ISSN 0973-8916

# Current Trends in Biotechnology and Pharmacy

Volume 4 Issue 4 October 2010


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**Current Trends in Biotechnology and Pharmacy**
**ISSN 0973-8916**

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ISSN 0973-8916

# Current Trends in Biotechnology and Pharmacy

(An International Scientific Journal)

Volume 4 Issue 4 October 2010


www.abap.co.in

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Current Trends in Biotechnology and Pharmacy
Vol. 4 (4) October 2010 ISSN 0973-8916

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Current Trends in Biotechnology and Pharmacy
Vol. 4 (4) October 2010. ISSN 0973-8916

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**ISSN 0973-8916**

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Current Trends in Biotechnology and Pharmacy
Vol. 4 (4) 850-854 October 2010. ISSN 0973-8916


# Identification of Complex Vertebral Malformation using Polymerase Chain Reaction–Primer Introduced Restriction Analysis in Karan Fries Bulls

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## Abstract

Complex Vertebral Malformation (CVM) is a hereditary lethal disease characterized by complex anomalies of the vertebral column and limbs in an aborted fetus and in prematurely born, stillborn, and neonatal calves. The mode of inheritance of CVM is autosomal recessive and it is caused by a point mutation from G to T at nucleotide position 559 of the bovine solute carrier family 35 member 3 (SLC35A3) gene. The aim of this study is to assess the frequency of the mutation in breeding bulls of Karan Fries. In the present investigation 52 Karan Fries bulls were examined by using Polymerase chain reaction–primer introduced restriction analysis (PCR-PIRA) technique. Reported primers were used to introduce *Pst* I cut site into PCR products and screening of wild-type animals and heterozygote CVM Carrier animals. In the study 12 animals were found to be carriers for this genetic disorder. The genotypic frequency of heterozygous carrier animals and undesired allele frequency were estimated as 23.08 % and 0.115 % respectively. These carrier bulls may be excluded from the herd and their progeny should also be screened for CVM disease.

**Key words :** CVM, Karan Fries, Missense mutation, SLC35A3 gene

## Introduction

The Karan Fries synthetic strain of dairy cattle was developed at the National Dairy Research Institute, Karnal (Haryana), by the crossing of indigenous Tharparkar females with imported semen of Holstein Friesian bulls. The breeding policy for improvement of the crossbred populations across the globe involves selective breeding, selection based on genetic markers and also universally screening breeding bulls for heritable diseases. Many heritable mutations and diseases have been traced to autosomal chromosomes of different cattle breeds, most of these mutations are lethal or bring about deficiencies in the defense mechanism if they occur in the homozygous recessive manner. Moreover, due to coverage of wild type allele on mutant allele in heterozygotes, carrier animals may not show any phenotypic abnormality but are most likely to transmit the mutant alleles to the next generation. The same problem may assume catastrophic significance if the carrier is a bull intended to be used in artificial breeding programs. One such heritable disease is Complex Vertebral Malformation (CVM) which is a recessively inherited disorder leading to frequent abortion of fetuses or vertebral anomalies and prenatal death (1, 2). The syndrome was discovered in the Danish Holstein population in 1999, but shortly thereafter it was reported in many other countries.

Genealogical records reveal that calves suffering with CVM are genetically related to the US Holstein sire *Penstate Ivanhoe Star* (US1441440, born in 1963), and its offspring *Carlin-M Ivanhoe Bell* (US1667366) which was used in dairy cattle breeding programmes worldwide for two decades due to the superior lactation performance of their daughters.

CVM is caused by a point mutation (missense mutation) from G to T at nucleotide position 559 of the bovine solute carrier family 35 member 3 (SLC35A3) gene which causes a valine to phenylalanine substitution (3). Bovine SLC35A3 plays a great role in the development of the axial skeleton, demonstrating that some of the molecular mechanisms that operate during formation of vertebrae and ribs depend on carbohydrate modification in the Golgi apparatus (4). In stillborn, aborted, and preterm calves, CVM has also been characterized by shortened cervical and thoracic regions of the vertebral column, symmetric arthrogryphosis (1, 5). Thomsen et al. (4) reported that the number of animals genetically related to the carrier bulls is very high, and the disease-causing mutation is widespread among Holstein cattle throughout the world. There are many reports concerned with CVM in Holstein- Friesian cattle population in different countries including India from the present study. The syndrome was first discovered in the Danish Holstein population in 1999 (A.H. Petersen, unpublished), but shortly thereafter reports documented the presence of CVM in United States (5), United Kingdom (6), Netherlands (7), in Japan (8), in Czech Republic (9), in China (10), in Belgium (11) and in Australia (12). The defective allele for CVM had spread in Holstein populations worldwide though extensive exploitation of sires that were later turned found to be carriers of the defect. Keeping this in view it is imperative to investigate CVM disease in Karan fries bulls as they were developed by crossing of HF bulls with Indian Tharparker heifers.

## Materials and Methods

The present study was done to detect CVM disease in Karan Fries bulls using PCR-PIRA technique. Blood samples (10 ml) were collected from 52 Karan Fries Bulls maintained at the Artificial Breeding Complex, National Dairy Research Institute-Karnal, Haryana, India. Genomic DNA was isolated from venous blood as per the standard procedure of DNA Isolation Midi Kit (OMEGA BIO-TEK *USA*) followed by quality and quantity checking of DNA. Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) is a reliable technique for detection of single nucleotide mutations and genetic polymorphisms because digestibility by restriction endonucleases strictly depends on nucleotide sequences of PCR products. However, there are no restriction sites around the 559th nucleotide of the SLC35A3 gene. Polymerase chain reaction– primer introduced restriction analysis (PCR-PIRA) is a method for detection of single nucleotide mutations by introducing artificial restriction endonuclease sites using primers containing mismatches (13). This method has been used for detection of single nucleotide mutations of a variety of genes including SLC35A3 gene (3, 14, 15). For CVM screening reported primer set (3) consisting of 23 bases each Forward- 5'- CAC AAT TTG TAG GTC TCA CTG CA -3' and Reverse-5'- CGA TGA AAA AGG AAC CAA AAG GG -3' was used. Forward primer included a *PstI* site in the amplified product from the wild-type allele.

The following PCR Condition was used: pre denaturation for 3 min at 95 °C, followed by35 cycles of: 30 s at 94 °C, 30 s at 56 °C, 30 s at 72 °C, and finished with 5 min at 72 °C. The PCR products were subjected to electrophoresis in 1.5

% agarose gel with ethidium bromide (Promega) and visualized under transilluminator to check the amplification. All the amplified PCR products were digested with *PstI* (New England BioLab) restriction enzyme as per standard protocol suggested by the supplier. The digested fragments were electrophoresed agarose gel stained with ethidium bromide and observed under an UV transilluminator. In order to check DNA sequence discrimination between wild-type and CVM alleles the PCR products from both groups were cloned (INSTANT Cloning, Banglore GeNei) and sequenced using an automated DNA sequencer (3100-Avant Sequence Analyzer).

**Results and discussion**

The PCR-PIRA was performed with genomic DNA samples extracted from the blood of Holstein calves as described above. After the digested products are subjected to electrophoresis, one band of 233bp indicates homozygote normal individuals and two bands of 233 and 212bp (Fig.1) indicate heterozygote carrier individuals. A total of 12 animals out of 52 animals showed heterozygous condition for CVM and no homozygous recessive animal were found. The genotypic frequency of heterozygous carrier animals and frequency of undesired allele were estimated as 23.08 % and 0.115 %respectively. In order to confirm the occurrence of SLC35A3 mutation, PCR Products of Wild and Carrier animals were cloned and sequenced (GenBank Accession Numbers: EU822945 and EU822946). The result justified the enzyme digestion for carrier animals.

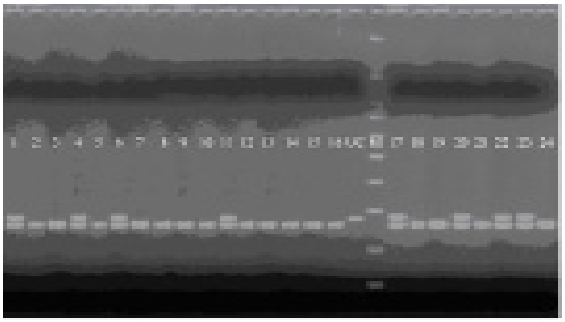

**Fig. 1** : Analysis of CVM allele by the PCR-PIRA method. The digested PCR products were analyzed by agarose gel electrophoresis. Lane no.1, 4, 6, 11, 17, 20, 22 & 23 showing carrier animals (two bands 233bp and 212bp) and others show normal animals. UC represents undigested PCR product (233bp) and M represents a molecular weight marker (100-bp ladder).

There are many reports concerned with CVM in Holstein- Friesian cattle population in different countries including India from the present study. The defective allele for CVM had spread in Holstein populations worldwide though extensive exploitation of sires that were later turned found to be carriers of the defect. Konersmann et al. (16) reported that 13.2 % of 957 sires used for insemination in Germany were diagnosed as carriers of CVM, while a prevalence of 31 % and 32.5 % was found in Denmark (4) and Japan (8) respectively. Also, the Holstein Association of USA reported in 2006 that of 11868 bulls examined 2108 were found to be carriers for CVM i.e., 17.76 %, which is less than the present study.

No productive and reproductive difference between carrier and normal animals were reported. The only difference which is very important is the increase in the rate of intra-uterine mortality. The risk of return to service was also significantly higher in carrier animals (17). In this context, different methods have been used for identification of single nucleotide polymorphism in SLC35A3 gene. Agreholm et al. (2) performed Genotyping of the CVM locus in a template-directed single-base extension assay and Kanae et al. (3) introduced “Polymerase chain reaction-primer introduced restriction analysis” (PCR-PIRA) for detecting a single nucleotide mutation in any gene without a restriction site. Rusc and Kaminski (18) used PCR-SSCP method

(polymerase chain reaction-single stranded conformation polymorphism) and Ghanem et al. (19) introduced DNA typing of CVM with allele specific PCR reaction (AS-PCR). Although an allele-specific polymerase chain reaction (AS-PCR) is a useful method for diagnosis of CVM, but the AS-PCR requires selected DNA polymerases and strictly controlled reaction conditions to obtain reliable results. Therefore, an alternative screening method of PCR-PIRA using a variety of DNA polymerases and PCR machines for the CVM gene was used.

## Conclusions

Inherited disorders are of great concern in cattle breeding as the breeding systems and the extensive use of genetically related sires predispose the animals to increased frequency of recessively inherited disease in the population and subsequently the occurrence of diseased animals. Furthermore, high numbers of defective animals may be reached due to international trade with semen, so the development of a genotyping test and its strategic use in selecting breeding sires can effectively reduce the number of affected calves and prevent continued uncontrolled spread of the defective allele. If cows and bulls that have a CVM gene are not used for breeding, the mutated gene can be removed from the cattle population in the same manner as a gene of bovine leukocyte adhesion deficiency has been successfully eliminated (20, 21). From the present study it is recommended that the use of semen from these 12 CVM carriers Karan fries bulls should be stopped and they should be removed immediately from the progeny testing programmes in India.

