ).

Results

For the first time, we report the detection of gallic acid in the fungal strain *Penicillium crustosum* AN₃ using natural forest waste of pine needles. By incubating the wastes directly with the crude enzyme tannase produced by *Penicillium crustosum* AN₃ for different time intervals ranging from 24 to 120 h, the amount of gallic acid formed was determined. Figure-1 shows clear increase of gallic acid yield up to 96 h (8.42 mg g⁻¹) and after that, the level was decreased gradually. The effect of different temperature ranges from 25-45^oC on gallic acid production is shown in Fig. 2. A temperature of 30^oC was found to be the optimum for gallic acid production (7.333 mgg⁻¹). Any increase decrease from optimum temperature affect the mycelial growth, hence, low levels of gallic acid were produced at high and low temperatures.

A pH of 5.5 was found to be the most suitable for high gallic acid production (8.21 mg g⁻¹). As the pH increases it results in gradual

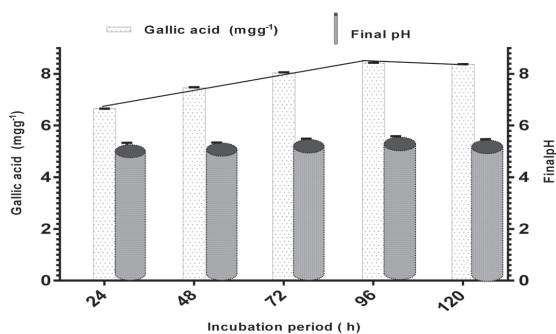


Fig. 1. Effect of incubation time on the production of gallic acid (GA) from pine needles using *P.crustosum* AN3. Error bars represent the standard error determined in the triplicate experiments

reduction of gallic acid (Fig.3). As shown in Fig.4, yield of gallic acid increases with an increase in moisture content up to 30% (8.29 mgg⁻¹). In case of tannic acid, its concentration of 0.5% (w/v) was found to be most optimum for gallic acid production. Further increase in tannic acid concentration causes a decrease in gallic acid production (Fig.5). Probably at low concentration of substrate molecule (tannic acid) some of the active sites of enzyme (tannase) remain unoccupied and less product (gallic acid) was formed whereas at high concentration, substrate molecules compete to bind with active site of the

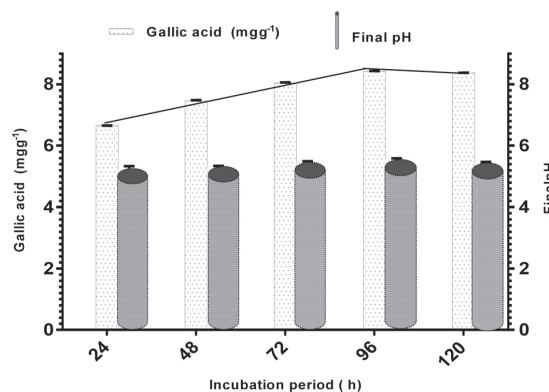


Fig. 2. Effect of temperature on the production of GA from pine needles using *P.crustosum* AN3. Error bars represent the standard error determined in the triplicate experiments

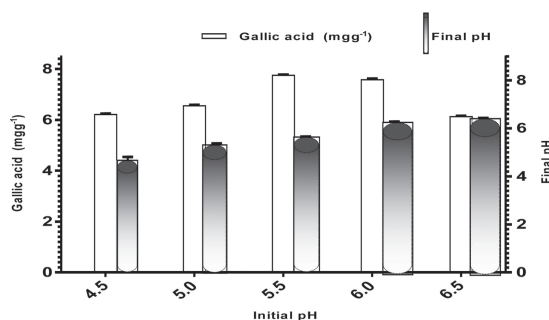


Fig. 3. Effect of initial pH on the production of GA from pine needles using *P.crustosum* AN3. Error bars represent the standard error determined in the triplicate experiments

enzyme which provide stearic hinderance and a less yield of product was observed.

Thin layer chromatography (TLC): Figure- 6 shows TLC separation of gallic acid from crude fermented fungal filtrate on silica gel plate with proper mobile phase have showed higher sensitivity in terms of standards. Spots were blue after spraying with $FeCl_3$. The precise measurement of Rf value of standard and gallic acid was found to be 0.56 and 0.57, respectively which confirmed presence of gallic in the fermented filtrate.

Fourier transform infra-red spectroscopy (FTIR): The FTIR spectrum for gallic acid in culture filtrate includes various infrared functional groups within the range of 3226.170 cm^{-1} , 1636.218 cm^{-1} , 1337.527 cm^{-1} , 1208.003 cm^{-1} , 1030.378 cm^{-1} (Fig.7).

High performance liquid chromatography (HPLC): HPLC was performed to detect the hydrolytic product of tannase (gallic acid) in culture filtrate. Solvent A (methanol) was increased to 10% subsequently while solvent B (water) to 90% at a flow rate of 0.5 ml min^{-1} up to 14 minutes. Compound was detected at a wavelength of 245 nm . Retention time (tR) for standard gallic acid solution and extracted gallic acid were 5.747 and 5.749 min, respectively. Tannase effectively hydrolyzed tannins of pine needles and produced gallic acid as main product

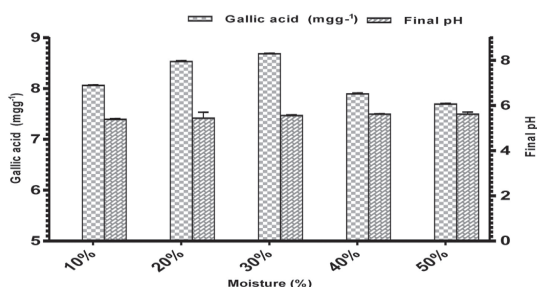


Fig. 4. Effect of moisture level (%) on the production of GA from pine needles using *P.crustosum* AN3. Error bars represent the standard error determined in the triplicate experiment

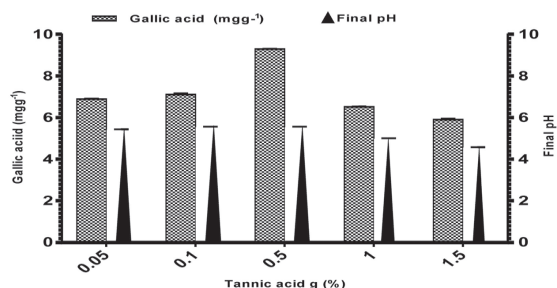


Fig. 5. Effect of tannic acid g (%) on the production of GA from pine needles using *P.crustosum* AN3. Error bars represent the standard error determined in the triplicate experiments



Fig. 6. TLC chromatogram of standard gallic acid and fermented filtrate
 A: Spot of standard gallic acid B: spot of gallic acid present in fermented filtrate

in the filtrate when compared with standard gallic acid peak. In control flasks no gallic acid production was observed as it was not inoculated with spore of *P. crustosum* AN₃ (Fig 8).

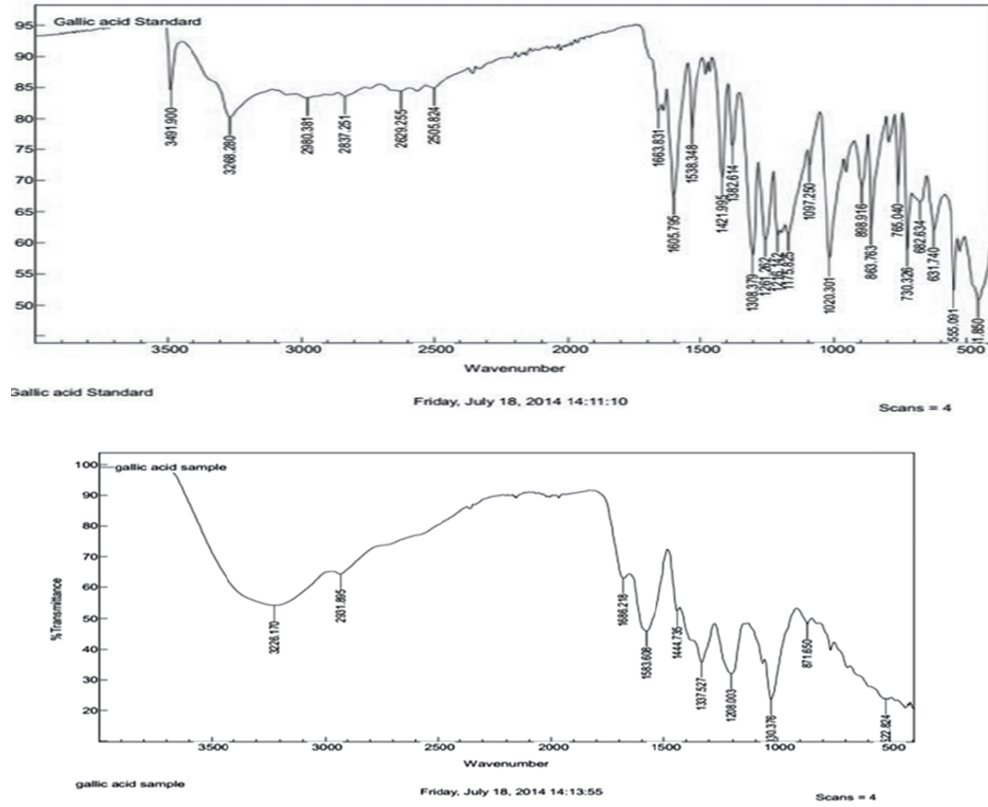


Fig. 7. FTIR spectra of standard gallic acid and fermented filtrate

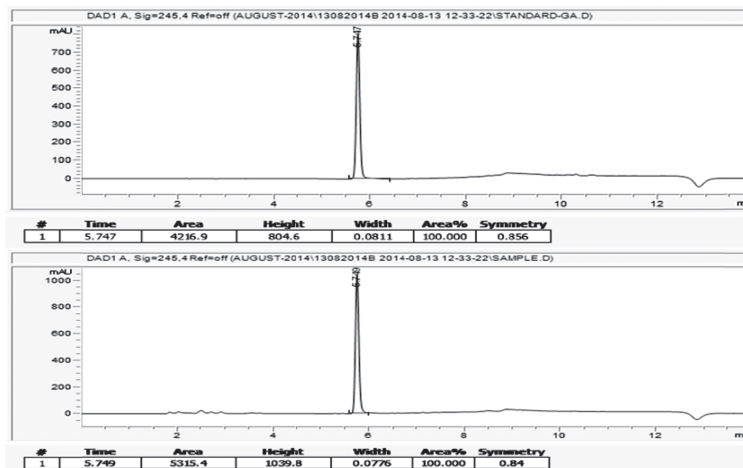


Fig. 8. HPLC chromatogram of standard gallic acid and fermented filtrate

Production of gallic acid from *Penicillium crustosum*

Discussion

Saxena *et al.* (20) explained that *Aspergillus* and *Penicillium* spp. could utilize catechin, gallotannin and gallic acid as carbon sources and thus cause decrease in gallic acid. Nalan and Merih (14) recorded that for different species of *Aspergillus* and *Penicillium*, the initial increase in gallic acid production, from gall nuts and sumac leaves, was followed by a clear decrease. The decrease in the rate of gallic acid formation may be either due to competitive inhibition of gallic acid or denaturation of the enzyme as a result of prolonged incubation period. Additionally, gallic acid may enter into other pathways catalyzed by other enzymes present in the crude preparation of tannase. Gallic acid is a soluble phenolic compound may also polymerize to form macromolecules (6). Vazquez-Duhalt *et al.* (25) stated that the decrease in measurable soluble phenolics could be in response to the polymerization of the released soluble phenolics at the late stage of incubation. According to Raimbault (18) increment of moisture level reduces the porosity of solid particles in the substrate which limits the transfer of oxygen whereas low moisture levels causes reduction in the solubility of nutrients of the substrate which results in high water tension.

Conclusion

For the first time, an attempt was made to produce gallic acid using pine needles as agrobased residue by *P. crustosum* AN₃ strain. We report here the extraction and enhanced production of gallic acid by varying the parameters which may help in large scale production.

Acknowledgments

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Protective Effects of Zinc and Vitamin-E for Arsenic Induced Mitochondrial Oxidative Damage in Rat Brain

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Abstract

This study was planned to determine the influence of age on arsenic (As; 10mg/kg body weight given through oral gavage) induced mitochondrial oxidative stress in three different age groups of rats; young (postnatal day [PND] 21), adult (3 months) and aged (18 months) at seven days post-acute exposure. Further, we also evaluated the therapeutic efficacy of essential metal Zinc (Zn; 0.02% through drinking water) and an antioxidant, α -Tocopherol (Vit-E; 125mg/kg body weight through oral gavage) against As-induced neurotoxicity. As exposure lead to a significant decrease in mitochondrial Superoxide Dismutase isoforms (Mn-SOD and Cu/Zn-SOD), Catalase (CAT), Glutathione reductase (GR), Glutathione peroxidase (GPx), non-enzymatic antioxidant levels of glutathione (GSH), total free sulfhydryl groups (TSG) with concomitant increase in lipid peroxidation and GST in different brain regions (cerebral cortex, cerebellum and hippocampus) of different age groups of rats. These effects were more pronounced in cerebral cortex followed by cerebellum and hippocampus. Among the three different age points, aged animals were found to be more vulnerable to the As-induced toxicity as compared to young and adult animals suggesting that As neurotoxicity is age dependent. These As-induced alterations were significantly reversed following supplementation with Zn or Vit-E. However, Vit-E was found to elicit greater protection as compared to Zn in restoring the

altered neurochemical perturbations, providing evidence for As induced oxidative damage.

Key words: Age, Arsenic, Zinc, Vitamin-E, Oxidative stress, Brain regions

Introduction

The mechanism underlying aging, an unavoidable biological process that affects most living organisms, is still an area of significant controversy. One of the factors responsible for these diseases includes heavy metals accumulation. Metals are of special concern because of their presence in the environment, and the metals studied, exposure to arsenic (As) contaminated drinking water represents one of the largest public health poisonings in the history of human civilization, affecting tens of millions of people worldwide (1-3).

As is known to be toxic to different organs, including hepatic, hematopoietic, renal, reproductive, nervous system, etc (4). Several recent reports suggest that children and elderly population share higher susceptibility to the toxic effects of As (5-10). Differential toxicity of As may be due to difference in absorption, distribution, metabolism and elimination that varies with age (11).

The exact mechanisms by which As induces toxicity are still being elucidated (12). One possible molecular mechanism involved in the As induced neurotoxicity is the disruption of the prooxidant/antioxidant balance which is

associated with increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that impair cellular antioxidant defense system and simultaneously damage the critical biomolecules, such as lipids, proteins and DNA (13).

Mitochondria have been a central focus of several theories of aging as a result of their critical role in bioenergetics, oxidants production, and regulation of cell death (14). Moreover, several studies have reported alterations in antioxidant enzyme activities such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) which can trigger apoptosis in As exposed animals (15). Therefore, the present study was focused on mitochondrial oxidative stress marker enzymes.

Despite the well established toxicity, yet no safe and effective therapeutic management of arsenicosis is available. Essential trace metal such as zinc (Zn) has been studied for the protective values against As. Earlier reports from our laboratory have established the protective role of essential metals (Zn and Ca) in reducing the impact of Pb and As-induced developmental neurotoxicity (14, 16, 17).

Alpha (α) tocopherol is the most abundant and active form of vitamin E *in vivo* (18, 19) and is an important lipophilic radical scavenging antioxidant. α -tocopherol is a term that encompasses a group of potent, lipid soluble, chain-breaking antioxidants that prevents the propagation of free radical reactions. A number of studies have been carried out to determine the protective effects of vitamin E in different biological models of injury (20, 21).

We hypothesized that As induced neurotoxicity is age dependent and driven by oxidative damage. Therefore antioxidants and essential metals will provide protection against As induced neurotoxicity. In view of this, the present study was designed to study the impact of age on As induced alterations in mitochondrial enzymatic and non-enzymatic oxidative stress

markers in the brain regions of albino rats at three different age points (PND [postnatal day] 21: young; 3 months: adult; 18 months: aged). Further, the present study was extended to compare the therapeutic efficacy of essential metal Zn and an antioxidant Vit-E against As induced neurotoxicity.

Materials and Methods

Procurement and maintenance of animals:

Three months (adult) and 18 months (aged) old male albino rats (Wistar) were purchased from Sri Venkateswara Traders, Bangalore, whereas PND21 (young) rats were obtained from our animal house by maintaining pregnant rats. Animals of all age groups were maintained in the animal house of Sri Venkateswara University, Dept. of Zoology, Tirupati for atleast one week for acclimatization before using for treatments. All the rats were kept in well cleaned, sterilized polypropylene cages lined with paddy husk (18" x 10" x 8"). The animals were maintained under a regulated light: dark 12 h (7:00–19:00) scheduled at $24 \pm 1^\circ\text{C}$ and relative humidity of $55 \pm 15\%$. Rats were provided standard rat chow (Sai Durga Feeds and Foods, Bangalore, India) and water *ad libitum*. The experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India (CPCSEA, 2003) and approved by the Institutional Animal Ethical Committee (Resoulution No: 09/2012-2013/i/a/CPCSE/IAEC/SVU/GRR-MRKdt.02-07-2012) at Sri Venkateswara University, Tirupati, India.