## Acknowledgment

The authors would like to thank the Director, National Dairy Research Institute (NDRI), and Indian Council of Agricultural Research, Government of India for providing funds to complete the present work. We are also thankful to Dr. M. S. Tantia, Principal Scientist of National Bureau of Animal Genetic Resources (NBAGR) for providing the required facilities.

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Current Trends in Biotechnology and Pharmacy
Vol. 4 (4) 855-861 October 2010. ISSN 0973-8916


# Assessment of Immunoprophylactic Efficacy of Recombinant Mid gut Antigen (Bm95) of *Rhiphicephalus microplus* in *Bos indicus*

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**Abstract**

Use of recombinant Bm95 isolated from an Argentinean strain of *Rhiphicephalus microplus* A as a vaccine candidate to control the tick infestation in India was investigated. Recombinant Bm95 expressed in yeast, *Pichia pastoris.* Purified protein blended into a vaccine using aluminium hydroxide as an adjuvant. An adverse effect on the reproduction of ticks was observed in vaccinated animals indicating reduced the environmental contamination and source of infestation indicating its use of Bm95 in the immunological control of ticks in India.

**Key words:** Tick, Bm95, *Rhiphicephalus*, tick-borne diseases.

## Introduction

Ticks are hematophagous ectoparasites belonging to the suborder Ixodida (1). In India, cattle ticks of the genera *Rhiphicephalus*, *Haemaphysalis*, *Hyalomma*, and *Argas* cause significant economic losses to the tune of US$ 498.7 million per annum (2). The use of chemical acaricides for the control of tick infestation has led to high incidence of acaricide resistance within tick populations and food safety concerns relating to the presence of toxic residues in milk and meat (3). Control of ticks by vaccination has the advantages of being cost effective, reducing environmental contamination and preventing the selection of drug-resistant ticks. In addition, development of vaccines against ticks using multiple antigens may also prevent or reduce transmission of pathogens (1). Several approaches have been used to actively immunize bovines against the cattle tick (4). The first approach was to evaluate the protective efficacy of a vaccine comprising of a large number of antigens derived from progressive fractionation of crude tick extracts against tick challenge (5). Such studies paved way for the isolation and characterization of Bm86, the first protective tick antigen (6). These studies led to the development of commercial vaccines incorporating Bm86 such as Tickgard Plus® and Gavac® which were successful in the control of tick populations, especially when appropriately combined with acaricidal treatments (7). With the advent of more modern technologies such as construction of cDNA libraries and expressed sequence tag (EST) databases from different tick tissues, analysis of developmental stages and from gene expression changes in response to various stimuli (i.e., tick feeding or infection with pathogens), new antigens were identified. Though a large number of potential vaccine candidates for control of tick infestations have been discovered, only a

few such as 64P (8), P29 (9) have been evaluated in animal trials which yielded mixed results. In this study, we have examined the efficacy of Bm95 gut antigen, a Bm86 homologue, isolated from an Argentine Bm86-resistant strain A of *R. microplus* (10) and demonstrate its utility as a potential anti-tick vaccine.

## Materials and Methods

***Vaccine formulations :*** Recombinant vaccine consisting of 200 mgs of recombinant Bm95 and 0.01% thiomersal were adjuvanted with 0.5% Aluminium hydroxide into doses of 1 ml each. The vaccine was stored at 4ºC till further use.

### Animal studies

***Determination of the efficacy of recombinant Bm95 in Bos indicus :*** A shed containing 48 calves from the "Mumbai Gow-Rakshak Mandali" with moderate tick infestation was selected for the experiment. All the gross ticks were removed from 24 randomly selected healthy calves which were treated with 200 mg/kg body weight of ivermectin in addition to (15 mg/kg body weight). The animals were deticked and the ticks were counted. .Immunized group containing 12 calves were vaccinated subcutaneously with 4 doses of vaccine at monthly intervals and control group containing 12 calves was kept as the unimmunized control. These calves were placed in a shed containing other 24 calves with moderate levels of tick infestation. Following live challenge, the efficacy of the tick vaccine was evaluated on the basis of comparison of degree of tick infestation and reproductive potential of female ticks collected on the 120$^{th}$ dpv.

***Indirect ELISA using Recombinant Bm95 :*** Humoral immune response against Bm95 recombinant antigen was measured by ELISA using serum samples collected from animals up to 150 dpv as described elsewhere (11). The absorbance values were recorded at 492 nm in ELISA reader (Microscan MS5605A, ECI Ltd., Bangalore).

## Results

***Assessment of humoral immune response :*** For the assessment of humoral immune response, serum samples (1:640 dilutions) from animals of immunized group and control group were subjected to indirect ELISA. The antibody response following immunization is depicted in Figure 1. Following vaccination, there was an increase in absorbance values till ~0.600 up to 35 dpv, following which the OD values were maintained till the 150 dpv. The humoral response in the unimmunized group showed values of <0.07 and remained unchanged during the course of the experiment.

***Tick counts :*** Following live challenge, the animals were examined for tick counts at monthly intervals. Though there was increase in the tick count over the period of study in calves of both the groups, the number of ticks found on the control animals was distinctly higher than on the animals of vaccinated group (Table 1). The percent rejection / reduction of tick count of the body of animals of the vaccinated group was estimated by comparing the average tick counts on the same day on the body of calves from the control group. It is evident from Table 1 that the percent rejection increased from 30.55 on the day 30 to 79.04 on day120 post immunization. Not only the tick counts but also the average weight of the female tick collected from the animals of vaccinated and control groups showed significant difference. There was steady decrease in average weight of the female tick from 30$^{th}$ day to 120$^{th}$ day. At the same time, the average weight of the female ticks feeding on the calves of control groups was similar throughout the period of study.

**Table 1:** Tick counts and average weight of female ticks recovered from vaccinated and unvaccinated calves.

| Immunized group | | | Control group | | |
|---|---|---|---|---|---|
| DPV | Average weight of females (mg) | Tick count | Average weight of females (mg) | Tick count | % reduction in tick counts |
| Initial screening | 398 | 10-32 (23.16) | 403 | 8-30 (21.16) | NA |
| 30th | 375 | 3-8 (4.25) | 386 | 5-9 (6.12) | 30.55 |
| 60th | 302 | 5-12 (7.69) | 375 | 9-21 (13.72) | 43.95 |
| 90th | 281 | 6-16 (9.12) | 390 | 16-39 (28.12) | 67.36 |
| 120th | 261 | 5-19 (11.8) | 411 | 14.79 (56.29) | 79.04 |

DPV: Days post vaccination
Figures in parenthesis indicate averages
NA: Not applicable
Initial screening: indicates counts at before treatment of animals with ivermectin and fenbendazole.

***Reproductive potential of females ticks :*** The engorged female ticks collected from the calves of vaccinated and control groups on day 120 of study were maintained in the laboratory to note their reproductive performance vis-a vis adverse effect of vaccination on the parameters. The preoviposition period of the ticks collected from vaccinated animals extended by three days as compared to that of ticks collected from control group. However, oviposition period of the ticks from vaccinated group was reduced to a day or two when compared with ticks collected from control group. The output of eggs expressed as weight of egg mass in milligram from the female ticks of vaccinated group was only 37.5% of the egg mass from the female ticks of control group. This indicates the positive effect of vaccination in the immunized animals wherein the egg mass was reduced by 62.5% when compared to the control group thereby leading to a decrease in the number of ticks. The hatchability of eggs laid by female ticks from vaccinated group was nil indicating 100% reduction. In contrast, female ticks from control animals produced total of 1680 larvae (61.8 larvae/female tick) (Table 2).

## Discussion

Cross-bred and exotic breeds of cattle are highly susceptible for tick infestation. Immunological intervention appears to be the best option for reducing tick burden. Hence, immunization of animals with tick antigen is drawing attention of many research groups to protect high productivity cross-bred dairy cattle.

**Table 2:** Reproductive performance of female *R. microplus* collected from vaccinated and unvaccinated calves.

| Reproductive parameters of ticks | Immunized group 120th dpv | Control group 120th dpv |
|---|---|---|
| No. of engorged females | 21 | 15 |
| Weight of engorged females | 5.49 | 5.94 |
| Preoviposition period (days) | 6-10 | 5-7 |
| Oviposition period (days) | 1-2 | 3-7 |
| Egg mass (mg) | 630 | 1680 |
| Hatchability Rate<br>No. of larvae/female | Nil | 927 |

The suggestion that the blood feeding ectoparasites may be damaged by an immunological reaction of the hosts against their internal organs is not new (12, 13). The first concealed gut antigen which was identified was Bm86 from *Rhiphicephalus microplus*. Commercial vaccines (TickGard and Gavac) have been developed by using recombinant Bm86 antigen in Australia (5) and Cuba (14). Other concealed gut antigens from *R. microplus* have been identified *viz.* Bm91 (15), BmA7 (16) and Bm95 (17), which have been used alone or in combination with Bm86 viz. Bm91 has been combined with Bm86 and has been commercialized as TickGard$^{Plus}$. Also, Bm95 has shown to induce protection even against Bm86 resistant *R. microplus* strains (17). Later, it was also noticed that combination of Bm86 and Bm95 antigens may provide better protection (5).

The immunoprotective properties of anti-tick antibodies is a well established fact in different tick-host systems (18, 19, 20, 21, 22, 23) In the present study, anti-Bm95 antibody titers peaked on the 35th day and were maintained till the 150th dpv. This rise in antibody titers was statistically significant ($P<0.01$) as early as day 10 dpv. A direct correlation between the anti-Bm95 antibody titers of vaccinated calves and its effect on feeding and reproductive parameters was noted. Similar results were reported by Ghosh and Khan (24), Rodriguez et al. (14), de la Fuente et al. (4), Andreotti (22), Opdebeeck et al. (23) by using different tick-derived antigens. However, Jackson and Obdebeeck, (19) reported a negative correlation between anti-gut membrane antibodies and its effect on reproductive index.

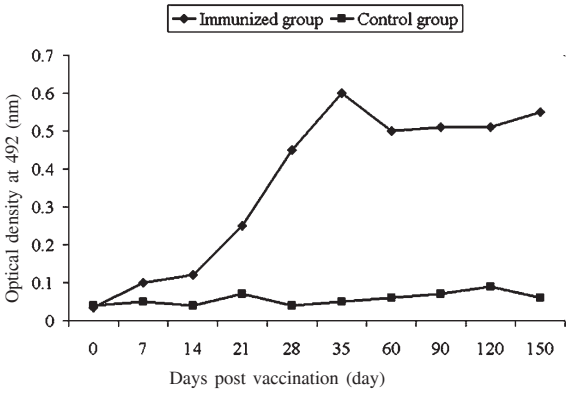
Cross-protection studies to evaluate the protection potential of Bm95 based vaccine against other tick species prevalent in India viz. *Hyalomma anatolicum anatolicum, Rhipicephalus haemaphysaloides* etc. needs to be carried out in India. Another most challenging scientific aspect that needs proper attention from the scientific community is the transmission limiting potential of tick pathogens. In a study, de la Fuente et al. (29) reported that the immunization of animals with Gavac significantly lowered the number of clinical cases of babesiosis. To develop an integrated pest management programme (IPM) for tick and tick-borne diseases suitable to Indian conditions, transmission limiting potentiality of the Bm95 based vaccines needs to be thoroughly studied.