Chemicals: The chemicals used in this study were purchased from Sigma Chemicals (St. Louis, MO, USA) and Merck, India. Sodium arsenite (NaAsO_2 , >99% purity) used in this study was purchased from Sigma–Aldrich (St Louis, MO, USA) and dissolved in sterile distilled water to the desired concentrations.

Animals exposure: After a week of acclimatization, rats of all age groups (PND21: young; 3 months: adult; 18 months: aged) were randomly divided into 4 groups and were treated

for a period of one week as follows:

Group II: Arsenic as sodium arsenite (10 mg/kg dissolved in deionized water, given orally through gavage) (n=6)

Group III: As (10 mg/kg, orally) + Zinc as ZnCl₂ (0.02% through drinking deionized water) (n=6)

Group IV: As (10 mg/kg, orally) + Vit-E, (125 mg/kg body weight, orally through gavage) (n=6).

The control animals which served as group I, received equivalent volumes of deionized water through oral gavage. The dose of sodium arsenite used in this study was based upon the reports of Itoh et al., (22) and Rodriguez et al., (23) in rats and it represents ¼ of the LD₅₀ dose for rats (24). Sodium arsenite solutions were prepared fresh daily and dissolved in deionized water. The Vit-E dose used in this study has no known side effects and is considered a supplemental dose (25). After completion of treatment, animals were sacrificed through cervical dislocation and the brain regions (cerebral cortex, hippocampus and cerebellum) were quickly isolated and stored at -80°C for biochemical analysis.

Preparation of mitochondrial fractions:

Mitochondrial fractions were prepared by following the method of Lai and Clark (26). The tissues were homogenized in 10 volumes (w/v) of SET buffer (0.25 M sucrose, 10 mM Tris-HCl, and 1 mM EDTA, pH 7.4). The homogenate was first centrifuged at 800 x g for 10 min at 4°C, and then the supernatant was centrifuged at 10,000x g for 20 min. Then the pellet of mitochondrial fraction was suspended in SET buffer.

Biochemical studies

Superoxide Dismutase (SOD) activity:

Measurement of total SOD activity was performed according to Misra and Fridovich, (27) based on the inhibition of auto-oxidation of epinephrine. The total reaction mixture contained of 880 µl of 0.05 M carbonate buffer (pH 10.2),

20 µl of 30 mM epinephrine and 100 µl of enzyme source and absorbance was recorded at 480 nm against reagent blank. The enzyme activity was expressed as micromoles of superoxide anion reduced/mg of protein. Mn-SOD activity was determined in the mitochondrial fractions by inhibiting the Cu/Zn-SOD with KCN and subtracting the values from total SOD activity.

Catalase (CAT) activity:

CAT activity was measured by following the method of Chance and Maehly, (28). The reaction mixture contained 1.9 ml reagent grade water, 1.0 ml of 0.059 M hydrogen peroxide (H₂O₂) in buffer. The reaction mixture was incubated in spectrophotometer for 4–5 min to achieve temperature equilibration and to establish blank rate if any. 0.1 ml of enzyme (mitochondrial fraction) was added and decrease in absorbance was recorded at 240 nm for 2-3 min. "A₂₄₀/min from the initial (45s) linear portion of the curve was calculated. The enzyme activity was expressed as mmol of H₂O₂ decomposed/mg protein/min.

Glutathione Peroxidase (GPx) activity:

GPx activity was determined by following the method of Rotruck et al., (29). Briefly, the reaction mixture contained 0.5 ml of 0.4 mol/l sodium phosphate buffer (pH 7.0), 0.1 ml sodium azide, 0.2 ml reduced glutathione, 0.1 ml H₂O₂, and 0.5 ml of 1:10 diluted aliquot of the enzyme extract and the total volume was made up to 2 ml with distilled water. The tubes were incubated at 37°C for 3 min and the reaction was terminated with 0.5 ml 10% TCA and centrifuged at 4°C for 10 min at 1500 rpm. The supernatant was collected and to this 4 ml of 0.3 mol/l disodium hydrogen phosphate and 1 ml DTNB (dithionitro benzoic acid) (0.004%) reagent were added. The color developed was read at 412 nm against the reagent blank containing only phosphate solution and DTNB reagent. The enzyme activity was expressed as µ moles of GSH oxidized/mg of protein/min.

Glutathione Reductase (GR) activity:

GR activity in the mitochondrial fraction of brain regions was assayed as described by Staal et

al., (30). The reaction mixture in a final volume of 3.0 ml contained 1.0 ml of 0.3 M Sodium phosphate buffer (pH-6.8), 0.5 ml of 250 mM EDTA, 0.5 ml of 12.5 mM GSSG, 0.7 ml of distilled water, 0.2 ml of 30 mM NADPH and 0.1 ml of enzyme extract. Changes in absorbance were recorded at 340 nm in a spectrophotometer. The enzyme activity was expressed as μ moles of NADPH oxidized/mg protein/min.

Glutathione-S-Transferase (GST) activity: GST activity in the mitochondrial fraction of brain regions was assayed by using 1-chloro-2, 4-dinitro benzene (CDNB) (at 340 nm) as described by Habiget al., (31). The reaction mixture in a final volume of 3.0 ml contained; 150 mM phosphate buffer (pH 7.5), 1 mM CDNB, 5 mM glutathione (GSH) and an appropriate amount of enzyme source. The reaction was initiated by the addition of GSH and incubated at 37°C. The formation of a thioether by the conjugation of CDNB to GSH was monitored at 340 nm in a spectrophotometer (Hitachi model, U-2001). Thioether concentration was determined from the slopes of initial reaction rates. The activity was expressed as μ moles of thioether formed/mg protein/min.

Glutathione (GSH) content: GSH content was determined according to the method of Ellman, (32). The mitochondrial fraction (720 μ l) was doubled diluted with distilled water and trichloroacetic acid (5%) was added to precipitate the protein content of the tissue homogenates. After centrifugation at 10000 g for 5 min, the supernatant was collected. To the supernatant, 5,5-dithiobis (2-nitrobenzoic acid solution (Ellman's reagent)) was added and the absorbance was measured at 412 nm. The values were expressed as millimoles/gm tissue.

Total Sulphydryl Group (TSG) content: TSG content was determined by the method of Sedlak and Lindsay, (33). Briefly, to the homogenate (100 μ l), 1 ml of tris-EDTA buffer (0.2 mol/L, pH 8.2) and 0.9 ml of EDTA solution (0.02 mol/L, pH 4.7) was added followed by the addition of 20 μ l of Ellman's reagent. After 30 min of incubation at

room temperature, samples were centrifuged and the absorbance was read at 412 nm in spectrophotometer.

Lipid Peroxidation (LPx): The lipid peroxides were determined by the TBA method of Ohkawa et al., (34). The reaction mixture contained 0.1 ml of tissue homogenate. 0.2 ml of 8.1% sodium dodecyl sulphate (SDS), 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid (TBA). The pH was adjusted to 3.5. The mixture was finally made up to 4.0 ml with distilled water and heated at 95°C for 60 min. After cooling under tap water, 0.1 ml of distilled water and 5.0 ml of a mixture of n-butanol and pyridine (15:1, V/V) were added and the mixture was shaken vigorously on a vortex mixture. After centrifugation at 2200 x g for 5 min the upper organic layer was separated and the absorbance was read at 532 nm in a spectrophotometer. The rate of lipid peroxidation was expressed as μ moles of malondialdehyde formed/gm wet wt. of tissue/hr.

Evaluation of As concentration in brain regions: Brain regional As levels were estimated according to the method of Ballentine and Burford, (35). To 50 mg of tissues, 0.5 ml of concentrated nitric acid was added, followed by 0.5 ml of perchloric acid. The sample was then digested over a sand bath until the solution turns yellow in color. If the color of the digest was brown, 0.25 ml each of nitric acid and perchloric acid were added and the oxidation was repeated. The digest was made up to 1 ml volume with deionized water. Aliquots of this were used to estimate arsenic by using atomic absorption spectrophotometer (AAS, Perkin Elmer model AAnalyst 100).

Statistical analysis of the data: Significance of each age group among different treatments was analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons test using Standard Statistical Software Package to compare the effects among various groups. The 0.05 level of probability was used as the criterion for significance.

Results

Our results showed that mitochondrial oxidative stress markers such as SOD isoforms (Mn-SOD and Cu/Zn-SOD), Catalase (CAT), Glutathione reductase (GR), Glutathione peroxidase (GPx), glutathione (GSH), total free sulfhydryl groups (TSG) were decreased with concomitant increase in lipid peroxidation and GST in different brain regions (cerebral cortex, cerebellum and hippocampus) of As exposed rats of all the different age points (young: PND21; adult: 3 months; aged: 18 months).

The activities of Mn-SOD and Cu/Zn-SOD in control rats were increased with age documenting maximum activity in adult rats compared with young rats. However, these enzyme activities were decreased in aged rats (18 months). Among the different brain regions studied, the cerebral cortex showed greater activities followed by cerebellum and hippocampus.

Exposure to As significantly decreased the activities of Mn-SOD and Cu/Zn-SOD in all the three brain regions. Decrease in their activities were more pronounced in aged rats (67.36% - cortex; 64.67% - cerebellum; 68.16% - hippocampus) followed by young (61.77% - cortex; 60.43% - cerebellum; 61.25% - hippocampus) and adult rats (47.72% - cortex; 46.12% - cerebellum; 51.75% - hippocampus) with cortex documenting maximum decrease in enzyme activity than cerebellum and hippocampus. The observed decrease in Mn-SOD and Cu/Zn-SOD activities in different brain regions at all the age points was significant at $P < 0.05$ (Fig.1a -2c).

Similar to SOD isoforms, exposure to As significantly decreased the CAT activity in all the three brain regions. Decrease in the CAT activity was more pronounced in aged rats (67.36% - cortex; 64.67% - cerebellum; 68.16% - hippocampus) followed by young (61.77% - cortex; 60.43% - cerebellum; 61.25% - hippocampus) and adult rats (47.72% - cortex; 46.12% - cerebellum; 51.75% - hippocampus)

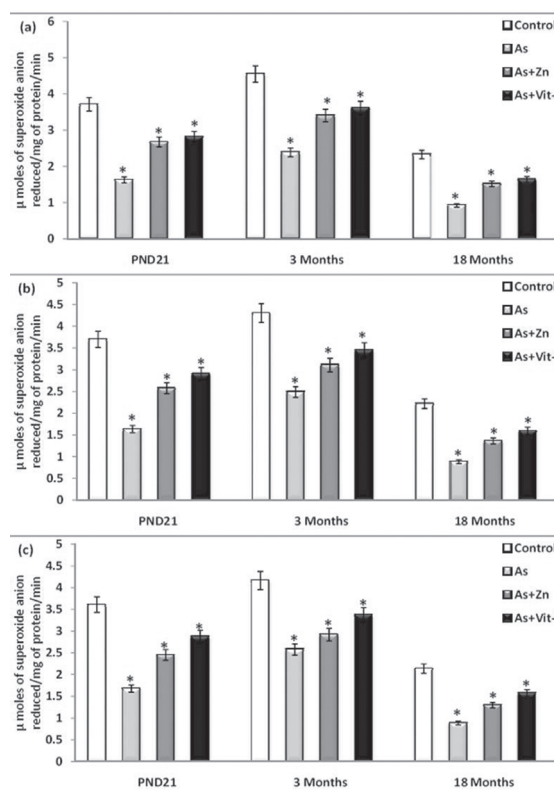


Fig. 1. Effect of arsenic exposure on Mn-SOD activity in three different brain regions (a) cerebral cortex, (b) cerebellum and (c) hippocampus and reversal response to administration of Zn and Vit E in young, adult and aged rats. Rats of all age groups were given deionized water (control), or arsenic (10mg/kg orally), zinc (0.02% through drinking water) and Vit E (125mg/kg orally) for a period of one week. All the values are mean \pm SD of six individual observations. All significant values are marked with asterisk (*); non-significant values are marked with (ns) as evaluated by the ANOVA followed by Bonferroni multiple comparisons test ($P < 0.05$).

with cortex documenting maximum decrease in enzyme activity than cerebellum and hippocampus. The observed decrease in CAT activity in different brain regions at all the age points was significant at $P < 0.05$ (Fig.3a – 3c).

The GPx activity in control rats was found to be maximum in adults followed by young and aged rats. Among the three brain regions, cortex

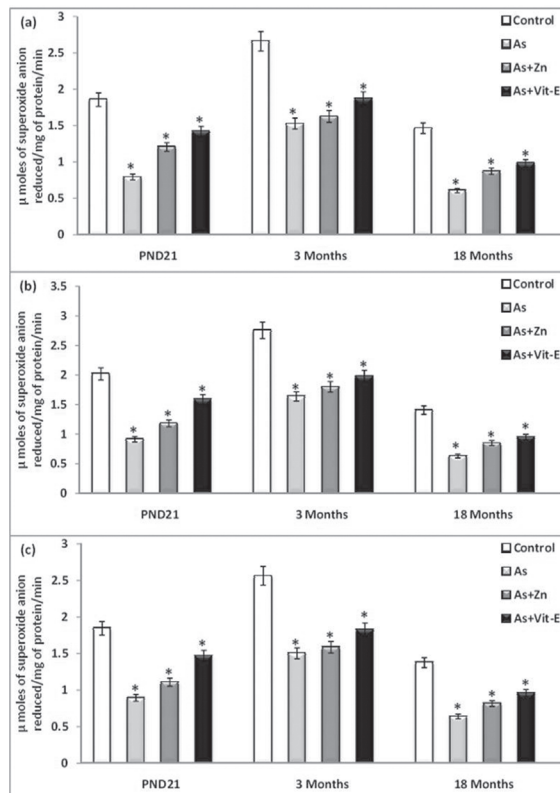


Fig. 2. Effect of arsenic on Cu/Zn-SOD activity in three different brain regions (a) cerebral cortex, (b) cerebellum and (c) hippocampus and its reversal response to administration of Zn and Vit E in young, adult and aged rats. Rats of all age groups were given deionized water (control), or arsenic (10mg/kg orally), zinc (0.02% through drinking water) and Vit E (125mg/kg orally) for a period of one week. All the values are mean \pm SD of six individual observations. All significant values are marked with asterisk (*); non-significant values are marked with (ns) as evaluated by the ANOVA followed by Bonferroni multiple comparisons test ($P < 0.05$).

showed maximum GPx activity followed by cerebellum and hippocampus (Fig.4) at all the age points studied. Exposure to As exhibited significant decrease in the specific activity of brain regional GPx and the decrease in activity was found to be greater in aged rats (60.67%, 61.44% and 59.49% decrease in cortex, cerebellum and hippocampus respectively) followed by young

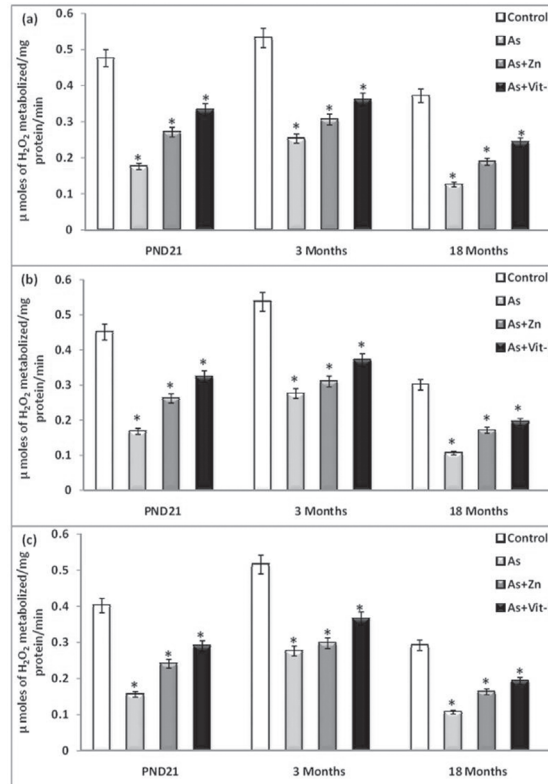


Fig. 3. Alterations in CAT activity in three different brain regions (a) cerebral cortex, (b) cerebellum and (c) hippocampus following As exposure and co-administration of Zn and Vit E in young, adult and aged rats. Rats of all age groups were given deionized water (control), or arsenic (10mg/kg orally), zinc (0.02% through drinking water) and Vit E (125mg/kg orally) for a period of one week. All the values are mean \pm SD of six individual observations. All significant values are marked with asterisk (*); non-significant values are marked with (ns) as evaluated by the ANOVA followed by Bonferroni multiple comparisons test ($P < 0.05$).

rats (55.07%, 55.20% and 54.86% decrease in cortex, cerebellum and hippocampus respectively) and adult rats (51.42%, 48.86% and 45.20% decrease in cortex, cerebellum and hippocampus respectively). From the data, it was found that cerebral cortex exhibited maximum decrease in enzyme activity than cerebellum and hippocampus. The observed decreases in

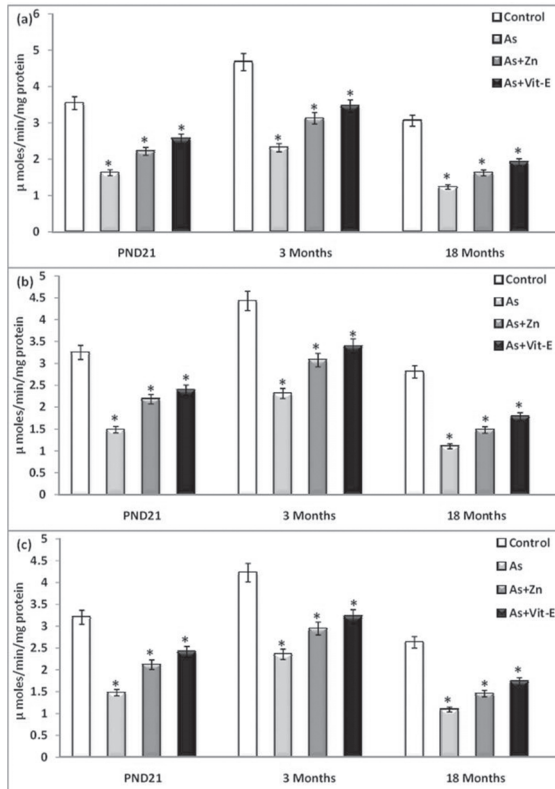


Fig. 4. Alterations in GPx activity in three different brain regions (a) cerebral cortex, (b) cerebellum and (c) hippocampus following As exposure and co-administration of Zn and Vit E in young, adult and aged rats. Rats of all age groups were given deionized water (control), or arsenic (10mg/kg orally), zinc (0.02% through drinking water) and Vit E (125mg/kg orally) for a period of one week. All the values are mean \pm SD of six individual observations. All significant values are marked with asterisk (*); non-significant values are marked with (ns) as evaluated by the ANOVA followed by Bonferroni multiple comparisons test ($P < 0.05$).

enzyme activity in different brain regions of all the age points were significant at $P < 0.05$ (Fig. 4a – 4c).

The GR activity in control rats was found to be maximum in adults followed by young and aged rats. Among brain regions, cortex showed maximum GR activity followed by cerebellum and

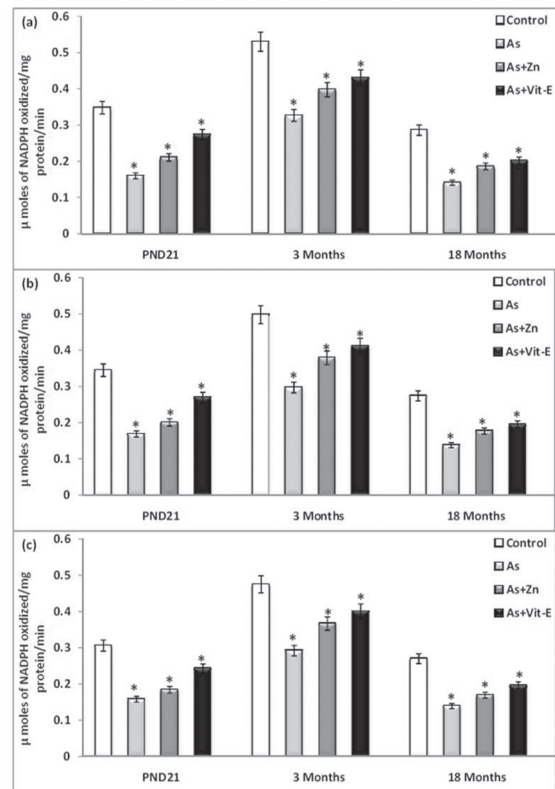


Fig. 5. Effect of arsenic on GR activity in three different brain regions (a) cerebral cortex, (b) cerebellum and (c) hippocampus and its response to administration of Zn and Vit E in young, adult and aged rats. Rats of all age groups were given deionized water (control), or arsenic (10mg/kg orally), zinc (0.02% through drinking water) and Vit E (125mg/kg orally) for a period of one week. All the values are mean \pm SD of six individual observations. All significant values are marked with asterisk (*); non-significant values are marked with (ns) as evaluated by the ANOVA followed by Bonferroni multiple comparisons test ($P < 0.05$).

hippocampus at all the age points studied. Exposure to As exhibited significant decrease in the specific activity of brain regional GR and the decrease in activity was found to be greater in aged rats (51.51%, 50.52% and 49.46% decrease in cortex, cerebellum and hippocampus respectively) followed by young rats (54.87%, 51.96% and 49.21% decrease in cortex,

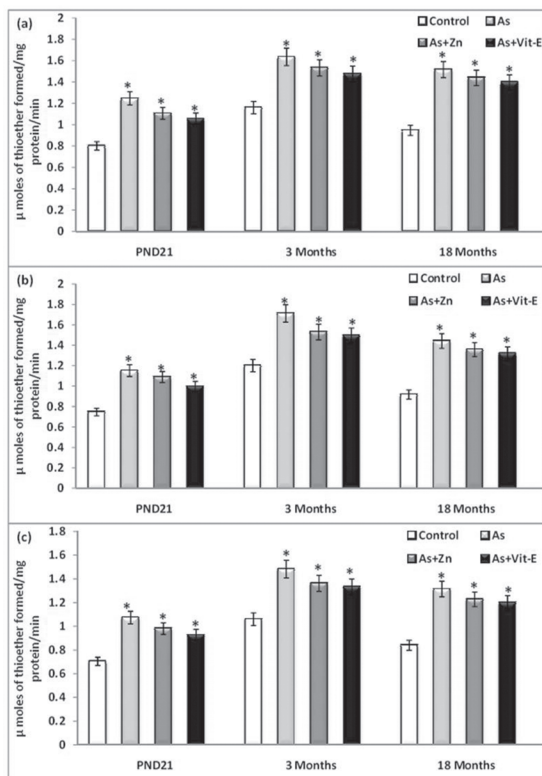


Fig. 6. Effect of arsenic on GST activity in three different brain regions (a) cerebral cortex, (b) cerebellum and (c) hippocampus and its response to administration of Zn and Vit E in young, adult and aged rats. Rats of all age groups were given deionized water (control), or arsenic (10mg/kg orally), zinc (0.02% through drinking water) and Vit E (125mg/kg orally) for a period of one week. All the values are mean \pm SD of six individual observations. All significant values are marked with asterisk (*); non-significant values are marked with (ns) as evaluated by the ANOVA followed by Bonferroni multiple comparisons test ($P < 0.05$).

cerebellum and hippocampus respectively) and adult rats (39.27%, 41.31% and 39.50% decrease in cortex, cerebellum and hippocampus respectively). From the data, it was found that cerebral cortex exhibited maximum decrease in enzyme activity than cerebellum and hippocampus. The observed decreases in enzyme activity in different brain regions of all

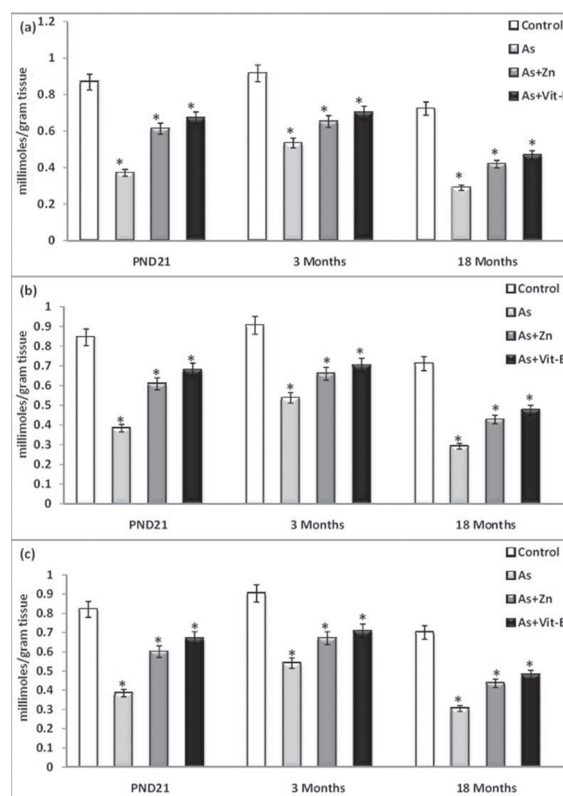


Fig. 7. Effect of arsenic exposure and supplementation with Zn and Vit E on GSH levels in three different brain regions (a) cerebral cortex, (b) cerebellum and (c) hippocampus of young, adult and aged rats. Rats of all age groups were given deionized water (control), or arsenic (10mg/kg orally), zinc (0.02% through drinking water) and Vit E (125mg/kg orally) for a period of one week. All the values are mean \pm SD of six individual observations. All significant values are marked with asterisk (*); non-significant values are marked with (ns) as evaluated by the ANOVA followed by Bonferroni multiple comparisons test ($P < 0.05$).

the age points were significant at $P < 0.05$ (Fig. 5a – 5c).

Exposure to As exhibited significant increase in the brain regional GST activity levels and the increase in levels was found to be greater in aged rats (61.23%, 58.51% and 57.60% increase in cortex, cerebellum and hippocampus

respectively) followed by young rats (56.85%, 54.95% and 53.41% increase in cortex, cerebellum and hippocampus respectively) and adult rats (42.06%, 43.79% and 40.94% increase in cortex, cerebellum and hippocampus respectively). From the results, it was found that cerebral cortex exhibited maximum increase in GST activity than cerebellum and hippocampus. The observed increases in GST activity in different brain regions at all the age points were significant at $P < 0.05$ (Fig. 6a – 6c).

Similar to enzymatic antioxidants; the non-enzymatic antioxidants, GSH and TSG levels in control rats were found maximum in the brain regions of adult rats followed by young and aged rats. Among the three brain regions, cortex showed maximum GSH and TSG levels followed by cerebellum and hippocampus (Fig. 7a – 8c) at all the age points studied. Exposure to As exhibited significant decrease in the levels of brain regional GSH and TSG and the decrease was found to be maximum in aged rats (61.03%, 61.58%; 59.83%, 59.30% and 57.46%, 58.07% decrease in cortex, cerebellum and hippocampus respectively) followed by young rats (58.24%, 55.46%; 55.49%, 53.29% and 54.08%, 52.23% decrease in cortex, cerebellum and hippocampus respectively) and adult rats (42.61%, 43.32%; 41.54%, 42.14% and 41.09%, 40.93% decrease in cortex, cerebellum and hippocampus respectively). From the data, it was found that cerebral cortex exhibited maximum decrease in GSH and TSG levels than cerebellum and hippocampus. The observed decreases in enzyme activity in different brain regions of all the age points were significant at $P < 0.05$ (Fig. 7a– 8c).

In the present investigation, thiobarbituric acid substances (TBARS) formation was recorded as a measure of lipid peroxidation in both control and experimental rat brain regions. The TBARS content increased with age in control brain regions with the cortex documenting the highest TBARS content followed by cerebellum and hippocampus at in aged (18 months old) rats.

Exposure to As exhibited significant increase in the brain regional TBARS levels and the increase in levels was found to be greater in aged rats (78.42%, 78.09% and 76.92% increase in cortex, cerebellum and hippocampus respectively) followed by young rats (74.02%, 72.22% and 72.09% increase in cortex, cerebellum and hippocampus respectively) and adult rats (51.15%, 50.12% and 50.76% increase

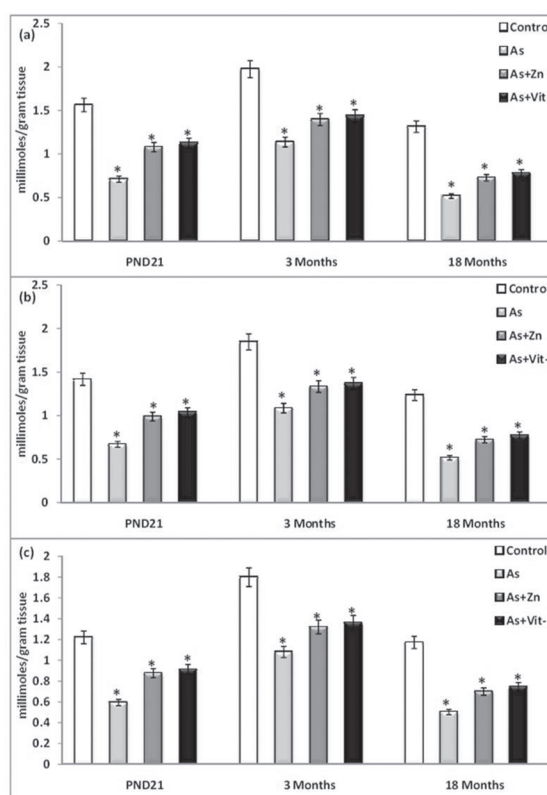


Fig. 8. Changes in TSG levels in three different brain regions (a) cerebral cortex, (b) cerebellum and (c) hippocampus following As exposure and the effect of administration of Zn and Vit E in young, adult and aged rats. Rats of all age groups were given deionized water (control), or arsenic (10mg/kg orally), zinc (0.02% through drinking water) and Vit E (125mg/kg orally) for a period of one week. All the values are mean \pm SD of six individual observations. All significant values are marked with asterisk (*); non-significant values are marked with (ns) as evaluated by the ANOVA followed by Bonferroni multiple comparisons test ($P < 0.05$).

in cortex, cerebellum and hippocampus respectively). From the results, it was found that cerebral cortex exhibited maximum increase in TBARS levels than cerebellum and hippocampus. The observed increase in TBARS levels in different brain regions at all the age points were significant at $P < 0.05$ (Fig. 9a – 9c).

Brain regional accumulations of As at different age points were shown in Fig. 10. As exposure lead to a significant increase in As content in all the three brain regions at all age points. Maximum increase in As content was observed at PND 21 followed by aged and adult rats. Among the brain regions maximum increase in As levels was found in cerebral cortex

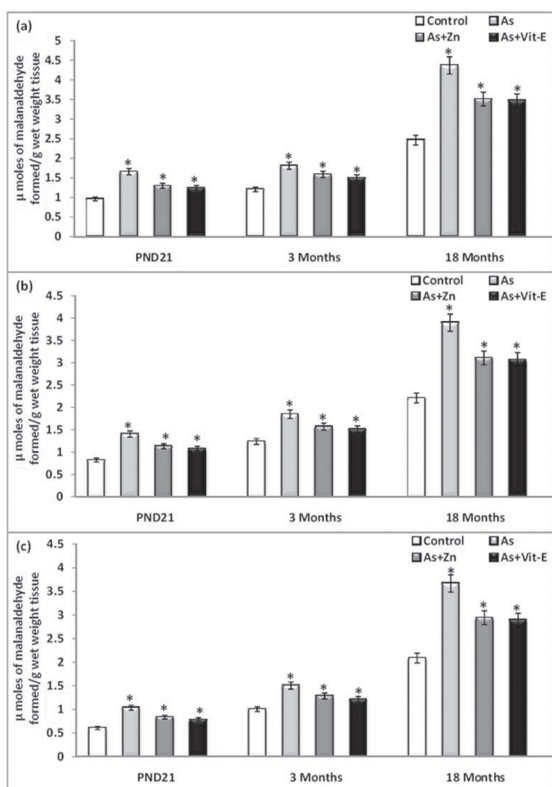


Fig. 9. Effect of arsenic on lipid peroxidation in three different brain regions (a) cerebral cortex, (b) cerebellum and (c) hippocampus and its response to administration of Zn and Vit E in young, adult and aged rats. Rats of all age groups were given deionized water (control), or arsenic (10mg/kg orally), zinc (0.02% through drinking water) and Vit E (125mg/kg orally) for a period of one week. All the values are mean \pm SD of six individual observations. All significant values are marked with asterisk (*); non-significant values are marked with (ns) as evaluated by the ANOVA followed by Bonferroni multiple comparisons test ($P < 0.05$).

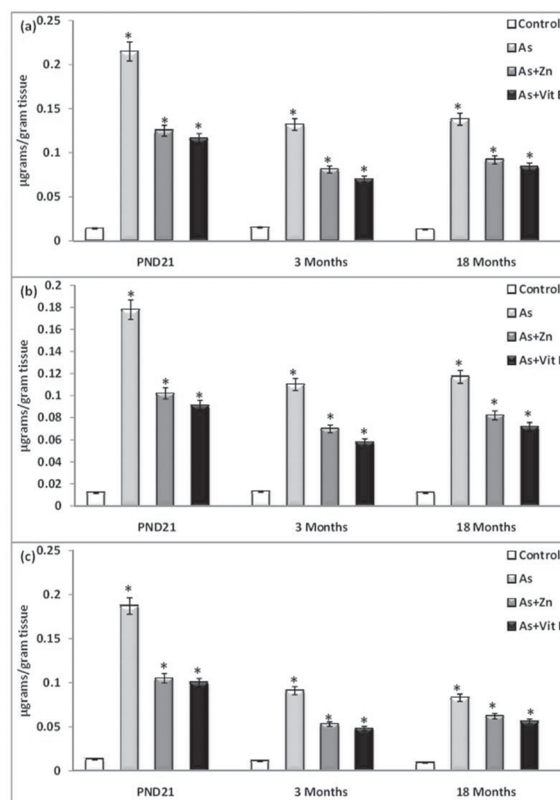


Fig. 10. Arsenic levels in three different brain regions (a) cerebral cortex, (b) cerebellum and (c) hippocampus of young, adult and aged rats. Rats of all age groups were given deionized water (control), or arsenic (10mg/kg orally), zinc (0.02% through drinking water) and Vit E (125mg/kg orally) for a period of one week. All the values are mean \pm SD of six individual observations. All significant values are marked with asterisk (*); non-significant values are marked with (ns) as evaluated by the ANOVA followed by Bonferroni multiple comparisons test ($P < 0.05$).

compared to cerebellum and hippocampus (Fig. 10a – 10c).