## Conclusions

The findings on immune response generated by Bm95 antigen in calves during the

present study are of great significance as they support the fact that when ticks feed on immunized animals, a cutaneous inflammatory response is generated at the site of bite followed by an increase in the titer of anti-tick antibodies in the serum. The cutaneous inflammation prevents further feeding and the antibodies that are consumed by ticks along with blood meal damage mid gut producing a deleterious effect, on their feeding and reproductive performances (25, 26, and 27) and eventually causing the death of ticks. Thus, immunization against ticks can have dual action in preventing tick attachment and also tick borne diseases (28).

**Fig. 1 :** Humoral response of calves following vaccination using recombinant Bm95 both in the controlled pen and field trial

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Current Trends in Biotechnology and Pharmacy
Vol. 4 (4) 862-870 October 2010. ISSN 0973-8916


# Antioxidative Potential of *Perna viridis* and its Protective role against ROS Induced Lipidperoxidation and Protein Carbonyl

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**Abstract**

The antioxidant potential of methanol extracts of green-lipped mussel (*Perna viridis*) were evaluated using tests such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, reducing potential, scavenging capacity of reactive oxygen species (ROS) such as hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($OH^-$), and inhibition of lipid peroxidation (LPX) and protein carbonyl (PC). The scavenging activity of DPPH radical, $H_2O_2$ and $OH^-$ radicals increased in dose dependent manner. The reducing power also increased significantly with increasing concentration but the effect was less sharp as compared to ascorbic acid as standard. Inhibition of LPX and PC levels shown by extract indicates its potential to protect the cell damage from ROS. Present investigation forms a first comprehensive report on the nutraceutical property of *P.viridis*.

**Key words:** *Perna viridis*; antioxidant potential; lipid peroxidation; protein carbonyl

## Introduction

Reactive oxygen species (ROS), which constitute free radicals such as superoxide anion ($O_2^{\cdot-}$), $OH^-$ and non free-radicals such as $H_2O_2$ and singlet oxygen ($^1O_2$) represent various forms of oxygen centered molecules (1). In living beings, ROS are formed endogenously during normal cellular metabolism. Exogenously ROS is obtained during exposure to ionization radiations and many xenobiotic substances (1). In cellular systems, ROS are balanced at physiological concentrations by varieties of antioxidants, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferase (GST), reduced glutathione (GSH) and ascorbic acids (ASA) (1). Under pathological conditions ROS are overproduced (2, 3). The imbalance between ROS and antioxidant defence mechanisms leads oxidative damages to biomolecules like lipids, proteins and nucleic acids leading to disruption of cellular functions (1,4). The cellular damage results in several human diseases, such as arthrosclerosis (5), cancer (6), gastric ulcer (7), aging and etc. (8). Their broad range of effects on biological and medicinal systems has aroused considerable interest among scientists to control these effects.

Much attention has recently been focused to search naturally occurring antioxidant for use in foods or medicinal purposes to replace synthetic antioxidants, which are being restricted due to their side effects of carcinogenicity (9, 10). Antioxidants acquired through diet are of great interest as possible protective agent against mutagen and oxidative damage (11). Hence, the studies on natural antioxidant have gained increasingly greater importance.

*Perna viridis,* an estuarine bivalve, is an excellent source of proteins, carbohydrates, lipids,

essential vitamins and serves as important sea food for human consumption (12). Earlier investigations are limited only to biological (13) and nutritional (12) status of this sea food. However, the present studies are intended to explore the medicinal properties of this bivalve *P. viridis* by understanding its antioxidant status and ROS scavenging potential.

## Materials and methods

***Collection and maintenance of mussels :*** Green lipped mussels (*Perna viridis*) were obtained from unpolluted areas of Goa coast and acclimatized for one week under laboratory condition (salinity 30 ‰, pH 8.0 and temperature range from 23-25°C). Mussels were fed with unicellular algal species (*Chlorella* sp.) twice daily at a constant initial density ($2x10^6$cells/l) during acclimatization. To avoid faecal contamination, the water was exchanged every 24 hours and oxygen provided by continuous air-bubbling system.

***Preparation of crude extract :*** After acclimatization period whole body tissue was carefully excised, thoroughly washed with distilled water and surface dried with tissue paper. A 10% (w/v) tissue homogenate was prepared in methanol, and homogenate was centrifuged at 8000 rpm for ten minutes. The supernatant was filtered through Whatman paper No 1, and the extract was concentrated through Rota evaporator. The condensed methanol extract containing 400 mg / ml of total dissolved solid (after blotted dry), was preserved at -20 °C until further use. All the experiments were conducted in triplicate.

***Free radical scavenging activity :*** Free radical scavenging potential was measured by 2, 2-diphenyl-1-picry-hydrazil (DPPH) by the method of Blois (14). The reaction mixture containing 2.5 ml of DPPH solution (0.1mM in methanol) and extract (0.1-0.4 ml) was adjusted to a total volume of 3 ml by adding methanol. The absorbance was measured at 0 min and after 30 min at 517 nm. Butlylated hydroxytoluene (BHT) was used as the control. Scavenging effect was calculated as below and expressed as percent

$[A_o - A_1 / A_o] \times 100$

$A_o$ was absorbance at 0 minutes

$A_1$ was absorbance at 30 minutes

***Measurement of reducing power :*** The reducing power of extract was determined by standard protocol, with a slight modification (15). The reaction mixture containing 2.5 ml phosphate buffer (0.2 M, pH 6.6), 2.5 ml potassium ferricyanide (1%) and different volumes of extracts (0.1 - 0.5 ml), was adjusted with distilled water to a total volume of 6.0 ml. Ascorbic acid was used as standard solution while blank was maintained with same reaction mixture without sample. The mixtures were incubated at 50°C in water bath for 30 minutes, allowed to cool at room temperature. Later 2.5 ml trichloroacetic acid (TCA, 10 %) was added to the reaction mixture and centrifuged at 2000 rpm for ten minutes. The upper layer of 2.5 ml of solution was separated in another test tube, 2.5 ml distilled water and 0.5 ml $FeCl_3$ (1.0 %) were added to it and allowed to react for ten minutes at room temperature. The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates increased reducing power.

***Hydrogen peroxide scavenging assay :*** The $H_2O_2$ scavenging power of extracts was estimated using the method of Ruch *et al*. (16) with some variations. A solution of $H_2O_2$ (10mM) was prepared in phosphate buffer (pH 7.4). Reaction mixture containing 2.5 ml of $H_2O_2$ solution and varying concentration of test samples (0.1-0.5 ml) was adjusted to 3 ml by adding phosphate buffer solution (0.1M, pH 7.4). The absorbance was measured at 0 min and after 60

min at 240nm. Ascorbic acid (ASA) was used as the control. The percentage of scavenging effect (%) was calculated as
$[A_o - A_1 / A_o] \times 100$
$A_o$ was absorbance at 0 minutes
$A_1$ was absorbance at 60 minutes

***Hydroxyl scavenging assay :*** Hydroxyl radical scavenging ability was measured according to procedure by Kaur and Saini (11), with few modifications. The degradation of deoxyribose by $OH^{\cdot}$ was measured colorimetricaly in presence and absence of test samples. The reaction mixture contained 100mM of $FeSO_4$, 2mM of $H_2O_2$, 3mM of deoxyribose and varying concentration of test sample. The test tubes were then incubated for 30 min at 37 °C. After incubation, TCA (0.5 ml, 5%) and TBA (0.5 ml, 1%) were added to reaction mixture and kept in boiling water bath for 45 minutes. The absorbance was measured at 532nm. The scavenging effect (%) was calculated as
$[1 - (A_o - A_1 / A_2)] \times 100$
Where Ao is the absorbance in the presence of extract, $A_1$ - absorbance without $FeSO_4$, and $H_2O_2$ and $A_2$ - absorbance of the control (without extract)

***Preparation of sheep liver fractions :*** Sheep liver was obtained from slaughter house (Panaji, Goa), washed with ice cold KCl (1.15%) and homogenized (10% w/v) with Teflon Potter-Elvejhem homogenizer. Homogenate was filtrated through cheese cloth and centrifuged at 10000 rpm for 10 min, at 4°C. Supernatant was used for LPX assay.

***LPX inhibition assay :*** Peroxidation of liver homogenate was induced by $FeSO_4$ solution. Liver homogenate was incubated with 100 mM of $FeSO_4$ for 30 minutes at 37 °C, the formation of thiobarbituric acid reactive substances (TBARS) in the incubation mixture was measured at 532nm (17). The percentage inhibitory effect was calculated as
$[1 - (A_o - A_1 / A_2)] \times 100$
Ao was the absorbance in the presence of extract, $A_1$ - absorbance without sheep liver homogenate and $A_2$ - absorbance of the control (without extract).

***Inhibition of protein carbonyl assay :*** Protein oxidations were carried as described earlier with minor modifications (18). Bovine serum albumin (BSA) was oxidized by a Fenton-type reaction. The reaction mixture (1.5 ml) containing Bovine serum albumin (BSA, 2mg), $FeCl_3$ (50 µM), $H_2O_2$ (1 mM) and ascorbic acid (100 mM) and varying of sample extracts (0.1-0.5 ml) was incubated for 30 minutes at 37°C. After incubation, 1ml of 10mM 2, 4-dinitrophenylhy-drazine (DNPH) in 2 M HCl was added to the reaction mixture. Samples were incubated for 30 min at room temperature.

Then, 1 ml of cold TCA (10%, w/v) was added to the mixture and centrifuged at 8000 rpm for 10 minutes. The protein pellet was washed three times with 2 ml of ethanol/ethyl acetate (1:1, v / v) and dissolved in 1 ml of guanidine hydrochloride (6 M). The carbonyl content was calculated from the absorbance measurement at 366 nm with the use of molar absorption coefficient of 22 000 mol $cm^{-1}$ and expressed as nmol / mg protein.

***Statistical analysis :*** All experiments were conducted with three replicate and results were expressed as mean ± standard deviation (SD). Differences among the means were analyzed by one-way analysis of variance (ANOVA) and post hoc tests (Newman-Keuls).Differences were considered statistically significant when $P < 0.05$.

## Results and discussion

***Free radical scavenging activity :*** DPPH is a stable free radical and this radical scavenging assay was introduced as a antioxidant property within the test samples (19). This free radical reacts with reducing agents, pairing electrons, and discolouring stochiometrically, depending upon the number of electrons utilized (14). The reducing capability of DPPH radical was monitored by decrease in its absorbance at 517 nm. BHT was used as standard, which is a known antioxidant. DPPH scavenging potential of the extract and standard depicted in Fig-1A reveals that increasing extract concentrations significantly enhanced the free radical scavenging capacity ($P < 0.05$). The action of an antioxidant is to scavenge free radicals by donating hydrogen to a free radical and reducing to an unreactive species (20). The decrease in the absorbance of DPPH solution caused by test samples is indicative of its reducing action. This suggests that scavenging potential of extract is directly proportional to extract concentration ($P < 0.05$).

***Reducing power :*** The reducing power of the test compound, which is a major indicator of antioxidant potential (21), is involved with various mechanisms, such as prevention of chain initiation, binding of metal ions, decomposition of peroxides and radical scavenging (22,23). Methanolic extract of *P. viridis* showed significant increase in reducing power with higher sample concentration (Fig-1B, $P < 0.05$). The reducing power of extracts is generally associated with presence of reductones, which react with certain precursors of peroxide and provide protection against peroxide damage (24).