We have also evaluated the protective effect of Zn / Vit E against the influence of age on As induced alterations in mitochondrial oxidative stress and brain As levels. Supplementation with Zn / Vit E, significantly reversed the As induced alterations in all the selected enzymatic antioxidant activities (Mn-SOD, Cu/Zn-SOD, CAT, GPx, GR, GST) non-enzymatic antioxidant levels (GSH and TSG), TBARS levels and As content in all brain regions (cerebral cortex hippocampus and cerebellum) of young (PND 21), adult (3 months) and aged (18 months) rats (Figures 1 to 10). However, among Zn and Vit E, Vit E produced greater recovery against As induced alterations in mitochondrial oxidative stress marker enzymes, non-enzymatic antioxidants and brain As levels compared to Zn, suggesting oxidative damage as the principal mechanism underlying As induced neurotoxicity.

Discussion

The current study was designed to determine the influence of age on As-induced alterations in brain regional (cerebral cortex, hippocampus, cerebellum) antioxidant system at different age group rats and further to compare the therapeutic efficacies of Zn and Vit-E against As induced neurotoxicity. As is known to cross blood–brain barrier and produce neurotoxic effects (23, 36). In the present study, As exposed rats showed a significant accumulation of As in brain compared to controls leading to possible neurotoxic effects. These increased As levels in rat brain regions exposed to As indicate that As and its metabolites are able to pass through the blood brain barrier (BBB), which is consistent with the results of Zheng et al., (37).

Among various anti-oxidative mechanisms in the body, SOD is thought to be one of the major enzymes which protects against tissue damage caused by the potentially cytotoxic activities of radicals (38, 39). In our current study, As exposure resulted in a decrease in brain regional

SOD isoforms activity at all age groups compared with respective controls. The decrease in SOD isoforms activity may be attributed to enhanced superoxide radical production during As metabolism (40, 41). Moreover the effect of As on SOD has also been attributed to (i) altered SOD expression (ii) modification of cellular antioxidant uptake, GSH and vitamin depletion or (iii) alteration in an antioxidant activity by affecting their structure (oxidation/reduction of thiol group and displacement of essential metals) (42).

Among the three different age groups studied, the activity levels of SOD-isoforms were significantly decreased in aged group rats followed by young and adult rats. This age-related decrease in SOD-isoforms activity in brain specific regions documented in the present study was in corroboration with the findings of Carrillo et al., (38). The possible mechanism suggested is that, an increase in arachidonic acid turnover (e.g. increase in prostaglandin synthase activity) may play a role in the increased oxygen radical load (43). Sawada and Carlson, (44) reported that superoxide radical formation increases with age, therefore a decreased protection against toxic radicals may have serious consequences for the aging brain.

By analysis of the Mn-SOD activity of the three brain regions at different ages, we found that the activity was significantly decreased in the cerebral cortex of aged rats. These results indicate that the cerebral cortex may suffer from higher oxidative stress during aging. Benzi et al., (45) and Carrillo et al., (38) also demonstrated that the activities of antioxidant enzymes, total SOD, and Mn-SOD decreased with aging in the cerebral cortex followed by cerebellum.

CAT has been shown to be responsible for the detoxification of significant amounts of H_2O_2 . From the present data, it is evident that As exposure significantly decreased CAT activity at all age groups compared with their respective controls. CAT requires NADPH for its regeneration from its inactive form. The activity

of glucose-6-phosphate dehydrogenase (G6PDH) decreases with advancing age. As the level of NADPH depends on that of G6PDH, a decrease in the activity of the latter affects the levels of the former. The paucity of NADPH production along with increased superoxide radicals during As exposure also decreases catalase activity (46, 47). The decreased NADPH production during As exposure also decreases catalase activity (46, 47). Among the different age groups, As exposure lead to a significant reduction in catalase activity of aged rats, followed by young and adult rats. The gradual decrease in catalase with increasing age appears to be due to an age-dependent change in the expression of the related genes (48).

Glutathione-related enzymes, such as glutathione peroxidase (GPx) and glutathione reductase (GR), function as antioxidants either directly or indirectly, whereas glutathione-S-transferase (GST) plays an important role in metabolic detoxification. GPx metabolizes peroxides such as H_2O_2 and protects cell membranes from lipid peroxidation. GPx is a Se-dependent enzyme and effect of As on synthesis and activity of selenoenzymes has been previously reported. Similarly, in the present study, a significant reduction in GPx activity was observed upon exposure to As at all age groups studied. As may directly interact with Se and form insoluble and inactive As-Se complex (49, 50) rendering it unavailable and ultimately resulting in the inhibition of GPx activity or alter the expression and synthesis of selenoproteins like GPx (51, 52).

The activity of GPx was found to be less in brain regions of aged rats suggesting that aged are most sensitive to oxidative stress than young and adult controls. Our results are in agreement with Brannan et al., (53) who observed a similar decrease in the activity of GPx as well as SOD during aging. In addition, during aging considerable increase in the production of H_2O_2 and a decrease in the activity of GPx were reported. The lowered GPx activity in aging may also be attributed due to the decline in glutathione

(GSH) concentration as observed in the present study.

GR is an important enzyme for maintaining the intracellular concentration of reduced glutathione. From the results, it is evident that, As exposure lead to a significant decrease in GR activity at all age groups compared with respective controls. Both arsenite and its methylated metabolites have been known to be potent inhibitors of GR *in vitro* (54-56); thus, exposure to arsenicals would compromise the antioxidant mechanisms by utilizing GSH and inhibiting the enzyme responsible for its recycling and eventually leading to cell death.

Our data on decrease of GR further gets support from the fact that trivalent arsenicals are potent inhibitors of GR (54), which can cause inhibition by the interaction of As with critical thiol groups in these enzymes (57). However, in the present study, a significant decrease in GR activity was observed in the brain regions of aged rats compared to the young and adult rats. The significant reduction in the activity of GR in the brain of aged rats may be associated with age-related depletion of GSH in various brain regions (58).

GST is believed to participate in the detoxification process by catalyzing the conjugation of electrophilic xenobiotics to GSH, by binding various ligands covalently and non-covalently and by expressing GPx activity towards lipid hydro-peroxides. Exposure to As lead to an increase in GST activity in all the brain regions of all the age groups studied. Increased GST activity following As exposure suggests a counteracting mechanism adopted by system to eliminate As. In one of the study conducted by Kim et al., (59), GST activity increased 1.7 fold in the brains of 9-month-old rats compared with those of 5-week-old rats. In our current study, GST was increased significantly in all the three brain regions of aged animals compared to other two age groups. Elevation in GST activity is assumed to respond to aging as an adaptive mechanism secondary to increases in oxidative stress.

Reduced glutathione (GSH), a non-protein thiol, plays an important role in the detoxification via facilitating removal of As from the cellular sites and stimulating excretion of methylated As (60), which is responsible for As-induced cytotoxicity. From the experimental results, it is evident that As exposure significantly depleted GSH levels at all age groups compared with their respective controls. Among the different age groups, As exposure lead to a significant reduction in GSH levels of aged rats, followed by young and adult rats. As-induced GSH depletion may be attributed to its direct binding with GSH due to its high thiol binding affinity and electrophilic nature of GSH. Indirect depletion of GSH by As may also due to its utilization in scavenging of As-induced free radical generation. This could be due to the utilization of GSH as electron donor for As metabolism or direct binding due to thiol preference. An age-associated decline in GSH level has been reported in the brain tissue of various species (61-63), which are consistent with the findings of current study. Maiti and Chatterjee, (64) also suggested that depletion of GSH may also be caused due to the GPx mediated excess utilization of GSH.

Glutathione and total thiols (sulfhydryl groups) non-enzymatically reduce peroxides and/or prevent occurrence of peroxidation. In the present study, a significant decrease in the sulfhydryl groups was noticed in aged rats. The recycling of GSH from GSSG (oxidised glutathione) is catalyzed by the enzyme glutathione reductase using NADPH as a cofactor. NADPH is generated in a pathway involving the enzyme, G6PDH. Age-associated decline in the activity of G6PDH leading to diminished production of NADPH may be the cause for the observed decrease in sulfhydryl groups.

LPx measured as TBARS in the present study forms the most common indices to assess oxidative stress. These peroxidation products of lipids, usually polyunsaturated fatty acids are susceptible to the attack of free radicals forming an important biomarker (65). In the present study,

significant increase in LPx was observed in young and old rats, compared to the adult rats. Increased lipid peroxidation in response to As is thought to be a consequence of oxidative stress, which occurs when the dynamic balance between peroxidant and antioxidant mechanism is impaired. The results of this study also support the correlation of increased TBARS level with the decreased antioxidant defense system due to arsenic toxicity.

Among the brain regions studied, the activity levels of oxidative stress marker enzymes were found to be depleted to a greater extent in cortex, followed by cerebellum and hippocampus, indicating cortex is highly sensitive to oxidative stress. Certain brain regions such as cortex and striatum are highly enriched with non-heme iron, which is catalytically involved in the production of free radicals (66). The relative sensitivity of cortex when compared to cerebellum and hippocampus may be attributed to the differences in the iron content which influence the generation of ROS.

The present investigation, however, showed that these alterations in mitochondrial oxidative stress markers induced by As could be reversed by administration of essential metal, Zn or antioxidant, Vit E. This may be attributed due to the fact that this essential trace element can affect toxicity of heavy metals by interacting at the primary site of action and/or this trace element could reduce the gastrointestinal absorption of As. Zn could be competing for binding sites and effectively reducing the availability of binding sites for metal uptake (16, 67). Treatment with vitamin E reduced As induced alterations, probably through its capacity to quickly and efficiently scavenge lipid peroxy radicals before they attack membrane lipids. The reaction of α -tocopherol with free radicals generates tocopherol radicals, which can be reduced by vitamin C or GSH (68), thereby reducing ROS. Since Vit-E is primarily located in cell membranes, it is possible that it modifies the kinetics of distribution of As in cell membranes,

such that its delivery to neuronal cells may be impeded.

Among Zn and vitamin E, Vitamin E supplementation produced significant reversal effect and found to be a better therapeutic agent against As induced neuronal oxidative damage at all the age points. Our findings are consistent with the view that protective effects of Vit-E against As exposure might be due to its antioxidant properties, and this may have a major impact on improving the quality of life of individuals suffering from neurotoxicity caused by As exposure. However, further studies have to be carried out to see whether this type of therapy can be advocated as safe and effective treatment for As induced neurotoxicity at all the age points of life.

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Green Synthesis of Silver Nanoparticles using two Apocyanaceae plants and Screening for their Catalytic activity

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Abstract

The catalytic activity of biosynthesized silver nanoparticles (AgNPs) gained an importance in removal of synthetic dyes from waste water released from textile industries. In this study aqueous leaf extract of *Cascabela thevetia* and *Wrightia tomentosa* were used to synthesize silver nanoparticles. Further the catalytic activity of newly synthesized particles in reducing 4-Nitrophenol, Methylene blue, Methyl orange and Methyl red using NaBH₄ was screened. It was observed that the respective amalgamated solutions of AgNO₃ and leaf extract turned reddish brown in colour after 48 hours incubation. The formation of AgNPs from *C. thevetia* and *W.tomentosa* were conformed by absorption maximum at 443.07nm and 440.05 nm in UV-Visible analysis. The absorption maxima results in UV-Visible analysis of respective dyes clearly indicate total degradation by NaBH₄ and the AgNPs of both plants have exhibited remarkable catalytic activity in dye reduction reactions.

Key words: Green synthesis, AgNPs, 4-Nitrophenol, Synthetic dyes, Catalytic activity

Introduction

Nanobiotechnology is an emerging research field applying for next generation development in agriculture, environment and medicine sciences. Nanoparticles are usually referred as particles with a maximum size of 1 to

100nm (1) and exhibit novel and improved properties with respect to their size, distribution and morphology (2). In general metallic nanoparticles are often prepared using Silver (Ag), Gold (Au), Zinc (Zn) and Platinum (Pt) etc. Indeed silver nanoparticles (AgNPs) gained prominent research interest among metal nanoparticles due to their *in vitro* antimicrobial activity and cytotoxic activity (3). Biological synthesis of AgNPs is ecofriendly and cost effective process compared to physical and chemical methods (4, 5).

Dyes are the major synthetic organic compounds used in textile industries. However 15% of dye used is wasted and released into water bodies resulting significant pollution (6). Waste water treatment by physical and chemical methods is costly process and require more energy. The nano silver particles have shown remarkable catalytic activity in dye reduction and removal and exhibited the degradation activity on certain toxic chemicals like 4-Nitrophenol (7, 8, 9).

The phenolic and polymorphic compound 4-Nitrophenol is used to darken the leather, in the manufacture of drugs, fungicides, insecticides and dyes. Ingestion and inhalation of 4-Nitrophenol leads to nausea, drowsiness, headache and cyanosis (10). Methylene blue or methylthioninium chloride is a synthetic

heterocyclic aromatic dye and dark green powder when dissolved in water turns in to blue colour (11). Methyl orange is a synthetic dye, pH indicator in acid-base titrations and causes irritation incase of skin contact and eye contact (12). Another one methyl red is an azodye and used in acid base titrations as pH indicator. It is prepared by diazotization of anthranilic acid and followed by dimethyl aniline (13). Green synthesis method of nanoparticles involves plant extracts which is easy, first and less complicated handling procedure (5). The present study was carried out to synthesize silver nanoparticles using the leaf extract from *Cascabela thevetia* and *Wrightia tomentosa* belongs to Apocyanaceae family. The catalytic activity was screened on 4-Nitrophenol, Methylene blue, Methyl orange and Methyl red.

Materials and methods

Chemicals, reagents and plant source : The leaves of *Cascabela thevetia* were collected from Acharya Nagarjuna University campus, Guntur, India and the leaves of *Wrightia tomentosa* were collected from Tirumala hills, Tirupathi, India. The plants were taxonomically identified and authenticated by Prof. M. Vijayalakshmi, Department of Botany, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India. The Silver nitrate (AgNO_3) with 99.5% purity, 4-Nitrophenol, Methylene blue, Methyl orange and Methyl red were purchased from Merck. Molecular grade water (Millipore, Milli Q) was used in this study.

Biosynthesis of AgNPs from the extracts of *C.thevetia* and *W.tomentosa* : The leaves of *C. thevetia* and *W. tomentosa* were washed thrice with distilled water, dried and coarsely powdered. The powder of *C. thevetia* and *W. tomentosa* leaf (3gms) dispensed into 100ml of distilled water, boiled at 100°C for 10 minutes and the extract was filtered through Whatman filter paper. The 10ml of aqueous plant extract was mixed with 190ml of already prepared 1mM silver nitrate solution. The suspension was stirred using a magnetic stirrer for 5 min and kept at incubation for 48h at room temperature. The change in color

of the solution from light yellow to dark brown indicates the termination of the reaction and synthesis of AgNPs.

UV-Visible spectrophotometric analysis of the biosynthesized AgNPs

The formation and stability of silver nanoparticles was confirmed by UV-Visible spectroscopic studies after 48hrs using AgNO_3 solution as blank. Spectral analysis of the AgNPs was executed using UV-Visible Double beam spectrophotometer (Thermo Fischer) and the values were recorded between the range from 200 to 800 nm.

Evaluation of catalytic activity of AgNPs

The leaf extract mediated AgNPs of *Cascabela thevetia* and *Wrightia tomentosa* were utilised as catalysts seperately in the degradation and removal of 4-Nitrophenol, Methylene blue, Methyl orange and Methyl red by NaBH_4 . The catalytic degradation of respective dye involves three reactions and carried out in a 3.5ml Quartz cuvette. The first reaction is prepared by adding 1.5ml of 1mM of synthetic chemical to 1.5ml of milli Q water. After UV-visible spectrophotometric analysis 1mg of solid NaBH_4 was added at first reaction for the preparation of second reaction. The third reaction is prepared by adding 20 micro litres of biosynthesized AgNPs to the second reaction. All three reactions were studied in Thermo scientific UV-Visible spectrophotometer using milli Q water as blank (10).

Results and Discussion

The aqueous leaf extracts of *C. thevetia* and *W. tomentosa* when mixed with AgNO_3 solution as seperate reactions, both the respective solutions showed pale yellow colour. However after 48 hours incubation, both the mixures turned in to deep reddish brown colour indicating the formation of AgNPs (14) (Fig1). UV-Vis analysis of the biosynthesized AgNPs of both the plants recorded and absorption maxima at 443.07nm and 440.05nm respectively due to surface plasmon resonance (15) and conforming the formation of AgNPs (Fig 2).