***Hydrogen peroxide scavenging assay :*** Although $H_2O_2$ is not a free radical, it can react with metals such as $Fe^{2+}$ or $Cu^{2+}$ as well as superoxide anions in the Haber-Weiss reaction, producing highly reactive hydroxyl radicals (25).

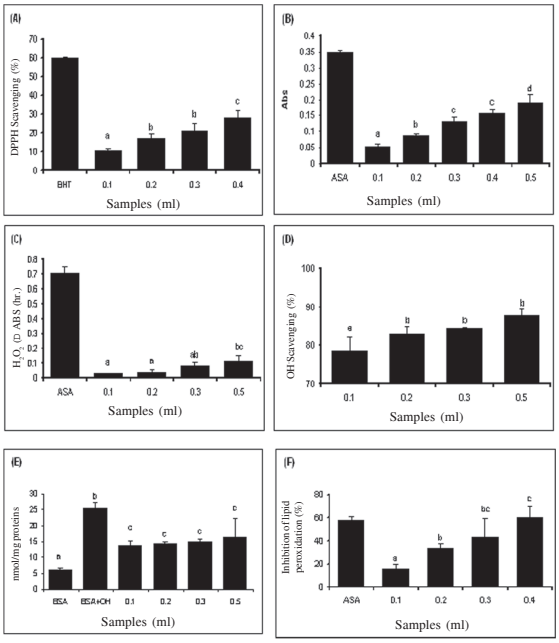


**Fig. 1.** (A) The DPPH radical-scavenging activity, (B) the reducing power, (C) the $H_2O_2$ scavenging activity, (D) the $OH^{\cdot}$ scavenging potential, (E) protein carbonyl (nmol / mg protein) and (F) inhibition of lipid peroxidation. Values are mean of triplicate determination ± SD (n = 3). Superscripts of different letters are significantly different from each other at $P < 0.05$.

Removal of $H_2O_2$ is therefore of utmost importance for cell or food systems to enable, prevention of several diseases. As shown in Fig-1C, methanolic extract demonstrated $H_2O_2$ scavenging activity in a dose dependent manner. However as compared with extracts, ascorbic acid showed more effective capacity for scavenging $H_2O_2$. It has been reported that electron donors may accelerate the conversion of $H_2O_2$ to $H_2O$ (26), which could possibly scavenge $H_2O_2$. A significant correlation observed between reducing power and $H_2O_2$ scavenging action (Fig-2A, $P < 0.01$) could support this statement.

***Hydroxyl radical scavenging assay :*** Hydroxyl radical is most reactive with a wide range of molecules found in living cells, such as sugars, amino acids, lipids and nucleotides. The resultant effect is lipid peroxidation (27), protein oxidation (28), DNA damage (29), and all other damages. Such consequences can either be prevented and /or reversed by increasing the cellular antioxidant capacity. It is vital therefore to explore for $OH^{\cdot}$ radical scavengers in food materials for avoidance of several dreadful diseases which are resultant of these oxyradicals.

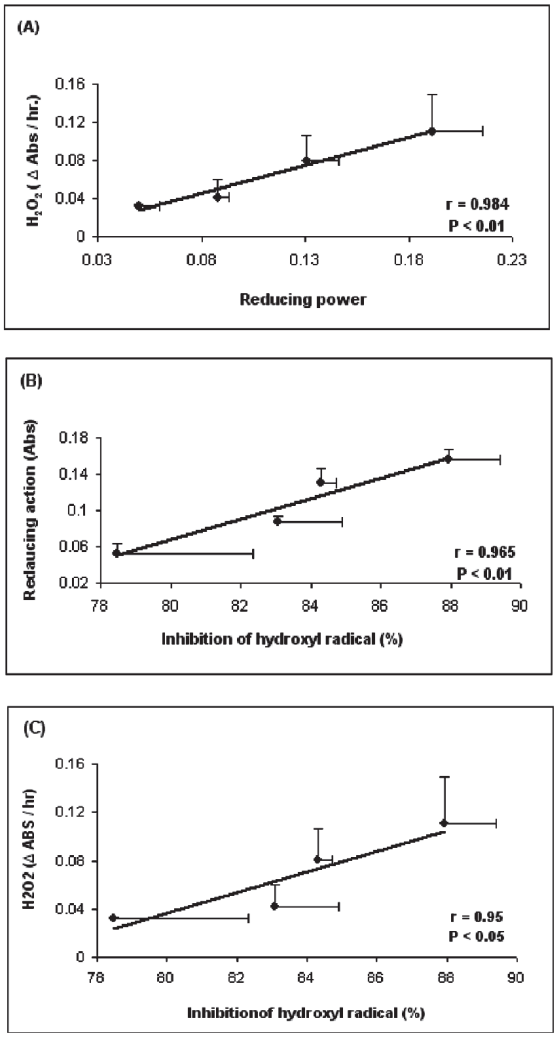


**Fig. 2.** Correlation between reducing action vs $H_2O_2$ scavenging potential (A), reducing action vs $OH^{\cdot}$ scavenging potential (B), $H_2O_2$ and $OH^{?}$ scavenging potential (C).

In biological systems formation of $OH^{\cdot}$ radicals occur in several pathways, the most important mechanism being the Fenton reaction (30). In the present study, the scavenging potential was measured by Fenton reaction. Our results reveal that methanolic extract of *P.viridis* has tremendous scavenging potential for $OH^{\cdot}$ radicals (Fig-1D), which could be due to reducing action and / or scavenging capacity for $H_2O_2$ or metals. Similarly the significant correlation between $OH^{\cdot}$ radicals v/s reducing action ($r = 0.965$, $P < 0.01$, Fig-2B) and $OH^{\cdot}$ radicals v/s $H_2O_2$ ($r = 0.95$, $P < 0.05$, Fig-2C) further strengthens our views on scavenging potential of $OH^{\cdot}$ radicals by *P.viridis* extract.

***Inhibition of protein carbonyl assay :*** Oxidation of proteins and carbonyl derivatives of proteins may result from oxidative modification of amino acids (31), which are responsible in aging and several pathological events (32). Carbonyl compounds possess toxic and carcinogenic properties and therefore their presence is of great concern to ill effects on health and food (33). Quantitative estimate of protein carbonyl derivatives is used as a sensitive assay for oxidative damage of proteins (34). Methanolic extract of *P.viridis* showed significant inhibitory effects on protein oxidation (Fig-1E, $P < 0.05$), indicating its effective use in pharmacology and food industry.

***LPX inhibition assay*** *:* Macromolecules such as carbohydrates, lipids, proteins and DNA, can undergo oxidative damage in presence of ROS. Among these, lipids are more susceptible to oxidative damage in physiological process (35). Oxidation of lipids generates various degradation products, such as malondialdehyde (MDA) which

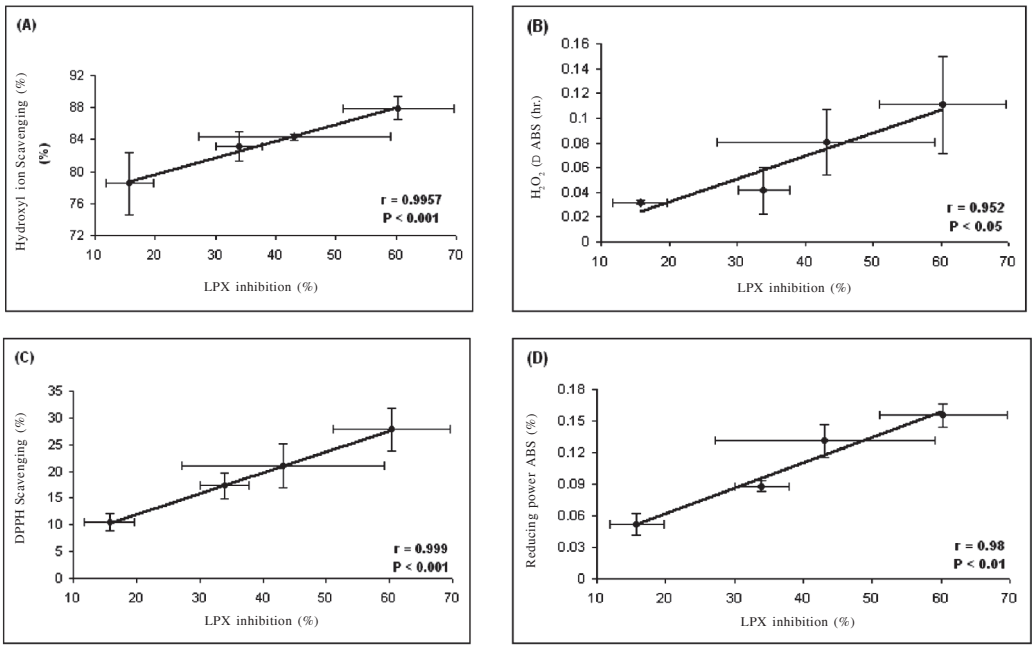


**Fig. 3.** Correlation between lipid peroxidation vs and $OH^{\cdot}$ scavenging potential(A), lipid peroxidation vs $H_2O_2$ (B), lipid peroxidation vs DPPH scavenging potential (C) and lipid peroxidation vs reducing action (D).

forms a major cause of cell damage (36). Inhibition of LPX in cells and food materials is therefore of significant importance in preventing several diseases. *In vitro* induction of lipid peroxidation by Ferrous ions is a tool for measuring antioxidant potential of extracts. Ferrous ion stimulates lipid peroxidation and supports decomposition of lipid peroxides, generating highly reactive intermediates such as hydroxyl radicals, perferryl and ferryl species (37). Our investigations indicated that, methanolic extracts of *P. viridis* significantly inhibited lipid peroxidation (Fig-1F, $P < 0.05$). Decrease in LPX by test sample is probably the evidence of the scavenging action of $OH^{?}$ radicals, $H_2O_2$, other free radicals and / or the reducing potential of the extract. Similarly, a significant correlation between $OH^{\cdot}$ radical scavenging capacity vs LPX ($r = 0.9957$, $P < 0.01$, Fig-3A), $H_2O_2$ scavenging action vs LPX ($r = 0.952$, $P < 0.05$, Fig-3B), DPPH scavenging potential v/s LPX ($r = 0.999$, $P < 0.001$, Fig-3C) and reducing action vs LPX ($r = 0.98$, $P < 0.01$, Fig-3D) are all in support of this hypothesis.

In conclusion, the present study determines that *P.viridis* is a rich source of natural antioxidants and has highly efficient protecting action against lipid peroxidation and protein oxidation. Hence *P.viridis* can be considered as the most suitable candidate for conducting further studies on purification, characterization and identification of antioxidant biomolecules for application in food industry and pharmaceutical investigations.

**Acknowledgment**

The authors are grateful to, Director, National Institute of Oceanography, Goa, India for his encouragement. KBJ thanks the Council

of Scientific & Industrial Research, (CSIR) Govt. of India for the award of Senior Research Fellowship (Ext.). These investigations were carried out under the grant of CSIR Net work project (NWP 0018).

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Current Trends in Biotechnology and Pharmacy
Vol. 4 (4) 871-880 October 2010. ISSN 0973-8916


# Xylanase Production using Alkalo-thermophilic *Bacillus halodurans* KR-1 by Solid-state Fermentation

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**Abstract**

Solid state fermentation has been carried out using *Bacillus halodurans* KR-1 for the production of xylanase enzyme. Among different solid materials such as wheat straw, rice bran, wheat bran, soybean meal, wheat bran served as a very good inducer of xylanase. Maximum enzyme production of 33.3 U/gm was observed with wheat bran moisturized with water (1:1 ratio, w/v) and 10% size of inoculums (v/w) incubated at 40ºC for 72h.

**Keyword:** Xylanase, *Bacillus halodurans*, Solid State Fermentation, Wheat bran

## Introduction

Xylanase (Endo-1,4 – b –D xylan, xylano hydrolase; EC 3. 2.1.8) acts on a -1,4 xylan and cleaves b-1,4 glycosidic linkage randomly (1). The products are xylose, xylobiose and xylo-oligosaccharides. The enzyme belongs to the glycoside hydrolase family. Xylan degrading enzymes occur ubiquitously in wide diversity of sources viz. plants, animals and microorganisms, however not in mammals. Xylanase producing microbes include aerobic and anaerobic mesophiles and thermophiles. Multiple xylanases are reported in numerous microorganisms (2). The enzyme is of industrial importance and is used in paper manufacturing to degrade xylan, to bleach paper pulp increasing its brightness, improving the digestibility of animal feed and for clarification of fruit juices (3). Use of xylanase avoids the use of chemical processes that are very expensive and cause pollution (4, 5).

The relatively high cost of enzyme production has hindered the industrial applications of enzymatic process (6). The use of abundantly available cost-effective agricultural by-products viz. wheat bran and other lignocelluloses in solid state fermentation for xylanase production will definitely be economical. The technique of Solid–State Fermentation (SSF) involves the growth and metabolism of microbe on the moist solid in the absence of any free flowing water. The fermentation system that is closer to the natural habitat of microbes has been shown to be more efficient in producing certain enzymes and metabolites (7, 8). SSF offers distinct advantages over submerged fermentation including economy of space, simplicity of the medium, no complex machinery equipments and control systems, greater compactness of the fermentation vessel owing to a lower water volume, more product yield, reduced energy demand, lower capital and recurring expenditure in industry. The easier scale up processes, volume of solvent needed for product recovery, superior yields, absence of built up foam, and easier control of contamination due to the low moisture level in the system are other advantages ( 7,9,10,11,12,13).

Most of the reports for xylanase production using solid state fermentation are with fungi and

Actinomycetes. Comparatively, there are only few reports for xylanase production using bacteria in the solid state fermentation (1, 14, 15). Here we report production of xylanase by alkalo-thermophilic bacteria; *Bacillus halodurans* strain KR-1 using wheat bran under solid state fermentation conditions.

## Materials and Methods

***Organism and growth conditions:*** The bacterium used in present study was isolated from soil near riverbed of Indore. The bacteria were identified as *Bacillus sp*. by morphological studies. It was confirmed as *Bacillus halodurans* by the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh and has been registered as *Bacilllus halodurans strain* KR-1, MTCC No. 9534. A medium (yeast extract, 0.5%; peptone, 0.5%; $K_2HPO_4$, 0.1%; $MgSO_4.7H_2O$, 0.02%; $FeSO_47H_2O$, 0.02% adjusted to 9.0 with 1% $Na_2CO_3$) supplemented with 1% xylan and 2.5% agar has been used for maintenance of the culture.

***Xylanase production by SSF:*** To 5 gm of wheat bran in a 250 ml capacity Erlenmeyer flask, 5 ml of tap water was added and sterilized by autoclaving at 15 psi for 20 min. After cooling to room temperature (25ºC), the flask was inoculated with 10% inoculum (freshly grown 24 h growth culture, w/v) and incubated in an Incubator at 40ºC for 72 hours.

***Xylanase extraction:*** The enzyme was squeezed from the semi-solid broth using two fold muslin cloths and collected in 50 mM glycine-NaOH buffer, pH 9.0 (50 ml of buffer was used for 5 g wheat bran). The squeezed enzyme collected in glycine-NaOH buffer was centrifuged at 10 x 000 *g* for 20 min in the cold condition (4 ºC) in a super speed cooling centrifuge model Sorvall RC-5B and the clear supernatant was used as enzyme extract.

***Enzyme Assay:*** Xylanase enzyme was assayed by estimating the release of the reducing sugar from birch wood xylan using dinitrosalicylic acid (DNS) method (16). A 0.9ml sample of 1% birchwood xylan dissolved in 50 mM glycine-NaOH buffer, pH 9.0 was pre- incubated at 50ºC for 5 min. To this, 0.1 ml of the enzyme was added and incubated at 50 ºC for 15 min. The reaction was stopped by adding 1.5 ml of DNS solution and the tubes were incubated in a boiling water bath for 15 min. A control was also run simultaneously where enzyme was added after the addition of DNS. A blank was also prepared where no enzyme was added and against the blank, zero was set in the colorimeter. D-Xylose was used as standard during the colorimetric estimation. One unit of the xylanase activity was taken as the amount of the enzyme required to release one ìmole of the reducing power as xylose equivalent per min under the conditions of the enzyme assay.

***Effect of various agricultural by-products on xylanase production:*** During solid state fermentation, various carbon and nitrogen sources in place of wheat bran viz. rice bran, wheat straw, and soybean meal were tested for xylanase production.

***Time course of enzyme production:*** Solid state fermentation was carried out upto 120 hours. An aliquot of the broth was taken out after every 24 hours for checking the amount of xylanase produced by the bacteria.

***Effect of moisture level:*** It was checked by varying the ratio of the wheat bran and water from 1: 0.5 to 1: 4.

***Effect of pH:*** It was checked by maintaining the pH of the broth varying from pH 5.0 to 12 before addition of the inoculum. The pH 5 and 6 was maintained by using citrate buffer, pH 7 and 8 by

using phosphate and pH 9 to12 was maintained by using glycine- NaOH buffer.

***Effect of temperature:*** It was checked by maintaining the temperature of the broth varying from 25ºC to 60ºC. The pH of the broth was maintained pH 9.0 and incubation period was 72 hours.

***Effect of various moisturizing agents:*** Different moisturizing agents viz. MS1 (yeast extract, 0.5%; peptone, 0.5%; $K_2HPO_4$, 0.1%; $MgSO_4.7H_2O$, 0.02% ; pH adjusted to 9.0 with 1% $Na_2CO_3$), MS2 (3 % NaCl , 0.075 % KCl, 0.7 % $MgSO_4.7H_2O$, 0.05 % $NH_4Cl$, 10% $K_2HPO_4$, 10% $KH_2PO_4$ , pH adjusted to 9.0 by using 1% $Na_2CO_3$), MS 3 (0.2 % $MgSO_4.7H_2O$ , 1 % $KH_2PO_4$, 1 %, $K_2HPO_4$ , 1 % $NH_4NO_3$, 0.02 % $CaCl_2$ , 0.05 % $FeCl_3$ pH adjusted to 9.0 with 1% $Na_2CO_3$), MS 4 (1.5 % $K_2HPO_4$, and 0.5 % $MgSO_4.7H_20$, pH adjusted to 9.0 with 1% $Na_2CO_3$), double glass distilled water, and tap water were used for semi-solid broth taken for xylanase production.

***Effect of inoculum size:*** Different inoculum sizes in the semi-solid fermentation broth viz.2%, 5 %, 10 %, 20 %, 30 % and 40 % were tested for maximum xylanase production. The fermentation was carried out at 40ºC for 72 hours.

***Effect on different additives:*** Different additives in 0.5% concentration viz. glucose, lactose, mannose and fructose as a carbon source, and $KNO_3$, $NaNO_3$ and casein as a nitrogen source were tested for maximum production of xylanase. The fermentation was carried out at 40ºC for 72 hours.

***Scaling up of enzyme production:*** Scaling up of xylanase production was tested by growing the bacteria with different amount (up to 25 g) of wheat bran supplemented with 0.5 % fructose as additive in a 2 l Erlenmeyer flask.

**Results and Discussion**

All the experiments were carried out at least thrice.

***Xylanase production on different agricultural by-products:*** Different agricultural by-products viz. wheat bran, rice bran, wheat straw, and soybean meal were tested for xylanase production (Fig.1). The results showed maximum production of xylanase in the presence of wheat bran compared to other agricultural by-products. The maximum production of xylanase may be due to low lignin and silica contents in wheat bran compared to other agricultural by-products. Battan et al. (2006) also reported maximum xylanase

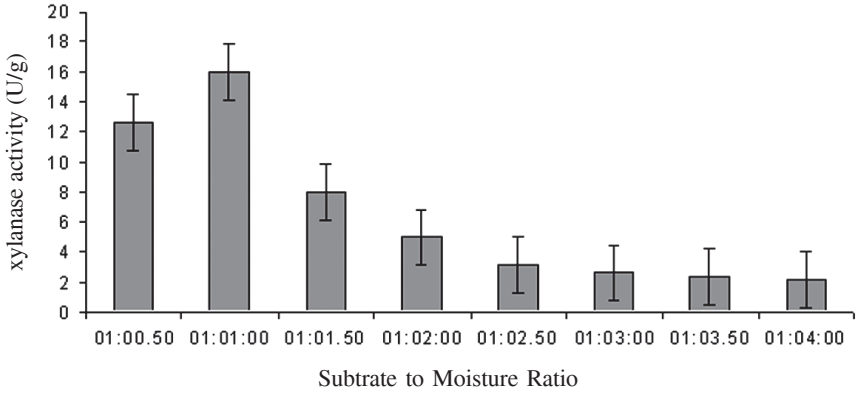


**Fig.1.** Production of xylanase from *Bacillus halodurans* on various agricultural by-products.

production (5300 U/gm) with wheat bran by *Bacillus pumillus*. Khandeparkar et al. (1) also reported xylanase activity by using different agricultural by-products in solid state fermentation. They reported xylanase activity with wheat bran 35.70 U/gm; rice husk, 25.04 U/gm; rice bran, 18.64 U/gm; and Bagassae 17.29 U/gm by using *Arthrobacter sp* MTCC 5214. Muthezhilan et al. (17) studied xylanase production from *Penicillium oxalicum* by using wheat bran rice bran, rice straw, sesame oil cake and wood husk and found maximum xylanase production with wheat bran followed by oil cake, rice bran, wood husk and rice straw, respectively. Gawande et al. (18) reported maximum xylanase production from *Aspergillus terreus* by using wheat bran. They reported xylanase production from *Aspergillus terreus*; 21.2 U/gm with wheat bran, 10.5 U/gm with rice straw, 10.2 U/gm with soybean hull, 3.5 U/gm with sugarcane bagasse. They also reported maximum xylanase production from *Aspergillus niger* with wheat bran, 26.7 U/gm. Antoine et al. (19) studied xylanase production by *Penicillium canescens* through solid-state fermentation with five different agro-industrial substrates viz. soya oil cake, soya meal, wheat bran, whole wheat bran and pulp beet, and found soya oil cake as best substrate in terms of enzyme production. Kavya and Padmavati (20) studied xylanase production by *Aspergillus niger* using different cheaper sources viz. wheat bran, rice bran, soya bran, ragi bran and dust, and found maximum xylanase production, 9.87 U/ml with wheat bran. Pal and Khanum (21) reported maximum xylanase production, 2596 U/gm by *Aspergillus niger* DFR-5 using wheat bran and soyabean cake in the ratio of 70:30. Sanghvi et al. (22) reported production of xylanase, 146 U/ml by *Tricoderma harzianum* through solid state fermentation using wheat straw. Murthy and Naidu (23) reported production of xylanase, 20388 U/gm by *Penicillium sp.* CFR 303 through solid state fermentation with coffee husk as substrate. Laxmi et al. (24) reported production of xylanase, 62480 U/ l by *Aspergillus sp*. RSP-6 using palm fibresl.