Catalytic activity of biosynthesized AgNPs: Reduction reactions of 4-Nitrophenol

The dye degradation reactions were monitored and depicted in the following order 4-Nitrophenol, Methylene blue, Methyl orange and Methyl red. All reactions were analysed by UV-Visible spectrophotometer. Figure 3 has shown the UV-Visible analysis of dye degradation of the

4-Nitrophenol using NaBH_4 with AgNPs of *C. thevetia* and *W. tomentosa* as catalysts. The reaction of 4-Nitrophenol when monitored in spectrophotometer the absorption maximum 1.262 was observed at 318nm (16). In addition of NaBH_4 to first reaction the absorption maximum shifted to 400nm with the solution appeared bright yellow colour because of the formation of sodium phenolate and no change in the absorption maximum with respect to time was observed. With the addition of AgNPs the solution turned colourless and absorption maxima suddenly decreased from 1.262 to 0.822 in the case of AgNPs synthesized with the extract of *C. thevetia* and from 1.262 to 0.534 with the AgNPs synthesized from the extract of *W. tomentosa* indicating the complete degradation of 4-Nitrophenol (9, 17).



Fig. 1: A) AgNPs of *C. thevetia* and
B) AgNPs of *W. tomentosa*

Reduction reactions of Methylene blue

The degradation and removal of methylene blue by NaBH_4 in the presence of biosynthesized AgNPs as catalyst were analyzed. The results related to the reduction reactions of methylene blue by AgNPs are illustrated in figure 4. Methylene blue showed maximum absorption 2.33 at 664nm (18). After the addition of 1mg NaBH_4 to the reaction mixture, the absorption maximum peak was shifted to 656.04nm. However the synthesized AgNPs of both the plants

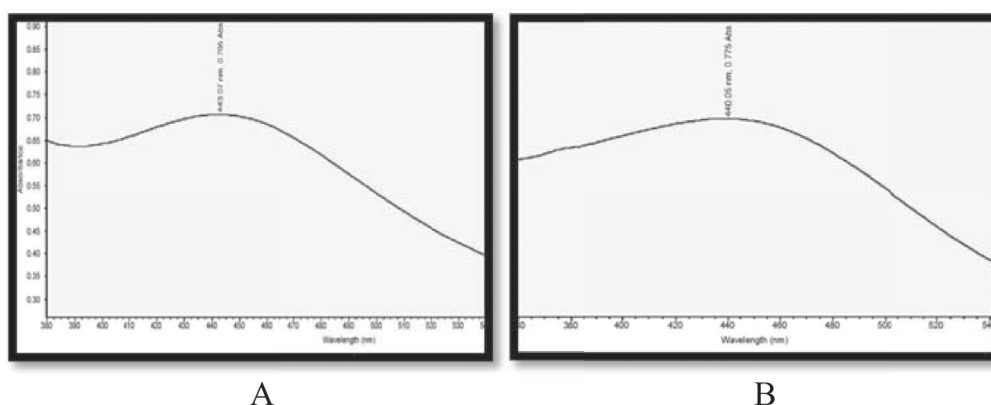


Fig. 2. UV- Visible analysis of A) *C. thevetia* AgNPs B) *W. tomentosa* AgNPs

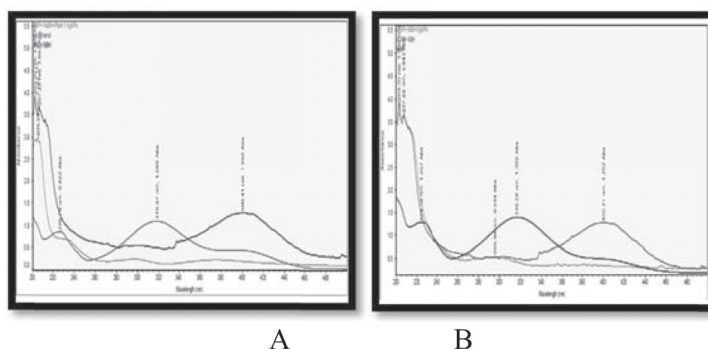


Fig. 3: Catalytic activity of A) *C. thevetia* AgNPs B) *W.tomentosa* AgNPs on 4-Nitrophenol

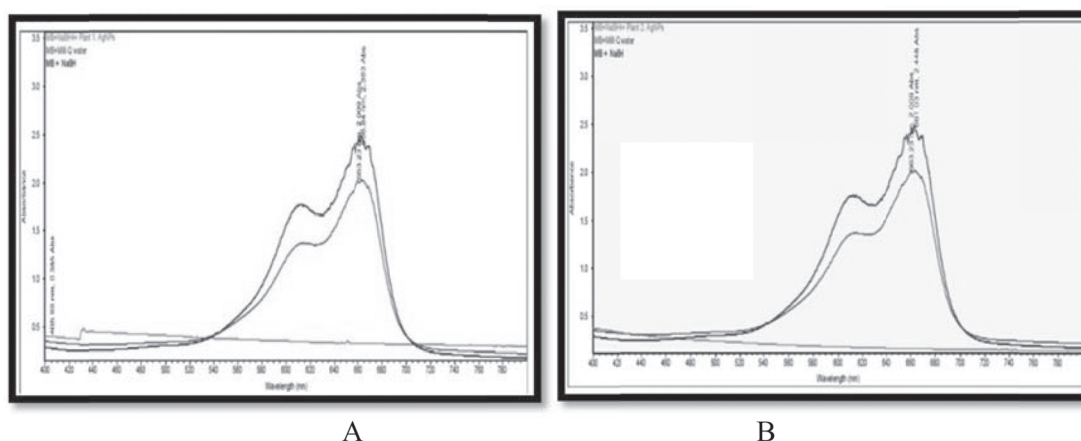


Fig. 4: Catalytic activity of A) *C. thevetia* AgNPs B) *W.tomentosa* AgNPs on Methylene blue

in separate reactions turned the solution colourless and the absorption was decreased from 2.33 to 0.385 and from 2.33 to 0.284 for *C. thevetia* and *W.tomentosa* respectively indicating that methylene blue was completely degraded (19).

Reduction reactions of Methyl orange : The degradation and removal reactions of methyl orange by NaBH_4 in the presence of AgNPs were studied by UV-visible spectrophotometer (Figure 5). The absorption maximum was found to be 0.752 at 462.22nm (8) and addition of 1mg NaBH_4 the maximum absorption was observed

at 463.89nm. After adding AgNPs of both the plants in separate reactions, the solution turned colorless with decrease in absorption maxima from 0.752 to 0.170 and 0.752 to 0.275 for *C. thevetia* and *W.tomentosa* respectively (20) indicating the reduction of methyl orange.

Reduction reactions of Methyl red : The UV-visible spectrophotometric analysis of the reduction reaction of methyl red by NaBH_4 in the presence of AgNPs as reducing agent is illustrated in figure 6. An absorption maximum of 2.651 was observed at 523.77nm (21). After addition of 1mg NaBH_4 the absorption maximum

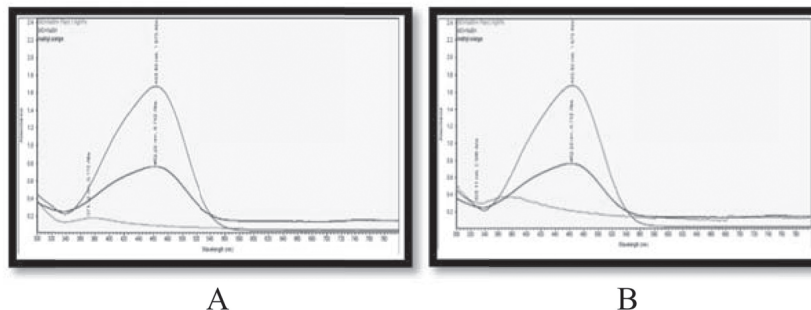


Fig. 5: Catalytic activity of A) *C. thevetia* AgNPs B) *W. tomentosa* AgNPs on Methyl orange

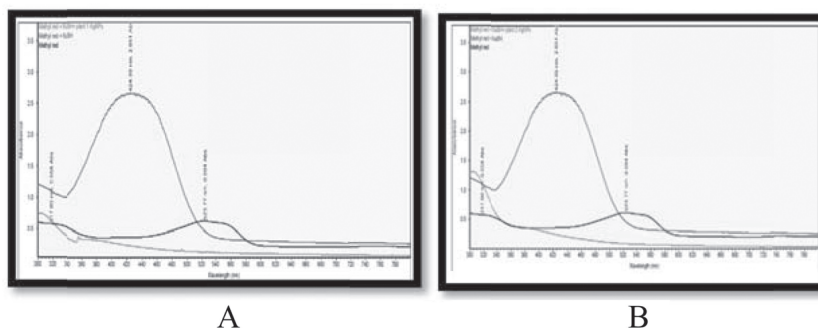


Fig. 6: Catalytic activity of A) *C. thevetia* AgNPs B) *W. tomentosa* AgNPs on Methyl red

S.No	Name of the Dye	Absorption Maxima of <i>C. thevetia</i>		Absorption Maxima of <i>W. tomentosa</i>	
		Before to the addition of AgNPs	After the addition of AgNPs	Before to the addition of AgNPs	After the addition of AgNPs
1	4-Nitrophenol	1.262	0.822	1.262	0.534
2	Methylene blue	2.33	0.385	2.33	0.284
3	Methyl orange	0.752	0.170	0.752	0.275
4	Methyl red	2.651	0.558	2.651	0.558

Table : UV-Visible Absorption maxima of synthetic chemicals in catalytic activity analysis.

was recorded at 424.89nm. With the addition of AgNPs of both the plants in separate reactions the solution turned colorless and the absorption completely decreased from 2.651 to 0.558 in the case of both plants indicating the complete reduction of methyl red (22, 23).

Conclusion

AgNPs were synthesized using the aqueous leaf extract of *Cascabela thevetia* and *Wrightia tomentosa* in separate reactions. The green synthesized AgNPs were used as catalysts in the reduction and degradation of 4-

Nitrophenol, Methylene blue, Methyl orange and Methyl red using NaBH_4 . All four reactions of respective dyes were analysed by UV-Visible spectrophotometer. In the reduction process of four dyes the absorption maxima of the four dyes decreased i) 4-Nitrophenol (from 1.262 to 0.822) with AgNPs of *C.thevetia* and (from 1.262 to 0.534) with AgNPs of *W. tomentosa*. ii) Methylene blue (from 2.33 to 0.385) with AgNPs of *C. thevetia* and (from 2.33 to 0.284) with the AgNPs of *W.tomentosa*. iii) Methyl orange (from 0.752 to 0.170) with the AgNPs of *C. thevetia* and (from 0.752 to 0.275) with the AgNPs of *W.tomentosa*. iv) Methyl red (2.651 to 0.558) with the AgNPs synthesized from both plants. From the above results it can be concluded that the AgNPs of both plants acted as catalyst in degradation of the above said dyes using NaBH_4 .

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Molecular Cloning of Partial Phytase Gene from *Bacillus subtilis* ATCC 6633 (GenBank: KT385665.1).

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Abstract

Phytase (*myo*-inositolhexakisphosphate phosphohydrolase) catalyzes the hydrolysis of *myo*-inositol hexakisphosphate (phytic acid) to inorganic monophosphate and lower *myo*-inositol phosphates, and in some cases to free *myo*-inositol. *Bacillus* species are known to produce a thermostable phytase. The *Bacillus subtilis* strain ATCC 6633 was chosen for cloning of *phy* gene. Primers were designed for *phy* gene amplification using the *phy* gene sequence of *B. subtilis* (AF298179). A sequence of 904 bp characteristic of *phy* gene was obtained through PCR amplification. Sequencing of PCR amplified fragments and further analysis using NCBI-BLAST online homology search program revealed that *phy* gene of *B. subtilis* (ATCC 6633) and *phy* gene of *B. subtilis* are 94% similar. The phylogenetic analysis indicated that *B. subtilis* (ATCC 6633) had high similarities with the phytases from AF292103.1 *Bacillus subtilis* strain YMN2f phytase (*phy*) gene and HQ730912.1 *Bacillus* sp. B5 phytase *PhyC1* gene. It was also found that *B. subtilis* (ATCC 6633) had relative high similarities with *Bacillus* sp. BAB- 1 *Phy* (*phy*) gene and FJ541287.1 *Bacillus subtilis* strain spizizenii strain phytase gene. Further 904 bp fragment was cloned in Insta T/A cloning vector and *E. coli* was successfully transformed and confirmed by colony PCR.

Key words: Phytase, PCR, *phy* gene, Molecular Cloning

Introduction

Phytase, or *myo*-inositol hexakisphosphate phosphohydrolase (EC 3.1.3.8), was first discovered by (8). The principal end products of phytase action are phosphoric acid and *myo*-inositol, but the phosphatidylinositols representing various degrees of dephosphorylation from inositol hexakisphosphate to inositol are generated as intermediates. Phytate, an inhibitor of iron absorption, can be degraded by phytase. Phytase is an ester-hydrolyzing enzyme with an estimated molecular mass of 35–700 kDa depending upon the sources of origin. Phytase genes have been isolated from plants (4), bacteria (5) and fungi (3).

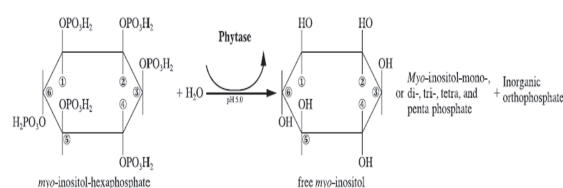


Fig.1. Hydrolysis of phytic acid by phytase enzyme

Although phytase shows a potential to be utilized for phytate bioconversion, the enzyme activities and yields need to be increased to make them possible for industrial application. Therefore, it is important to isolate a variety of different microorganisms and their enzymes for phytate degradation (1).

Phytic acid can be reduced through supplementing phytase enzyme in animal feeds as it is capable of hydrolyzing phytic acid to less-phosphorylated *myo*-inositol derivatives (2). Phytases also reduce the antinutritional effect of food having high phytate content. Phytase is widely present in nature, occurring in microorganisms, plants as well as in some animal tissues. Several phytases have been identified and cloned from *Pseudomonas* sp., *Escherichia coli*, *Klebsiella* sp. Phytase *appA* from *E. coli* is known to have the greatest specific activity but it lacks desired thermostability. In order to produce a more stable enzyme based on the *E. coli* phytase, it is necessary to have a set of homology to *E. coli* phytase sequence. However, this protein has only a few homologies with low sequence identity not exceeding beyond 30% to other known phytases. The phytase (*phyC*) from *Bacillus* sp. are \hat{a} -propeller phytase which exhibit an optimum pH from 6.0-9.0, suitable for neutral animal tracts such as trout and cyprinids with more thermostability.

As the primary sequence is emerging, gene cloning would be the next step to redesign this enzyme for novel substrate specificity; thus, genetic manipulation for this enzyme would lead to better catalytic performance with higher turnover rates and this would be industrially important. Bacterial phytase has been cloned and characterized from *B. subtilis* as well as *E. coli*. *A. niger* phytase has been well characterized and the gene encoding it, *phyA*, has been cloned and sequenced.

Hence, in the present investigation, the *Bacillus subtilis* ATCC 6633 strain was chosen to isolate and clone *phy* gene with a view to its overexpression in future endeavours.

Materials and Methods

Strains and growth media : *Bacillus subtilis* ATCC 6633 strain was procured from HiMedia. It was grown on the nutrient agar media and incubated at 37 °C for 16 h. A single colony was inoculated in the nutrient broth medium and incubated at 37° C for 16 h at 180 rpm.

Amplification of phy gene using PCR:

Genomic DNA extraction from bacterial strain was carried out according to the standard protocol (6). A set of forward and reverse primer to amplify the *phy* gene was designed by primer express software using the published *phy* gene sequence (AF298179). These primers were synthesized from Eurofins scientific laboratory, India. The primers were dissolved in TE to make a final working concentration of 20 pmol. The PCR amplification was carried out in 20 μ l of total reaction volume using programmable thermal cycler (Eppendorf, Germany). PCR was optimized by testing various components of the reaction mixture in different concentrations *viz.*, MgCl₂ (1.5 mM), forward and reverse primers PhyF (5'CTGTCTGATCCTTATCATTT3') and PhyR (5'TCCGCTTCTGTCGGTCA 3') (10 pmols) and annealing temperature (40-55°C). Amplification of the *phy* gene region was successfully achieved with initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation, annealing and extension for 1 min at 95°C, 55°C and 72°C, respectively and the final extension was carried out at 72°C for 8 min. The PCR product was visualized on 1.0% (w/v) agarose gel.

Ligation of phy gene in cloning vector and transformation:

The ligation of amplified *phy* gene was carried out in InsT/A cloning vector (pTZ57R/T) (Fermentas, USA) which is a convenient system for direct one step cloning of PCR amplified DNA fragment. The ligation reaction mix was prepared as follows: 10.0 μ l of purified PCR product, 1.5 μ l of InsT/A cloning vector, 2.5 μ l of ligase buffer, 1.0 μ l of T₄ ligase enzyme and 2.5 μ l of PEG. The final volume was made to 25 μ l with nuclease free water. Ligation reaction was carried out at 22 °C for 12 h in thermomixer (Eppendorf, Germany) and the ligation mix was subsequently used for transformation. Transformation was carried out by mixing 10 μ l of ligation product to 200 μ l of the *E. coli* DH5 α competent cells. The mixture was kept on ice for 1 h. Heat-shock was given for 45 s at 42 °C and rapidly chilled on ice for 10 min.

Upon transformation, 20 µl glucose and 10 µl MgCl₂ were added in to it and incubated at 37 °C for 2 h with 180 rpm shaking. The transformed cells were spread on ampicillin (75 µg ml⁻¹) containing LB agar plate and incubated at 37 °C for 18 h (10).

Screening of recombinant (*InsT/A*) clones having *phy* gene: The colonies growing on LB plates containing ampicillin (75 µg ml⁻¹) were used to isolate plasmid DNA. The plasmid DNA was extracted adopting miniprep plasmid isolation method. The confirmation of positive

recombinant clones was carried out by colony PCR.

Result and Discussion

Amplification of *phy* gene: The genomic DNA was extracted from *B. subtilis* (ATCC 6633). The data observed by the molecular characterization were in accordance with the biochemical characterization. Primers designed by (10) were used to amplify *phy* gene specific sequence. Size of amplified product was 904 bp (Fig.1). Amplified products were eluted from the gel and purified followed by sequencing for further analysis.

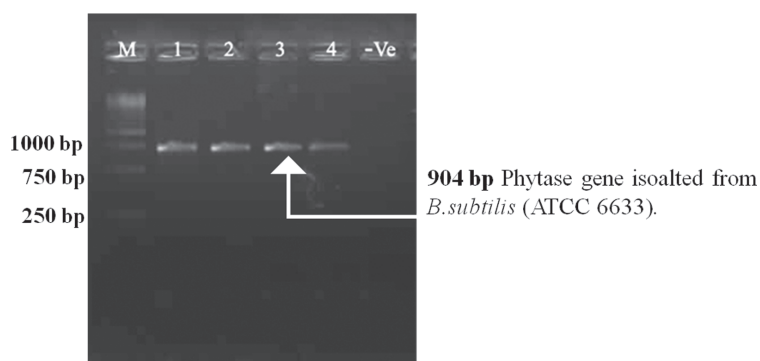


Fig. 1. 1.2% agarose gel electrophoresis showing PCR amplified 904 bp *phy* gene. M - (Marker), Lane 1, 2, 3,4 - PCR amplified product of *phy* gene, -ve – Negative control.

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GCTCATTATGTGAATGAGGAACATCATTTCAAAGTGACTGCACACACGGAGACAGATCCG 60
CGATGACGCAGCAGATGACCCGGCCATATTACAACAAATAAGAAAGTCAGGGCTCGTTGTG120
TATGATTTAGACGGAAAACAGCTTCATGAGTGTGCGATCTGCGCTATGATTTTCCATTGAA 180
CGGCGAAATTGCTGCTGCATCCAACCGGTCCGAAGGAAAAAATACAATTGAAGTATATGC 240
AATAGACGGGGATAAAGGAAAATTGAAAAGCATTACAGATCCGAAACATCCTATTTCCAC 300
CAATATTTCTGAGGTTTATGGATTTCGGCCTGTATCACAGCCAGAAAACAGGAGCATTTTA 360
CGCATTAGTGACAGGCAACAAGGGGAATTTGAGCAGTATGAAATTGTTGATGGTGGAAA 420
GGGTTATGTAACAGGGAAAAAGGTGCGTGAATTTAAGTTGAATTCTCAGACCGAAGGCCT 480
TGTTGCGGATGATGAGTATGGGAACCTATACATAGCAGAGGAAGATGAGGCCATCTGGAA 540
ATTTAACGCTGAGCCCGGCGGAGGGTCAAAGGGACAGGTTGTTGACCGTGCGACAGGAGA600
TCATTTGACAGCTGATATTGAAGGACTGACAATCTATTATGCACCAAATGGCAAAGGATA 660
TCTCATGGCTTCAAGTCAAGGAAATAACAGCTATGCAATGTATGAACGGCAGGGGAAAAA 720
TCGCTATGTAGCCAACCTTTGAGATTACAGATGGCGAGAAAATAGACGGTACTAGTGACAC 780
GGATGGTATTGATGTTCTCGGTTTCGGACTTGGCCCTATCCGTACGGGAGTGGCGCAGGA 84
OTGGCGAAAATATCGATAACGGACAAACCGTCAATCAAATTTCTTGTAACGTG 904
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Fig. 2. The sequence of *B.subtilis* (ATCC 6633).

Sequencing and phylogenetic analysis: Sequencing of PCR amplified fragments and further analysis using NCBI-BLAST online homology search program revealed that *phy* gene of *B. subtilis* (ATCC 6633) and *phy* gene of *B. subtilis* BC5 was 94% similar. A sequence of 904 bp of gene was obtained and the sequence is shown in fig. 2:

ClustalW analysis: The ClustalW analysis results of *B. subtilis* (ATCC 6633) and five other phyatse genes from AF453255.1 *Bacillus amyloliquefaciens* phytase (*phyB*) gene, AF292103.1 *Bacillus subtilis* phytase (*phy*) gene, HQ730912.1 *Bacillus* sp. B13 phytase *PhyC1* gene, GU143090.1 *Bacillus* sp. MD2 *Phy* (*phy*) gene and FJ541287.1 *Bacillus subtilis* strain E20 phytase gene, are listed in Fig. 3.

gi 345128268 gb HQ730912.1 gi 220067269 gb FJ541287.1 gi 15419530 gb AF292103.1 gi 10444418 gb AF298179.1 gi 270000210 gb GU143090.1 l214_278_003_2_Bsphy-F1-D02.ab	----- ----- ATTCACATTTGACCATTTTCACAAAACCTAACAC TGAACCTCCTGTATG ----- -----
gi 345128268 gb HQ730912.1 gi 220067269 gb FJ541287.1 gi 15419530 gb AF292103.1 gi 10444418 gb AF298179.1 gi 270000210 gb GU143090.1 l214_278_003_2_Bsphy-F1-D02.ab	-----ATGAATC -----ATGAATC TATTTTACAATTAAGTACAC TTTTCATAAAAGGAGGAAGGAAATGAATC ----- -----
gi 345128268 gb HQ730912.1 gi 220067269 gb FJ541287.1 gi 15419530 gb AF292103.1 gi 10444418 gb AF298179.1 gi 270000210 gb GU143090.1 l214_278_003_2_Bsphy-F1-D02.ab	ATTCAAAACAC TTTTGTAAACC GCGGCAGCCGGATTGGTGC TCACATGC ATTCAAAACAC TTTTGTAAACC GCGGCAGCCGGATTGGTGC TCACATGC ATTCAAAACAC TTTTGTAAACC GCGGCAGCCGGATTGGTGC TCACATGC ----- -----
gi 345128268 gb HQ730912.1 gi 220067269 gb FJ541287.1 gi 15419530 gb AF292103.1 gi 10444418 gb AF298179.1 gi 270000210 gb GU143090.1 l214_278_003_2_Bsphy-F1-D02.ab	GGTGCGGTTTCTTCCAGGCCAAGCATAAGCTGTCTGATCCTTATCATTT GGTGCGGTTTCTTCCAGGCCAAGCATAAGCTGTCTGATCCTTATCATTT GGTGCGGTTTCTTCCAGGCCAAGCATAAGCTGTCTGATCCTTATCATTT -----CTGTCCGATCCTTATCATTT -----CTGTCTGATCCTTATCATTT -----GCTCATTATGTGAATGAGGAACATCATTT ***** *:*
gi 345128268 gb HQ730912.1 gi 220067269 gb FJ541287.1 gi 15419530 gb AF292103.1 gi 10444418 gb AF298179.1 gi 270000210 gb GU143090.1 l214_278_003_2_Bsphy-F1-D02.ab	TACC GTGAATGCGGCGGCCGAAACGGAACCGGTTGATACAGCAGGTTGATG TACC GTGAATGCGGCGGCCGAAACGGAACCGGTTGATACAGCAGGTTGATG TACC GTGAATGCGGCGGCCGAAACGGAACCGGTTGATACAGCAGGTTGATG TACC GTGAATGCGGCGGCCGAAACGGAACCGGTTGATACAGCAGGTTGATG TACC GTGAATGCGGCGGCCGAAACGGAACCGGTTGATACAGCAGGTTGATG CAAAGTGACTGCACACACGGAGACAGATCCGGTCCGATCTGGCGATGACG *:*
gi 345128268 gb HQ730912.1 gi 220067269 gb FJ541287.1 gi 15419530 gb AF292103.1 gi 10444418 gb AF298179.1 gi 270000210 gb GU143090.1 l214_278_003_2_Bsphy-F1-D02.ab	CAGCTGATGATCCTGCGATTTGGCTGGACCCCAAGAATCCTCAGAACAGC CAGCTGATGATCCTGCGATTTGGCTGGACCCCAAGAATCCTCAGAACAGC CAGCTGATGATCCTGCGATTTGGCTGGATCCCAAGAATCCTCAGAACAGC CAGCTGATGATCCTGCGATTTGGCTGGACCCCAAGAATCCTCAGAACAGC CAGCTGATGATCCTGCGATTTGGCTGGACCCCAAGAATCCTCAGAACAGC CAGCAGATGACCCGGCCATAT-----CAAGAATCCTCAGAACAGC *****
gi 345128268 gb HQ730912.1 gi 220067269 gb FJ541287.1 gi 15419530 gb AF292103.1 gi 10444418 gb AF298179.1 gi 270000210 gb GU143090.1 l214_278_003_2_Bsphy-F1-D02.ab	AAATTGATCACAACCAATAAAAAATCAGGCTTAGTGTGTACAGCCTAGA AAATTGATCACAACCAATAAAAAATCAGGCTTAGTGTGTACAGCCTAGA AAATTGATCACAACCAATAAAAAATCAGGCTTAGTGTGTACAGCCTAGA AAATTGATCACAACCAATAAAAAATCAGGCTTAGTGTGTACAGCCTAGA AAATTGATCACAACCAATAAAAAATCAGGCTTAGTGTGTACAGCCTAGA -----TACAACCAATAAGAAGTCAAGGCTCGTTGTGTATGATTTAGA *****
gi 345128268 gb HQ730912.1 gi 220067269 gb FJ541287.1 gi 15419530 gb AF292103.1 gi 10444418 gb AF298179.1 gi 270000210 gb GU143090.1 l214_278_003_2_Bsphy-F1-D02.ab	GGGAAAGATGCTTCATTCCTATCCTACCGGGAAGCTGAACAATGTTGATA GGGAAAGATGCTTCATTCCTATCCTACCGGGAAGCTGAACAATGTTGATA GGGAAAGATGCTTCATTCCTATCCTACCGGGAAGCTGAACAATGTTGATA GGGAAAGATGCTTCATTCCTATCCTACCGGGAAGCTGAACAATGTTGATA GGGAAAGATGCTTCATTCCTATCCTACCGGGAAGCTGAACAATGTTGATA CGGAAACACAGCTTCATGAGTGTCGATCTG----- *****

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gi|10444418|gb|AF298179.1|      TCCGCTATGATTTTCCGTTGAACGGAAAAAAAGTCGATATTGCGGCGGCA
gi|270000210|gb|GU143090.1|      TCCGTATGATTTTCCGTTGAACGGAAAAAAAGTCGATATTGCGGCGGCA
I214_278_003_2_Bsphy-F1-D02.ab  --CGCTATGATTTTCCATTGAACG-----GCGAAATTGCTGCTGCA
                                ** *****.*****                                **:***** ** **

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gi|15419530|gb|AF292103.1|      TCCAATCGGTCTGAAGGAAAGAATACCATTGAGATTTACGCCATCGACGG
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I214_278_003_2_Bsphy-F1-D02.ab  TCCAACCGGTCCGAAGGAAAAATACAAATTGAAGTATATGCAATAGACGG
***** ** ** *****.*****.*****.*** ** ** *****

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..*:*:*:*.*.***..*****.*****..*.* ** ** * :

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gi|220067269|gb|FJ541287.1|      CAGCAATTGATGAAGTATACGGTTTCAGCTTGTACCACAGTCAAAAAACA
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gi|270000210|gb|GU143090.1|      CAGCAATTGATGAAGTATACGGTTTCAGCTTGTACCACAGTCAAAAAACA
I214_278_003_2_Bsphy-F1-D02.ab  CCAATATTTCTGAGGTTTATGGATTCCGGCTGTATCACAGCCAGAAAAACA
*...*:*: : ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** * * * * *

gi|345128268|gb|HQ730912.1|      GGAAAGTATTACGCGATGGTGACAGGGAAGAAGGCCGAATTTGAACAATA
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gi|10444418|gb|AF298179.1|      GGAAGATATTACGCGATGGTGACAGGGAAGAAGGCCGAATTTGAACAATA
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I214_278_003_2_Bsphy-F1-D02.ab  GGAGCATTTTACGCATTAGTGACAGGCCAAACAAGGGGAATTTGAGCAGTA
***. *:*:*****. *.*.***** ** ** ** ** ** ** * * * * *

gi|345128268|gb|HQ730912.1|      CGAATTAATGCTGATAAAAAATGGATACATATCCGGCAAAAAGGTAAGGG
gi|220067269|gb|FJ541287.1|      CGAATTAATGCTGATAAAAAATGGATACATATCCGGCAAAAAGGTAAGGG
gi|15419530|gb|AF292103.1|      CGAATTAATGCTGATAAAAAATGGATACATATCCGGCAAAAAGGTAAGGG
gi|10444418|gb|AF298179.1|      CGAATTAATGCTGATAAAAAATGGATACATATCCGGCAAAAAGGTAAGGG
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***. *:*:***. *.*.***** ** ** ** * * * * *

gi|345128268|gb|HQ730912.1|      CGTTTAAAATGAATTCAGACAGAAGGGATGGCAGCAGACGATGAATAC
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gi|15419530|gb|AF292103.1|      CGTTTAAAATGAATTCAGACAGAAGGGATGGCAGCAGACGATGAATAC
gi|10444418|gb|AF298179.1|      CGTTTAAAATGAATTCAGACAGAAGGGATGGCAGCAGACGATGAATAC
gi|270000210|gb|GU143090.1|      CGTTTAAAATGAATTCAGACAGAAGGGATGGCAGCAGACGATGAATAC
I214_278_003_2_Bsphy-F1-D02.ab  AATTTAAGTTGAATTCAGACAGGAAAGGCTTGTGCGGATGATGAGTAT
..*****.:*****.*****. * * :*:* ** ** **

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gi|15419530|gb|AF292103.1|      GGCAGTCTTTATATCGCAGAAGAAGATGAGGCCATCTGGAAGTTTCAGCGC
gi|10444418|gb|AF298179.1|      GGCAGTCTTTATATCGCAGAAGAAGATGAGGCCATCTGGAAGTTTCAGCGC
gi|270000210|gb|GU143090.1|      GGCAGTCTTTATATCGCAGAAGAAGATGAGGCCATCTGGAAGTTTCAGCGC
I214_278_003_2_Bsphy-F1-D02.ab  GGAACCTATACATAGCAGAGGAAGATGAGGCCATCTGGAATTTAACGC
** * . **:* ** ** *****.*****.***** ** * ** **
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gi|15419530|gb|AF292103.1| TGAGCCGGACGGCGCAGTAACGGAACGGTTATCGATCGTGCCGACGGCA
gi|10444418|gb|AF298179.1| TGAGCCGGACGGCGCAGTAACGGAACGGTTATCGATCGTGCCGACGGCA
gi|270000210|gb|GU143090.1| TGAGCCGGACGGCGCAGTAACGGAACGGTTATCGATCGTGCCGATGGCA
I214_278_003_2_Bsphy-F1-D02.ab TGAGCCC GGCGAGGTC AAGGGACAGGTTGTTGACCGTGCCGACAGGAG
***** * . * * * * * : * * * * * . * * * * * * * * * * * * * * * *

gi|345128268|gb|HQ730912.1| GGCATTTAACCCCGATATTGAAGGACTGACGATTTACTACGCTGCTGAC
gi|220067269|gb|FJ541287.1| GGCATTTAACCCCGATATTGAAGGACTGACGATTTACTACGCTGCTGAC
gi|15419530|gb|AF292103.1| GGCATTTAACCCCGATATTGAAGGACTGACGATTTACTACGCTGCTGAC
gi|10444418|gb|AF298179.1| GGCATTTAACCCCGATATTGAAGGACTGACGATTTACTACGCTGCTGAC
gi|270000210|gb|GU143090.1| GGCATTTAACCCCGATATTGAAGGACTGACGATTTACTACGCTGCTGAC
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. * * * * * . * * * * * * * * * * * * * * * * * * * * * * * * * * * *

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gi|15419530|gb|AF292103.1| GGGAAAGGTTATTTGCTTGCATCAAGCCAGGGTAACAGCAGCTACGCGAT
gi|10444418|gb|AF298179.1| GGGAAAGGTTATTTGCTTGCCTCAGCCAGGGTAACAGCAGCTACGCGAT
gi|270000210|gb|GU143090.1| GGGAAAGGTTATTTGCTTGCCTCAGCCAGGGTAACAGCAGCTACGCGAT
I214_278_003_2_Bsphy-F1-D02.ab GGCACAAAGGATATCTCATGGCTCAGTCAAGGAAATACAGCTATGCAAT
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