***Time course of enzyme production:*** Solid state fermentation broth was incubated up to 120 hours. Aliquots were taken out at every 24 hours intervals for testing xylanase production. The results are shown in Fig. 2. The results indicated maximum production of xylanase after 72 hours incubation. Thereafter, a slight decrease in xylanase production was observed**.** The incubation time required depends on the growth rate of the

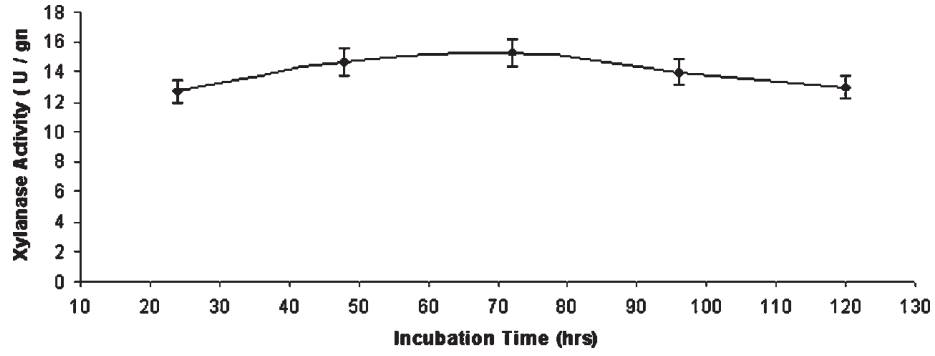


**Fig.2.** Xylanase production from *Bacillus halodurans* at different incubation times using wheat bran.

bacteria and its enzyme production pattern. Maximum xylanase production has been reported at 72 hours of incubation in solid state fermentation using *Bacilllus pumillus* (25) and *Arthrobactor,* (1). Muthezhilan et al. (17) reported xylanase production in *Penicillium oxalicum* at various time incubation and found maximum activity after 144 hours. Simoes et al. (26) reported maximum xylanase production from *Aspergillus japonicus* after 120 hours of incubation using wheat bran. Murthy and Naidu (23) studied production of xylanase by *Penicillium sp.* CFR 303 through solid state fermentation with coffee husk as substrate and found optimum fermentation time of 5 days. From these results, it is indicative that bacteria showed maximum xylanase production at 72 hours of incubation, whereas fungi showed at or after 120 hours of incubation.

***Effect of moisture level:*** Initial moisture contents are considered to be one of the key factors influencing xylanase production. In the present study, maximum xylanase production was observed at the wheat bran and tap water ratio 1:1 (Fig. 3). The results indicated drastic decrease in xylanase production on increasing the ratio of tap water. There was about 80% xylanase production when wheat bran and tap water ratio was 1:0.5. It has been assumed that increase in the moisture level reduces the porosity of wheat bran and therefore limits oxygen transfer (27). On the other hand, Feniksova et al. (28) assumed that on decrease in the moisture level than the optimum ratio reduces the solubility of nutrients. Battan et al (25) reported maximum xylanase production from *Bacillus pumillus* on keeping the ratio of wheat bran and moisture at 1:2.5. Khandeparker et al (1) reported maximum xylanase production from *Arthrobacter sp* MTCC 5214 on keeping the ratio of wheat bran and moisture at 3:1. Gessesse and Mamo (15) reported maximum xylanase production from *Bacillus sp*. AR-009 at wheat bran to moisture ratio of 1:1.

***Optimum pH for xylanase production:*** In the present study, xylanase production was tested at pH of the broth ranging from pH 5.0 to 12. The results showed maximum xylanase production at pH 9.0 (Fig. 4). However, there was not drastic decrease in xylanase production at other pH tested. In this experiment, we found lesser activity (nearly 12 U/ gm wheat bran) compared to while testing various agro-byproducts (nearly 16 U/ gm of wheat bran). It may be due to enzyme handling. Khandeparker et.al (1) also reported maximum xylanase production from *Arthrobacter sp* MTCC 5214 at pH 9.0. Muthezhilan et al. (17) reported maximum xylanase production from *Penicillium oxalicum* at pH 8.0. Yang et al. (29)

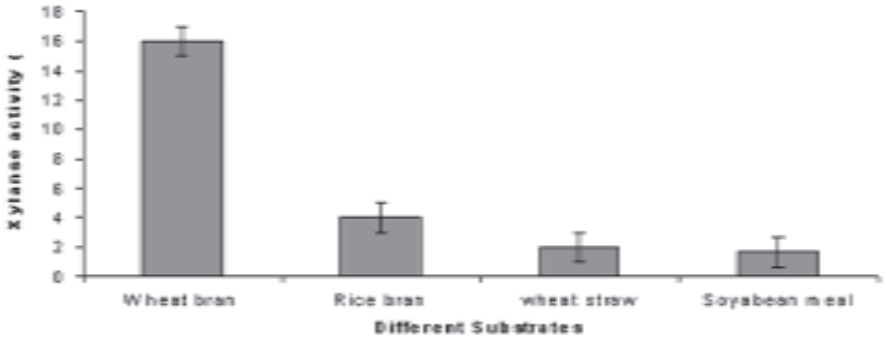


**Fig. 3.** Production of xylanase from *Bacillus halodurans* on different wheat bran to tap water ratio.

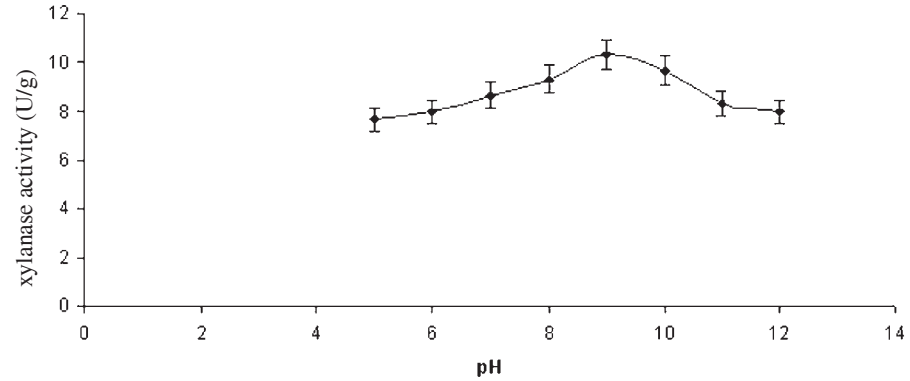


**Fig. 4.** Effect of pH on xylanase production from *Bacillus halodurans* in solid state fermentation using wheat bran.

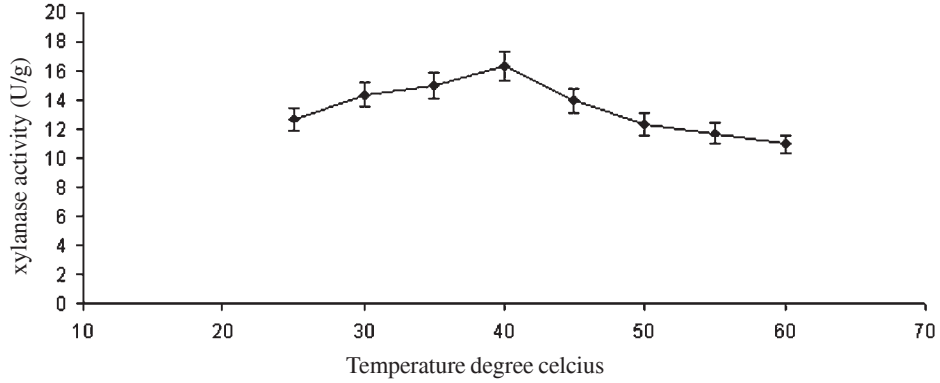


**Fig.5.** Effect of temperature on xylanase production from *Bacillus halodurans* in solid state fermentation using wheat bran.

reported maximum xylanase production from *Paeclomyces themophila* J18 at pH 7.0 using wheat straw. Sanghvi et al. (22) reported maximum xylanase production was observed at pH 5.0 by *Tricoderma harzianum* through solid state fermentation. Murthy and Naidu (23) reported production of xylanase *Penicillium sp.* CFR 303 at pH 5 using solid state fermentation and coffee husk as substrate.

***Optimum temperature for xylanase production:*** Optimum incubation temperature for maximum xylanase production under SSF was found to be 40ºC (Fig.5). There was about 80% xylanase production at 55ºC. Battan et al. (25) reported optimum temperature for xylanase production from *Bacillus pumillus* at 37ºC. Khandeparker et al. (1) reported maximum xylanase production from *Arthrobacter sp* MTCC 5214 at 28ºC. Muthezhilan et al. (17) reported xylanase production from *Penicillium oxalicum* at 45ºC. Simoes et al. (26) reported maximum xylanase production from *Aspergillus japonicus* at 25ºC using wheat bran. Yang et al. (29) reported maximum xylanase production from *Paeclomyces themophila* J18 at 50ºC using wheat straw. Antoine et al. (19) reported maximum xylanase production by *Penicillium*

*canescens* through solid-state fermentation at 30ºC. Kavya and Padmavati (20) reported maximum xylanase production by *Aspergiillus niger* at 28ºC. Pal and Khanum (21) reported maximum xylanase production by *Aspergillus niger* DFR-5 at 37ºC. Sanghvi et al. (22) reported maximum xylanase production by *Tricoderma harzianum* through solid state fermentation at 28ºC . Murthy and Naidu (23) reported production of xylanase by *Penicillium sp.* CFR 303 through solid state fermentation at 30ºC using on coffee husk as substrate.

***Effect of different moisturing agents on xylanase production:*** Among various moisturizing agents tested for xylanase production, tap water was found to be best in whose presence; xylanase production was 23.31U/gm wheat bran. There was 21.31 U of xylanase production per gm wheat bran in the presence of distilled water as moisteurizing agent. There was decrease in xylanase production with other moisteurizing agents, least being with MS4 (Table 1). Higher amount of xylanase production by tap water indicated that the present *Bacillus halodurans* does not require supplementation of mineral salts for xylanase production. Battan et al. (25) also reported maximum xylanase production with tap water (5984U/gm) compared to other mineral salt solutions. Yang et al.(29) reported maximum xylanase production from *Paeclomyces themophila* J18 in the presence of tap water with wheat straw. Therefore, our results coincided with other reported results. Here, we tested different combinations of mineral salts to check whether different combinations of mineral salts may be helpful in enhancing xylanase production. The results indicated that supplementation of any mineral salt is not required for enhancement in production of xylanase from *Bacillus halodurans* KR-1.