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gi|15419530|gb|AF292103.1| TTATGAAAGACAGGGACAGAACAATATGTTGCGGACTTTTCAGATAACAG
gi|10444418|gb|AF298179.1| TTATGAAAGACAGGGACAGAACAATATGTTGCGGACTTTTCAGATAACAG
gi|270000210|gb|GU143090.1| TTATGAAAGACAGGGACAGAACAATATGTTGCGGACTTTTCAGATAACAG
I214_278_003_2_Bsphy-F1-D02.ab GTATGAAAGCGCAGGGGAAAAATCGCTATGTAGCCAAC TTTGAGATTACAG
***** * * * * * . * * * * * . * * * * * * * * * * * * * * * * * * * * * *

gi|345128268|gb|HQ730912.1| ACGGGCCTGAAACAGACGGCACAAGCGATACAGACGGAATTTGACGTTCTG
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gi|15419530|gb|AF292103.1| ACGGGCCTGAAACAGACGGCACAAGCGATACAGACGGAATTTGACGTTCTG
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gi|270000210|gb|GU143090.1| ACGGGCCTGAAACAGACGGCACAAGCGATACAGACGGAATTTGACGTTCTG
I214_278_003_2_Bsphy-F1-D02.ab ATGGCGAGAAAATAGACGGTACTAGTGACACGGATGGTATTGATTTCTC
* * * * * . * * * * * * * * * * * * * * * * * * * * * * * * * * * *

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gi|220067269|gb|FJ541287.1| GGTTTTGGGCTGGGGCCTGAATATCCGTTCCGGCTTTTTGTCGCACAGGA
gi|15419530|gb|AF292103.1| GGTTTTGGGCTGGGGCCTGAATATCCGTTCCGGCTTTTTGTCGCACAGGA
gi|10444418|gb|AF298179.1| GGTTTTGGGCTGGGGCCTGAATATCCGTTCCGGCTTTTTGTCGCACAGGA
gi|270000210|gb|GU143090.1| GGTTTTGGGCTGGGGCCTGAATATCCGTTCCGGCTTTTTGTCGCACAGGA
I214_278_003_2_Bsphy-F1-D02.ab GGTTCGACGATTCGTTCCGGCTTTTTGTCGCACAGGA
GGTTTTGGGCTGGGGCCT----ATCCGTACG----GGAGTGGCGCAGGA
***** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

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gi|15419530|gb|AF292103.1| CGGAGAAAATATAGATCACGGCCAAAAGGCCAATCAAAAATTTTAAATGG
gi|10444418|gb|AF298179.1| CGGAGAAAATATAGATCACGGCCAAAAGGCCAATCAAAAATTTTAAATGG
gi|270000210|gb|GU143090.1| CGGAGAAAATATAGATCACGGCCAAAAGGCCAATCAAAAATTTTAAATGG
I214_278_003_2_Bsphy-F1-D02.ab TGGGAAAATATCGATAACGGACAAACCGTCAATCAAAAATTT----CTTG
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

gi|345128268|gb|HQ730912.1| TGCCATGGGAAAGAATCGCTGATAAAAATCGGCTTTCACCCGCGAGGTC AAT
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gi|15419530|gb|AF292103.1| TGCCATGGGAAAGAATCGCTGATAAAAATCGGCTTTCACCCGCGAGGTC AAT
gi|10444418|gb|AF298179.1| TGCCATGGGAAAGAATCGCTGATAAAAATCGGCTTTCACCCGCGAGGTC AAT
gi|270000210|gb|GU143090.1| TGCCATGGGAAAGAATCGCTGATAAAAATCGGCTTTCACCCGCGAGGTC AAT
I214_278_003_2_Bsphy-F1-D02.ab TATCGTG-----
* . * * *

gi|345128268|gb|HQ730912.1| AAACAGGTTGACCCGAGAAAACGACCGACAGAAAGCGGAAAATAA
gi|220067269|gb|FJ541287.1| AAACAGGTTGACCCGAGAAAACGACCGACAGAAAGCGGAAAATAA
gi|15419530|gb|AF292103.1| AAACAGGTTGACCCGAGAAAACGACCGACAGAAAGCGGAAAATAA
gi|10444418|gb|AF298179.1| AAACAGGTTGACCCGAGAAAACGACCGACAGAAAGCGGAAAATAA
gi|270000210|gb|GU143090.1| AAACAGGTTGACCCGAGAAAACGACCGACAGAAAGCGGAAAATAA
I214_278_003_2_Bsphy-F1-D02.ab -----
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Fig 3. ClustalW analysis of *B. subtilis* (ATCC 6633) and other five sequences with high similarities with *B. subtilis* (ATCC 6633).

Phylogenetic analysis: The phylogenetic analysis indicated that *B. subtilis* (ATCC 6633) had high similarities with the phytases from AF292103.1 *Bacillus subtilis* strain YMN2f phytase (*phy*) gene and HQ730912.1 *Bacillus* sp.

B5 phytase *PhyC1* gene. It was also found that *B. subtilis* (ATCC 6633) had relative high similarities with *Bacillus* sp. BAB- 1 Phy (*phy*) gene and FJ541287.1 *Bacillus subtilis* strain spizizenii strain phytase gene.

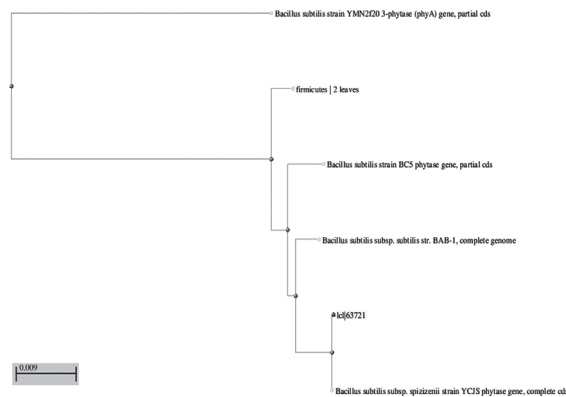


Fig.4. The phylogenetic tree of *B. subtilis* (ATCC 6633) phytase gene.

Cloning of phy gene in InsT/A cloning vector: PCR amplified 904 bp phytase gene was ligated in InsT/A cloning vector as shown in Fig. 12. Construct was designated as InsT/A-phy. In the present study, transformation was done by using heat-shock method. Initial confirmation of the insertion of amplified *phy* gene into the cloning vector was done by colony PCR. The colony PCR with *phy* gene specific primers revealed a PCR product of 904 bp indicating successful transformation (Fig. 5).

Conclusion

The identified and confirmed InsT/A-phy clone with phytase gene of *B. subtilis* will be

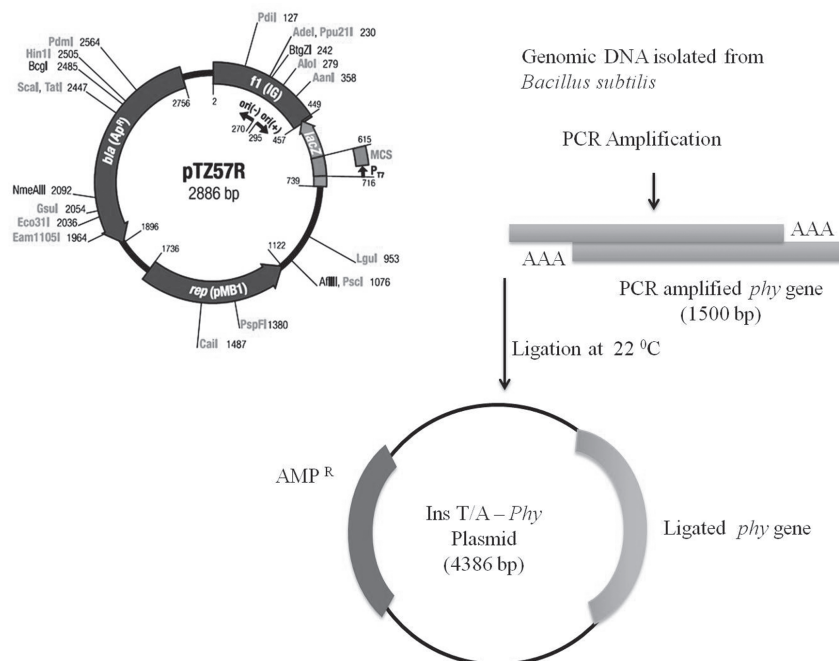


Fig.5. Cloning strategy for inserting PCR amplified *phy* gene in InsT/A cloning vector [Source: www.fermentas.com]

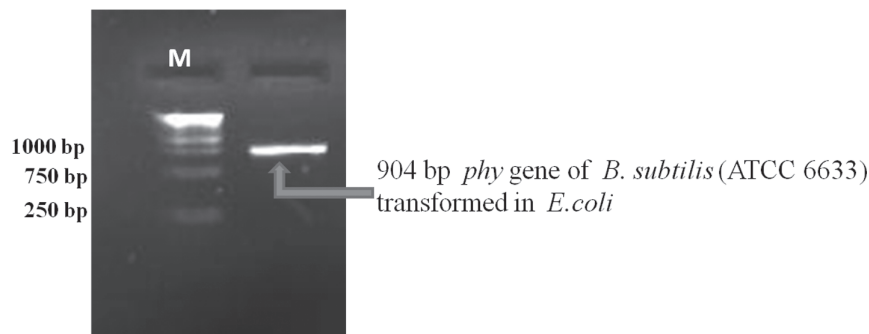


Fig.6. The agarose gel electrophoresis of the colony PCR products showing 904 bp *phy* gene of *B. subtilis* (ATCC 6633)

helpful for further over-expression for large scale production of cost effective and thermostable phytase enzyme through fermentation and their utilization in animal feed and other purposes.

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***Mucorimietiana* sp. nov.**

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Abstract

A thermophilic, heterothallic species of *Mucor* - *M. imietiana* A. Swathi Sri.& A.Subrahm.sp. nov.isolated from garden soil from MIET campus is described and illustrated. It grows well at 50^o C on synthetic mucor agar and potato-dextrose agar from pH 3.0-9.0 with an optimum pH at 7.0. The specific epithet refers to its habitat.

Key words: *Mucorimietiana* sp. nov., Thermophilic, Heterothallic, Garden soil MIET Meerut

Introduction

During study on fungal flora of Meerut district a novel species of *Mucor* optimally growing at 50^o C was isolated from MIET garden soil. Its morphology and culture characters observed on monosporic culture grown on synthetic mucor agar at 50^oC are presented.

MucorimietianaA. Swathi Sri.& A. Subrahm.sp. nov. (Fig 1-6; Plate 1-5).

Colonies on synthetic mucor agar at 50^o C fast growing cover 80 mm plate in 48 hrs. White at first, slowly turn mouse gray with age and sporulation; turf 2mm in height; sporulation abundant; reverse colony white with black areas here and there, heterothallic.

Mycelium hyaline, smooth, aseptate and branched; sporangiophores hyaline occasionally swollen at the base, one septate below sporangium and branched; branching sympodial to irregular, more or less umbellate at the distal end. Occasionally compound umbels are also

present or one of the branches of sporangiophores may become sterile spine. Sporangia spherical, multispored, hyaline when young, pale golden yellow when mature, 14.0-48.0µm, columellate; columella variable in shape, globose to ellipsoid, collarate 17.0-34.0 X 14.0-28 µm; sporangial wall smooth, hyaline, distal part of the sporangial wall becomes deliquescent at maturity to release sporangiospores; sporangiospores globose, small, hyaline, one celled, smooth 2.0 – 3.0 µm often agglutinated. Rhizoids absent; Zygospores absent even after prolonged incubation at lower temperatures like 5-20^oC.

Habitat : Garden soil, MIET campus, Meerut, Uttar Pradesh, India. Temperature relations : Optimum-50^o C; Minimum 28^o C; Maximum- 60^o C

pH relations : Grows from pH 3.0 - 9.0; Optimum pH 7.0

Date of isolation : 22 October, 2009

Culture deposited at : NFCCI2251 (Agarkar Institute), Pune, India and MTCC, Chandigarh- 10744

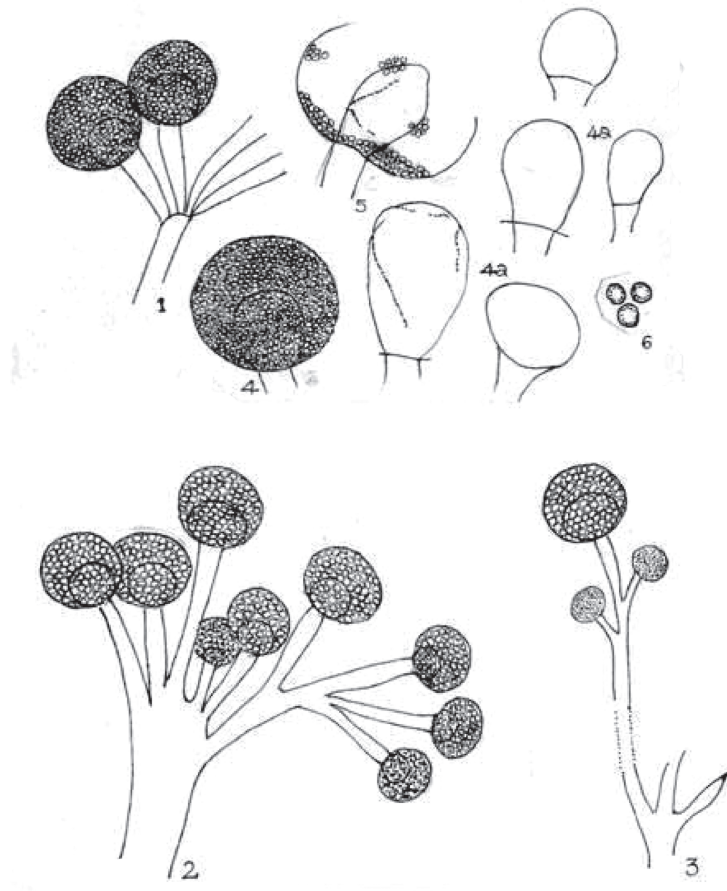
***Mucorimietiana* sp. nov** (Fig 1-6; Plate 1-5)

Sporangiophores stipes, stipes sympodial utincompositemagisvel minor umbellate proculcolus terminus sporangia globose columellate, teres, rutilus crocus, parietisteres tenuis deliquiscent 14.0 – 48.0 µm columella variable in vultus, teresglobose ut ellipsoid collarate 17.0 – 34.0 X 14.0 – 28.0 µm. Rhizoids

adversus ut sporangiophores nunquam tendo. Sporangiospores ve grandis smooth, globose, unis celled 2.0 - 3.0 μm zygosporas non produced. Habitat: Ortus Terra MIET Castra Meerut.

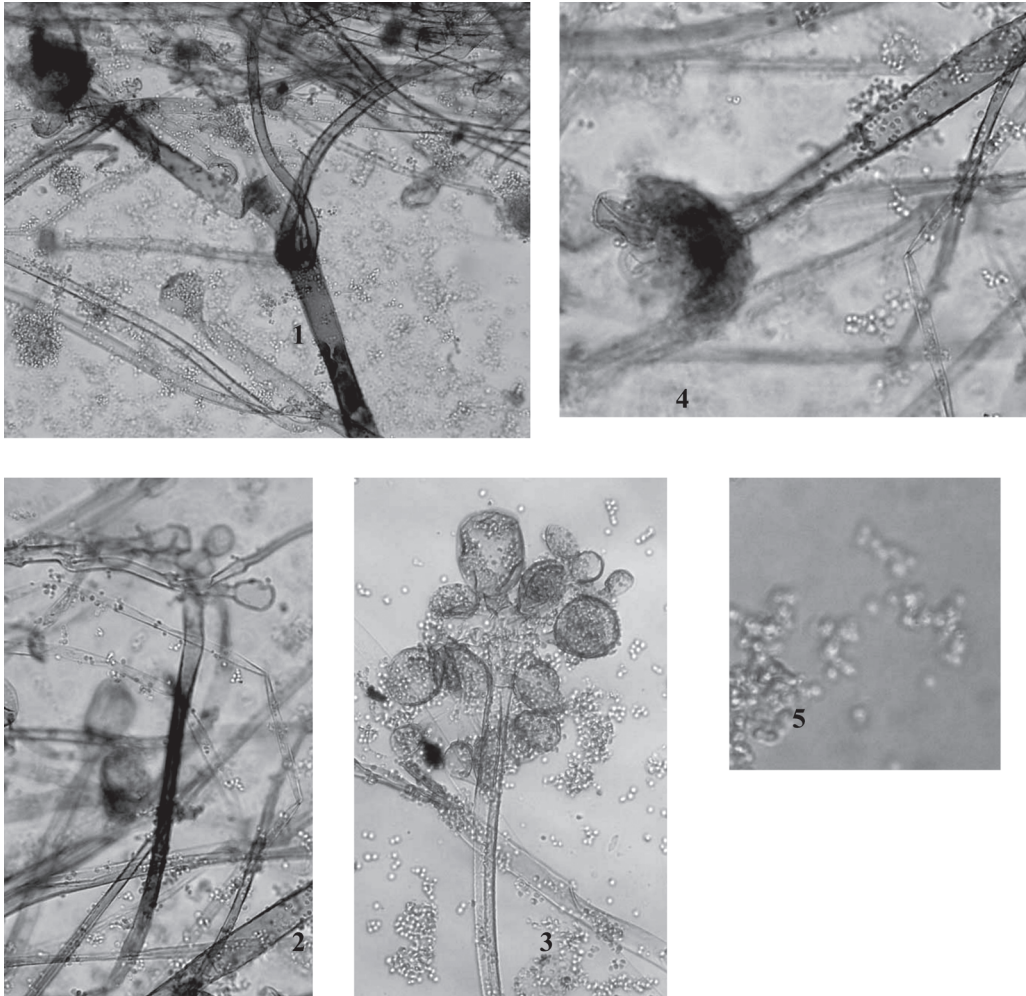
Discussion

Thermophilism in fungi was first reported in *Mucorpusillus* (6). Since then few more thermophilic species of *Mucor* were reported (1-3, 5). But Schipper (4) transferred all thermophilic



Figs. 1-6. *Mucorimietiana* sp. nov

- | | |
|---|-------|
| 1. Umbellate arrangement sporangiophores | 400X |
| 2. Compound umbel | 400X |
| 3. One of the branches of sporangiophores modified into a sterile spine | 400X |
| 4. Mature sporangium | 400X |
| 4a. Different shapes of columella | 400X |
| 5. Mature sporangium after dissolution of sporangial wall | 400X |
| 6. Sporangiospores | 1500X |



Plates 1-5. *Mucorimietiana* sp. nov.