***Effect of inoculum size on xylanase production:*** In the present study, xylanase production increased on increasing the inoculum size from 5 % to 10 % and thereafter decreased up to 40 % (Fig.6). Battan et al. (25) reported maximum xylanase at 15 % of innoculum size.

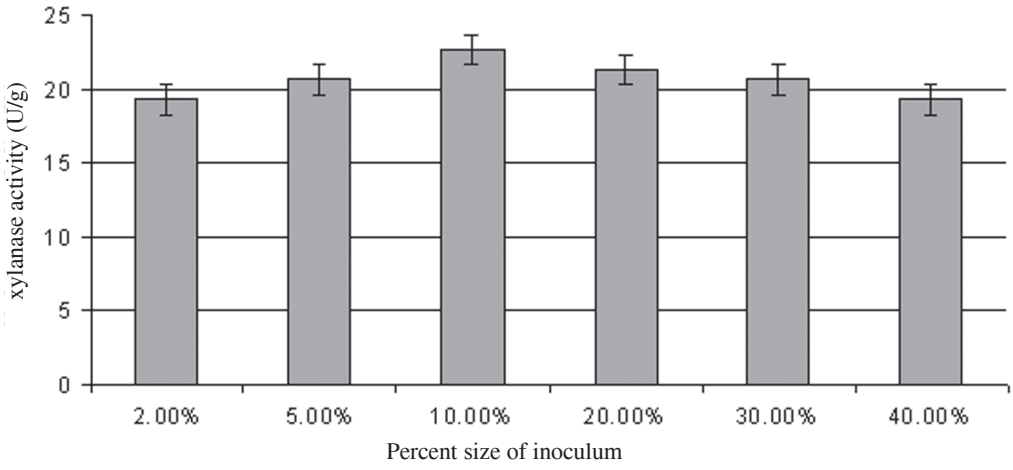


**Fig. 6.** Effect of inoculum size on xylanase production from *Bacillus halodurans* in solid state fermentation using wheat bran.

**Table 1.** Effect of various moisturizing agents on xylanase production in solid state fermentation using wheat bran

| Moistening agents | Xylanase production (U/gm) |
|---|---|
| Moistening agent 1 | 17.98 ± 0.017 |
| Moistening agent 2 | 16.65 ± 0.024 |
| Moistening agent 3 | 12.65 ± 0.022 |
| Moistening agent 4 | 11.64 ± 0.011 |
| Tap water | 23.31 ± 0.038 |
| Distilled water | 21.31 ± 0.035 |

**Table 2.** Effect of different additives on production of xylanase

| 0.5% Additives | Xylanase production (U/gm) |
|---|---|
| None | 21.97 ± 0.010 |
| Glucose | 29.97 ± 0.053 |
| Fructose | 33.3 ± 0.018 |
| Lactose | 27.30 ± 0.046 |
| Maltose | 25.97 ± 0.022 |
| Casien | 23.97 ± 0.030 |
| KNO3 | 21.64 ± 0.030 |
| NaNO3 | 24.64 ± 0.017 |

Gessesse and Mamo (15) reported maximum xylanase production from *Bacillus sp*. AR-009 at 10% inoculum size. Murthy and Naidu (23) reported production of xylanase by *Penicillium sp*. CFR 303 through solid state fermentation at inoculum size 20% using coffee husk as substrate.

***Effect on different additives on xylanase production:*** Different additives viz lactose, glucose, maltose, and fructose were tested as carbon source; and potassium nitrate, sodium nitrate, and casein were tested as a nitrogen source. The results are presented in Table 2. The results showed nearly 80% in xylanase production in the presence of fructose followed by lactose and glucose. However, there was no significant change in xylanase production in the presence of various nitrogen sources tested. Battan et al. (25) reported 20% increase in xylanase production in the presence of 4% peptone. Yang et al. (29) reported maximum xylanase production from *Paeclomyces themophila* J18 in the presence of yeast extract and wheat straw. Murthy and Naidu (23) reported production of maximum xylanase by *Penicillium sp*. CFR 303 with peptone as nitrogen source in solid state fermentation and coffee husk as carbon source. Laxmi et al. (24) reported maximum production of xylanase by *Aspergillus sp*. RSP-6 using palm fibres as carbon source and beef extract as nitrogen source.

***Scaling up of enzyme production:*** On five times scale up, increase in xylanase production was not observed linearly. During scale up, fructose was also used as additive. There was about two times increase in enzyme production upon five times scale up. Enzyme production with 25 gm of wheat bran was found to be 30.20 U/gm. There may be limitation of effective aeration upon scale up.

## Conclusion

In the present study, conditions for xylanase production from *Bacillus halodurans* strain KR-1 under SSF have been optimized. The data showed economical production of xylanase. The process may be exploited for industrial applications

## Acknowledgement

The authors acknowledge the facilities of the Department of Biotechnology, Ministry of Science and Technology, Government of India, New Delhi (DBT) under M.Sc. Biotechnology program. Facilities of DBT's Bioinformatics sub centre used in the preparation of the manuscript are also acknowledged. KKM acknowledges

DBT-TWAS post graduate fellowship awarded to him.

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Current Trends in Biotechnology and Pharmacy
Vol. 4 (4) 881-899 October 2010. ISSN 0973-8916


# Protocol to Isolate Sponge-associated Fungi from Tropical waters and an Examination of their Cardioprotective Potential

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## Abstract

Fungi represent an essential component of biodiversity not only because of their high species richness at multiple scales, but also because of their ecological, evolutionary and socio-economic significance. Despite poorly understood natural history and uncertain estimates of diversity, marine fungi have been identified as a major source of new natural products with pharmacological applications. The aims of this study were (1) to characterize fungi associated with marine sponges in protected areas of the Pacific and Caribbean coasts of Panama, and (2) to examine their effects through radioligand binding assays on endothelin $ET_A$ ($ET_A$) and neuropeptide Y $Y_1$ ($Y_1$) receptors, which provide an indication of cardioprotective potential. A total of 369 marine sponges were collected in areas of high biodiversity along the Panamanian coasts, including 156 from the western Caribbean and 213 from the eastern Pacific. From these, 2,747 and 2,263 fungal isolates were recovered, respectively, with variable isolation frequencies when sponge fragments were cultivated on five media. After determining the seasonality, geographic stucture, and taxonomic diversity of these fungal assemblages, we identified five strains that inhibited by > 50% the binding of [$^3$H] BQ-123 and one the binding of [$^3$H] neuropeptide Y to the $ET_A$ and $Y_1$ receptors, respectively, at 100 µg/ml. Further studies are required to determine whether these interactions are agonistic or antagonistic. Drawing from our methods for isolating and screening these fungi we propose a general protocol for capturing, cataloguing, and assessing the pharmacological potential of previously undiscovered fungi associated with marine sponges.

**Key words**: Ascomycota, fungi, sponges, endothelin $ET_A$, NPY $Y_1$

## Introduction

As a function of their complex architecture for secondary metabolism, marine sponges produce thousands of previously unknown chemical structures of special interest for chemo-discovery and biotechnological applications (1). Sponges frequently harbor microbial symbionts, including diverse fungi, with the sponge-microbe interaction providing defense against predators, pathogens, and biofouling (1). Molecules produced by microbial symbionts protect hosts directly in

some cases, and also may serve as precursors of defense metabolites (2) that greatly expand the chemical repertoire of their hosts.

Fungi represent an essential component of biodiversity not only because of their high species richness at local, regional, and global scales (3), but also because of their ecological, evolutionary and socio-economic significance (4). Especially in the last four decades, the pharmacological potential of fungi – particularly microfungi – has gained increasing attention (5-9). Examples of important products from microfungi include lovastatin, an efficient antilipidemic produced by *Aspergillus terreus* and used to reduce blood cholesterol level (10), and the promising marine-derived fungal metabolite sorbicillactone A, which is active against leukemia cells (11).

Marine fungi are under-explored in terms of their diversity, ecological associations, and secondary metabolites, but are increasingly recognized as a trove of new natural products with pharmacological applications (8): they have been considered one of the world´s greatest unexploited resources for new bio- and chemodiversity (12). Several recent studies have evaluated the pharmacological potential of marine fungi, including those associated with marine sponges (6, 8, 9). However, relatively little is known regarding the geographic distributions, host affinity, species diversity, or best practices for isolating fungi associated with sponges (9, 13-17).

Recently, compounds isolated from two species of marine sponges and one marine bacterium were shown to inhibit the binding of endothelin-1 to the $ET_A$ receptor and the activity of the endothelin-converting enzyme (18-20). Endothelins are a family of important vasoconstrictive peptides that can cause hypertension through activation of the $ET_A$ receptor (21). Neuropeptide Y (NPY), one of the most abundant neuropetides known, exerts direct pressor efffects and potentiates effects of other constrictors through activation of the $Y_1$ receptor (22). Because morbidity due to cardiovascular diseases (including cardiopathies and hypertension) is fourfold higher than that caused by AIDS, tuberculosis and malaria combined (23), we are interested in discovering novel metabolites with antihypertensive properties. Here we focus on screening marine sponge-associated fungi for their binding properties to the $ET_A$ and $Y_1$ receptors.

The specific goals of the present study were (1) to characterize fungi associated with diverse marine sponges in protected and biotically rich areas of the Pacific and Caribbean coasts of Panama, and (2) to examine their effects through radioligand binding assays on $ET_A$ and $Y_1$ receptors.

## Materials and Methods

Living, apparently healthy marine sponges were collected by SCUBA at depths of up to 30 m from 11 primary sites along the Caribbean and Pacific coasts of the Republic of Panama (Figure 1, Table 1). Three individuals of each species were collected in an area of ca. 3 $m^2$ at each site. To prevent cross contamination among samples, each sponge was sealed *in situ* in an individual plastic bag after removing excessive water and were processed for fungal isolation (below) within 2 h. Overall, 369 sponges were collected.

Sponges were identified following Zea, 1987; Hooper and Van Soest, 2002; and Collin et al., 2005 (24-26). A total of 369 collections represented at least 71 species, 36 genera and 28 families belonging to the orders Chondrosida, Dendroceratida, Dictyoceratida, Hadromerida, Halichondrida, Haplosclerida, Homosclerophorida, Lithistida, Poecilosclerida, Spirophorida, and Verongida (Table 1).