- | | |
|--|------|
| 1 & 2 Umbellate arrangement of sporangiophores | X200 |
| 3 Compound umbel | X200 |
| 4 Mature sporangium after dissolution of sporangial wall | X200 |
| 5 Sporangiospores | X400 |

Mucor species developing rhizoids and stolens to *Rhizomucor*.

Looking at the sympodial branching of the sporangiophores which appear more or less umbellate at the distal end, thermophilic nature, heterothallic behavior of the present isolate one may consider it as *Rhizomucorpusillus*.

But the present isolate although thermophilic and heterothallic never produces rhizoids and stolans. Therefore it is considered as species of *Mucor* rather than *Rhizomucor*. Other differences are presented in Table-1.

The present isolate is *Mucor* like and resembles the mesophilic species *M. hemilis* in

Mucorimietiana sp. nov.

Table 1. Comparative characters of *M. imietiana* and *M. hemilis*

Character	<i>M. imietiana</i>	<i>M. hemilis</i>
Temperature	Thermophilic	Mesophilic
Rhizoids & Stolons	Absent	Absent
Sporangiophores	Branching sympodial to irregular, upper part umbellate, colorless	Sympodial, colorless
Compound umbels	Occasionally present	Absent
Sporangia	Spherical golden yellow 14.0 - 48.0 µm in diameter	Yellow to dark brown up to 70 µm, in diam.
Sporangial wall	Smooth golden yellow deliquescent at maturity	Deliquescent at maturity
Columella	Globose to ellipsoidal 17.0-34.0 X 14.0-28.0 µm	Globose 38.0-30.0 µm
Sporangiospores	Globose, hyaline 2.0-3.0 µm	Ellipsoidal flat at one end 5.5-8.5 X 2.5-5.5 µm
Zygospores	Heterothallic	Homothallic

characters like sympodial branching of sporangiohores, globose to ellipsoid, hyaline, smooth columella and sporangiospores. However the present isolate appears distinct in being thermophilic, smaller dimensions of sporangia, columella, sporangiospores and absence of oidea. Occasional presence of compound umbels, modification of one of the branches of sporangiophores into sterile spine, are the additional features of distinction. Therefore it is described as new species under the name *M. imietiana*. The specific epithet refers to its habitat.

Acknowledgements

Thanks are due to P.V. Brahmachari, Head, Biotechnology and Prof.M.V. Basaveswara Rao, Director, for their interest and encouragement for carrying out this work. Thanks are also due to Shree Vishnu Saran, Chairman, MIET, Lt. Gen. J. M.Garga, D.G. MIET; Dr. V.N. Sharma, Director Biotechnology for Laboratory facilities, ShriSudheerKumar Bhatt for typing script and Shri Shariq Iqbal, Lab Supervisor for photography.

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NEWS ITEM

SCIENTIFIC NEWS



104th Indian Science Congress: Prime Minister Shri Narendra Modi on 3 January 2017 inaugurated the five-day annual 104th Indian Science Congress (ISC-2017) at the Sri Venkateswara University in Tirupati. The focal theme of 104th ISC-2017 was "Science & Technology for National Development". Prime Minister Modi emphasized that the government was committed to supporting different streams of scientific knowledge from fundamental science to applied science with an emphasis on innovations. He said that the country's best science and technology institutions should further strengthen their basic research in line with leading global standards. The prime minister also touched upon the importance of social responsibility of science. He also specifically addressed Cyber-Physical Systems and expressed that there "is a need to develop and exploit these technologies in services and manufacturing sectors."

Assam Government designates Majuli to be India's first ever carbon neutral district: Assam government has initiated a project to make river Island Majuli the country's first ever Carbon Neutral district by 2020. The project titled 'Sustainable Action for Climate Resilient Development in Majuli' (SACReD, Majuli) has been initiated by Assam's department of Environment and Forest and was launched by Additional Principal Chief Conservator of Forest

(Biodiversity and Climate) at Majuli. The project, SACReD, Majuli has been initiated to combat climate change and reduce greenhouse gas emissions. Mitigation through forestry activities and biodiversity conservation will be the starting points for the carbon neutral agenda followed by other interventions over the next three years. The project will be designed and implemented in partnership with other departments of the district for which a district level committee under the chairmanship of Deputy Commissioner has been constituted.

MIT researchers use Jets to clean Delhi air: A team of experts from Massachusetts Institute of Technology (MIT), and Jawaharlal Nehru Technological University, is pursuing an idea to improve Delhi's winter air quality. The scientists plan to pilot a project under which decommissioned jet engines will be placed close to a power plant. The turbojets and turboprop engines will be directed upwards to form powerful updrafts (air moving upward) and disrupt "winter inversion" - a phenomenon during which air temperature near the ground is less than above - which traps pollutants close to the ground.

SCIENTIFIC FINDINGS

Regeneration of the external layer of a human heart by Stem cells: A process using human stem cells can generate the cells that cover the external surface of a human heart -- epicardium cells -- according to a multidisciplinary team of researchers from biomedical engineering and biology, Penn State, USA. Myocardium, the middle of the heart's three layers, is the thick, muscular part that contracts to drive blood through the body. The Wnt signaling pathway is a group of signal transduction pathways made of proteins that pass signals into a cell using cell-surface receptors. The group's results, published in Nature Biomedical Engineering, bring them one step closer to regenerating an entire heart wall. Through morphological assessment and

functional assay, the researchers found that the generated epicardium cells were similar to epicardium cells in living humans and those grown in the laboratory. The group's method of generating epicardium cells could be useful in clinical applications, for patients who suffer a heart attack. According to the Centers for Disease Control and Prevention, every 43 seconds, someone in the United States has a heart attack.

Researchers report in vitro tissue engineering of functional component of human stomach: Scientists from the Cincinnati Children's Hospital Medical Center report in *Nature* using pluripotent stem cells to generate human stomach tissues in a petri dish that produce acid and digestive enzymes. They grew tissues from the stomach's corpus/fundus region. The study comes two years after the same team generated the stomach's hormone-producing region (the antrum). The discovery means investigators now can grow both parts of the human stomach to study disease, model new treatments and understand human development and health in ways never before possible. Now both antral- and corpus/fundic-type human gastric mini-organs can be grown and it is possible to study how these human gastric tissues interact physiologically, respond differently to infection, injury and react to pharmacologic treatments, as in investigated in their study.

Novel approach to diagnose retinal diseases developed by Indian Scientists: Early diagnosis of certain eye diseases and studying the early progression of the diseases has now become possible, thanks to the work carried out by a team of researchers from three institutes — IISER, Kolkata, L.V. Prasad Eye Institute, Hyderabad, and BARC, Visakhapatnam. The researchers used the retinal data captured by a well-established imaging method in ophthalmology (optical coherence tomography or OCT) and applied an algorithm based on a statistical biomarker tool for early detection of diabetic macular edema. The results were published in

the *Journal of Biomedical Optics*. Biological tissues have complex geometrical patterns, which are called multifractals. The OCT images are light intensity-based and so the multifractal information gets hidden. Using the software, the researchers are able to find a peak at the junction between two layers from the refractive index data extracted from the OCT images. The thickness of a layer can be calculated by measuring the distance between two successive peaks. When the thickness of a layer becomes more as the disease progresses the distance between two successive peaks increases. This approach can be used for other eye diseases such as age-related macular degeneration (AMD) too.

Improving longevity of functionally integrated stem cells in regenerative vision therapy: Researchers from the Buck Institute report one of the first demonstrations of long-term vision restoration in blind mice by transplanting photoreceptors derived from human stem cells and blocking the immune response that causes transplanted cells to be rejected by the recipient. Publishing in the *Cell Stem Cell*, this work highlights immune system rejection as one of the key issues that needs to be addressed to improve efficiency of stem cell regeneration therapies. The findings support a path to improving clinical applications, specifically for restoring vision in humans by allowing photoreceptors derived from human stem cells to integrate and thrive in the eye. They showed that these mice can now perceive light as far out as 9-months following injection of these cells. The team used a specific mouse strain that is healthy but it is lacking in a specific immune cell receptor, which makes the mouse unable to reject transplanted foreign cells, called immunodeficient IL2 receptor gamma (IL2r1) null mice. These animals lack the IL2ry receptor that humans also have as part of a functional immune system. In these mice, the team showed that without the rejection process, there was a 10-fold increase of living human embryonic stem cell-derived donor retinal cells that matured and integrated into the retina.

Congenital defects associated with Zika are more severe than thought: Zika-linked abnormalities that occur in human foetuses are more extensive and severe than previously thought, according to scientists who found that microcephaly is not the most common congenital defect caused by the infection. The study suggests that damage during foetal development from the mosquito-borne virus can occur throughout pregnancy and that other birth defects are more common than microcephaly, when babies are born with very small heads. These defects may only be detected weeks or months after the baby is born as reported by a professor from University of California, Los Angeles (UCLA). This is the largest study till date of Zika-affected pregnancies. The findings were published in the New England Journal of Medicine.

Novel Ebola Vaccine gives absolute protection: In a scientific triumph that will change the way the world fights a terrifying killer, an experimental Ebola vaccine tested on humans in the waning days of the West African epidemic has been shown to provide 100 per cent protection against the lethal disease. The vaccine has not yet been approved by any regulatory authority, but it is considered so effective that an emergency stockpile of 3,00,000 doses has been created for use should an outbreak flare up again. The test results of the trial in Guinea were released Thursday in *The Lancet*. The vaccine was not ready in time to stop the outbreak, which probably began in a hollow, bat-filled tree in Guinea and swept Liberia and Guinea before being defeated. But the prospect of a vaccine stockpile has brought optimism among public health experts. "While these compelling results come too late for those who lost their lives during West Africa's Ebola epidemic, they show that when the next outbreak hits, we will not be defenseless," said Marie-Paule Kieny, World Health Organization's assistant director-general for health systems and innovation and the study's lead author. "The world can't afford the confusion and human disaster that came with the last epidemic. "It's certainly good news with regard to

any new outbreak — and one will occur somewhere," said Anthony S. Fauci, director of the National Institute for Allergy and Infectious Diseases, which makes many vaccines and did some early testing on this one. "But we still need to continue working on Ebola vaccines." The *Lancet* study was done in 11,841 residents of Guinea last year. Among the 5,837 people who got the vaccine, none came down with Ebola 10 or more days later. There were 23 Ebola cases among the thousands of others not immediately vaccinated.

Indian Pepper may serve as a Potential Cancer Drug: The Indian long pepper, widely popular for spicing up food, may soon be used as a potential cancer treatment drug, according to a new study. The Indian long pepper contains a chemical that could stop your body from producing an enzyme that is commonly found in tumours in large numbers, according to the study in *Journal of Biological Chemistry*. UT Southwestern Medical Enter scientists have uncovered the chemical process behind anti-cancer properties of a spicy Indian pepper plant called the long pepper, whose suspected medicinal properties date back thousands of years. The secret lies in a chemical called Piperlongumine (PL), which has shown activity against many cancers including prostate, breast, lung, colon, lymphoma, leukaemia, primary brain tumours and gastric cancer. Using X-ray crystallography, researchers were able to create molecular structures that show how the chemical is transformed after being ingested. PL converts to hPL, an active drug that silences a gene called GSTP1. The GSTP1 gene produces a detoxification enzyme that is often overly abundant in tumours, the study said.

Scientists hear voice of ancient humans in baboon calls: The barks, yacks and wa-hoos of the Guinea baboons reveal distinct human-like vowel sounds. Baboon grunts and mating calls may hold secrets about human speech, according to a new study suggesting that the origins of human language could reach back as much as

25 million years. The barks, yacks and wa-hoos of the Guinea baboons reveal distinct human-like vowel sounds, according to the study published on Wednesday in the journal Plos One by scientists from six universities in France and Alabama. The authors from the Grenoble Alpes University studied the acoustics of 1,335 baboon sounds and the animals' tongue anatomy. Researchers suggest that the human vocal system developed from abilities already present in ancestors such as the Guinea baboon. The scientists also found similar muscles in baboon tongues as human tongues which are key to our ability to make vowel sounds. "The evidence developed in this study does not support the hypothesis of the recent, sudden, and simultaneous appearance of language and speech in modern Homo sapiens," the study says. "It suggests that spoken languages evolved from ancient articulatory skills already present in our last common ancestor ... about 25 million years ago."

Study says Hindu Kush – Himalayan Water Supplies may be affected by ongoing Climate change: More than a glacial retreat in the Hindu Kush-Himalaya region (HKH), the shifts in rain and snow due to climate change are likely to have an impact on regional water supplies and groundwater recharge, a study said. The study was conducted by two Kathmandu-based experts from International Centre for Integrated Mountain Development (ICIMOD). It showed that at lower elevations, glacial retreat is unlikely to cause significant changes in water availability over the next couple of decades, but other factors, including groundwater depletion and increasing human water use, could have a greater impact. Higher elevation areas could experience altered water flow in some river basins if current rates of glacial retreat continue. "The shifts in the location, intensity, and variability of rain and snow due to climate change impacts will likely to have a greater impact on regional water supplies and groundwater recharge than glacial retreat," the study said. The Hindu Kush-Himalaya is one of the most dynamic, diverse, and complex

mountain systems in the world, with several rivers and glacial systems making the region a "Third Pole" of the earth, providing fresh water resources to more than 210 million people in the mountains and 1.3 billion people downstream. Scientific evidence shows that most glaciers in the Hindu Kush-Himalaya region are shrinking, but the consequences of this melt for the regional water systems, especially groundwater, is not clear.

OPPORTUNITIES (Post-doctoral Fellowships)

IISER, Mohali: IISER Mohali invites applications at the level of Postdoctoral Research Associates. Interested applicants who have either a PhD degree or have submitted their PhD thesis can apply for these positions. It is preferred that the candidate has some overlap with the research interests of one or more faculty members at the Institute. This is a rolling advertisement and the applications will be reviewed from time to time. The salary for Postdoctoral Research Associates will be as per the MHRD norms. Applications should be sent by email to deanfaculty@iisermohali.ac.in with the subject clearly stating "Application for Postdoctoral position at IISER Mohali".

IIT – Bhubaneswar: Candidates preferably below 35 years having a Ph.D. Degree and willing to carry out advanced research in the fields of Basic Sciences; Earth, Ocean & Climate Sciences; Electrical Sciences; Infrastructure; Mechanical Sciences; Humanities, Social Sciences & Management may apply for this position. For further information please contact to Assistant Registrar (Academic Affairs), Contact No – 0674-2576019, E-mail id – ar.acad@iitbbs.ac.in

IIT – Jodhpur: Candidates with consistently good academic record and interdisciplinary research potential, as full-time Institute Post-Doctoral Fellowships (PDFs) to carryout research in the following areas at the Institute: Computer Science & Engineering, Electrical Engineering, Mechanical Engineering, Biology and others may

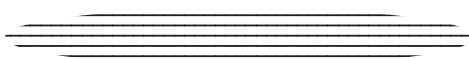
apply. For more details visit the website <http://iitj.ac.in/PDFs>.

IIT – Gandhinagar: Indian institute of Technology Gandhinagar is seeking applications for postdoctoral positions in Cognitive Science. Candidates of any nationality with a PhD degree ('all but defense' are eligible) in any discipline related to Cognitive Science can apply. We will also consider applications of exceptionally meritorious candidates who would like to switch their focus of research from another discipline into Cognitive Science. For more details visit the website <http://cogs.iitgn.ac.in/>

Indian Institute of Science: Applications are invited for a Research Associate/Post-Doctoral

Fellow to work on a Wellcome Trust DBT-India Alliance funded project at the laboratory of Dr. Sridharan Devarajan, at the Centre for Neuroscience at IISc, Bangalore. Please send a CV with two references by e-mail to sridhar@cns.iisc.ernet.in. Short listed candidates will be called for an interview.

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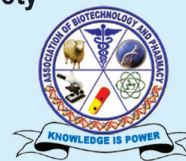
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