***Isolation of sponge-associated fungi :*** Because many surface sterilization methods are destructive to sponge tissue and may compromise associated fungi (6) we followed Höler *et al.* (27) with modifications to prepare sponge samples for fungal isolations. Under sterile conditions in a laminar flow cabinet (Portable Clean Air Unit, Liberty Industries), whole fresh sponges were placed on a sterilized strainer and washed thoroughly with sterile artificial seawater (ASW, adjusted to 36 g $l^{-1}$ for Caribbean sites and 32 g $l^{-1}$ for Pacific sites) (P. Gondola and E. Peña, personal communication). Samples were pressed on sterile, absorbent paper and cut with a sterile scalpel into ca. 1 x 0.5 cm pieces, making a cross section from the osculum to the holdfast. Pieces were washed three times with sterile ASW and dried as above. From the cleaned mesohyl of each sample, 50 cubes of 2-3 $mm^3$ were cut. Ten cubes were placed onto each of five solid isolation media, prepared prior to sterilization in 1 l volumes of distilled water: P15: 1.25 g $l^{-1}$ peptone, 1.25 g $l^{-1}$ yeast extract, 3 g $l^{-1}$ D-glucose, 20 g $l^{-1}$ agar, 15 g $l^{-1}$ marine salt; P30: 1.25 g $l^{-1}$ peptone, 1.25 g $l^{-1}$ yeast extract, 3 g $l^{-1}$ D-glucose, 20 g $l^{-1}$ agar, 30 g $l^{-1}$ marine salt; EM: 10 g $l^{-1}$ malt extract, 20 g $l^{-1}$ agar; PD: 20 g $l^{-1}$ potato dextrose agar, 10.00 g $l^{-1}$ agar; and SNA, a standard nutrient medium previously described by Höller *et al.* (6). Salinity of EM, PD and SNA was adjusted by adding 36 or 32 g $l^{-1}$ marine salt as above. A small number of isolates also was recovered on two additional media in preliminary surveys (Appendix 1). For samples presenting bacterial growth, 0.010 g $l^{-1}$ streptomycin and 0.100 g $l^{-1}$ penicillin G were added to the corresponding isolation media. All plates were monitored daily for hyphal growth for 8 weeks, and emergent fungi transferred to axenic culture on the same medium as that used in isolation.

Negative controls to detect contamination from sea water were prepared using a sterilized syringe to obtain 3 ml of sea water from sponges at collection. This water was spread on a Petri dish containing EM isolation media and allowed to stand at room temperature for 12 weeks. No fungal growth was observed (data not shown).

***DNA Extraction, PCR, and sequencing :*** Total genomic DNA was extracted from 100 representative isolates following Arnold and Lutzoni (28). The nuclear internal transcribed spacers (ITS1, ITS2) and 5.8S gene, and a ca. 500 base pair portion of the nuclear ribosomal large subunit (LSU) were amplified as a single fragment (ITS-LSU) using primers ITS5 and LR3 (18). Reactions were performed in a Gene Amp PCR System 2700 with 25 µl of reaction per sample: 1 µl of DNA template (1:10 dilution of extracted genomic DNA), 9.5 µL of DNAse and RNAse-free ultrapure water, 1 µl of each primer (10 µmol), and 12.5 µl of RedTaq® Ready Mix™ PCR reaction mix. A non-template control was included in each run. The cycling parameters were 3 min at 94°C, 34 cycles of 30 sec at 94°C, 30 sec at 54°C, and 1 min at 72°C, and 72°C for 10 min. PCR products were resolved by electrophoresis in a 1.5% agarose gel (Promega, USA) stained with 0.5 $\mu g^{-1}$ml ethidium bromide. Positive products showing single bands were sequenced bidirectionally at the University of Arizona Genomic Core facility on an ABI 3700. Basecalls and assembly were performed by *phred* and *phrap* (29, 30) with orchestration by Mesquite (Maddison and Maddison 2009: http://www.mesquiteproject.org), and contigs edited by eye in Sequencher 4.6 (Gene Codes, USA). Consensus sequences were compared against the NCBI GenBank database using BLAST to estimate taxonomic placement, and assembled into operational taxonomic units approximating species based on 95% sequence similarity (31) using Sequencher 4.6.

***Culture extracts :*** Sixty-two representative isolates were cultivated in 100 ml of their

respective liquid culture media (prepared as above, but without agar) on an orbital shaker (Thermo, USA) at 175 rpm for 10, 18 or 30 days at 28°C. To assess the importance of agitation, liquid cultures for 26 of the selected isolates, chosen haphazardly, also were allowed to grow on shelves as stationary cultures in their respective culture media at room temperature (22°C) for 3 weeks. At harvest each culture was homogenized using a laboratory blender (Waring, USA.) for 1 min, and resultant mixture extracted with ethyl acetate (2 x 100 ml). Organic fractions were combined and the solvent was removed at reduced pressure at <40°C. Dried extracts were dissolved in DMSO/water with a final concentration of 10 µg $ml^{-1}$ (0.1% DMSO) and 100 µg $ml^{-1}$ (1% DMSO) for the assays described below.

***Cell culture and [$^3$H]-NPY and [$^3$H]-BQ-123 binding assays :*** Human neuroblastoma SK-N-MC cells expressing the $ET_A$ and the $Y_1$ receptors (HTB-10, American Tissue Culture Collection) were maintained in 75 $cm^2$ flasks in Eagle's Minimum Essential Medium (EMEM) supplemented with 2 mM L-glutamine and 2% of a stock solution containing 5000 I.U. $ml^{-1}$ penicillin and 5000 ㎎ $ml^{-1}$ streptomycin (American Tissue Culture Collection), and 10% fetal bovine serum (Invitrogen). Cells were grown in a humidified atmosphere containing 5% $CO_2$ at 37℃ until confluent and then used in assays described below.

Radioligand-binding assays were carried out following Caballero-George *et al.* (32). Cells were grown in 24-well plates until confluent and then were washed with 500 µl of PBS. To each well, 500 µl of binding buffer (EMEM containing 0.5% BSA for neuropeptide Y [NPY] binding and 0.1% BSA for BQ-123 binding) was added, and the mixture left for 15 min at room temperature. The plate then was preincubated with 50 µl of buffer (for total binding) or test substance (for competition binding) or 0.1 µM of either human NPY or human endothelin 1 (for nonspecific binding). Incubation started after addition of 50 µl of [$^3$H]-NPY or [$^3$H]-BQ-123 (final concentration 1 nM). Preincubation and incubation steps each were conducted at 38°C and lasted for 30 min. At the end of 60 min the plate was placed on ice, the binding buffer removed, and the cells washed with 500 µl of PBS buffer.

To measure total cell binding of the radioligand, 200 µl of 1 M NaOH was added to each well. After 30 min, 300 µl of distilled water was added, and the resulting solution was placed into scintillation vials with scintillation liquid (Ready Safe, Beckman Coulter) and radioactivity measured on a Beckman Coulter model LS 6500. Extracts that inhibited the binding of radioligands by > 50% at 100 µg $ml^{-1}$ or more of total binding were considered active.

***[$^3$H]-thymidine uptake :*** Cell viability was measured by the active uptake of thymidine following Fierens *et al.* (33). Briefly, confluent cells were incubated on 24 well plates overnight with sterile EMEM that was supplemented with L-glutamine and antibiotics but did not contain serum, which arrested cell growth. Cells then were washed twice with 0.5 ml EMEM buffer and left for 15 min with 400 µl of supplemented EMEM containing serum, which then induced cell growth. The effect of test substances on [$^3$H] thymidine uptake was measured by pre-incubating the cells for 30 min with 100 µg $ml^{-1}$ of each extract. Non-specific binding was assessed using 10% ethanol, which causes total cell death. EMEM medium was used for control. Cells then were incubated with 50 µl of 70 nM [$^3$H]-thymidine for 30 min, placed on ice, and washed twice with ice cold HEPES buffer. Radioactivity was measured as above.

## Results and Discussion

Three hundred sixty-nine marine sponges were collected in 11 primary sites representing areas of high biodiversity along the coasts of the Republic of Panama (Figure 1). A total of 156 sponges was collected in the Caribbean and 213 in the Pacific (Table 1). These yielded 2,747 and 2,263 fungal isolates respectively (Table 2). Overall, fungi were isolated from 27.7 % ± 26.0 (mean ± SD) of sponge fragments, and from all sponge species examined (Table 2).

***Effect of media on isolation frequency*** **:** Isolation frequency of fungai from the three most thoroughly sampled orders of sponges differed in sensitivity to different media significantly among five media, ranging from 22.6% (P30) to 33.1% (EM) ($F_{4, 1773} = 6.38$, P<0.0001; Table 2). Isolation frequencies on each medium were positively correlated with one other (P<0.0001 in each case), with $r^2$ values for pairwise comparisons ranging from $r^2 = 0.40$-$0.57$ (mean ± SE, $r^2 = 0.49 \pm 0.02$).

Isolation frequency of fungi from the three most thoroughly sampled orders of sponges differed in sensitivity to different media. Isolation frequency was consistent regardless of medium for sponges representing Verongida and Poecilosclerida (range, 15.0-21.4%; $c^2 = 4.60$, df = 1, P = 0.3308 and 30.6-41.8, $c^2 = 4.07$, df = 1, P = 0.3962, respectively), but differed significantly for Haplosclerida (22.0-38.4%; $c^2 = 17.30$, df = 1, P = 0.0017) with the highest isolation frequency on EM (38.4% ± 3.4).

***Effects of ocean, season, and taxonomy on isolation of fungi :*** Multiple regression was used to assess the effect of ocean (Caribbean vs. Pacific), sampling season (dry vs. wet), and taxonomy (sponge order) on the overall isolation frequency of fungi. Fungi were isolated more frequently from Caribbean sponges (35.8% ±1.9 of fragments yielded a fungus in culture, N = 155 collections; one outlier removed) than from Pacific sponges (21.3% ± 1.7; N = 213) (P<0.0001)

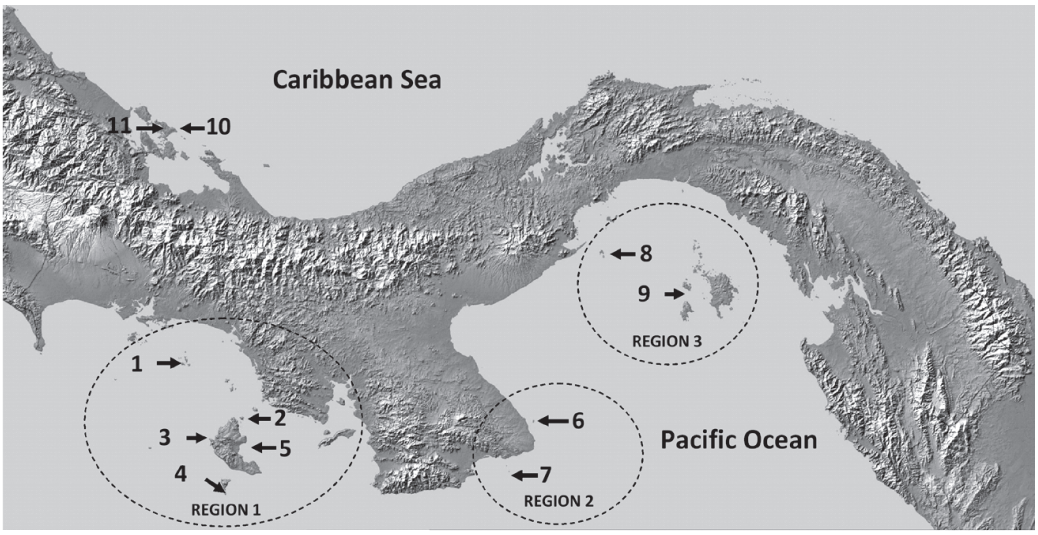


**Fig. 1.** Distribution of sites for collection of sponges in the Republic of Panama. 1) Secas Islands, Gulf of Chiriqui; 2) Ranchería Island, Coiba National Park; 3) Punta Hermosa, Coiba National Park; 4) Jicarón & Jicarita Islands, Coiba National Park; 5) Bahía Damas, Coiba National Park; 6) Isla Iguana, Los Santos; 7) Frailes & Monjas Islands, Los Santos; 8) Otoque & Boná, Gulf of Panama; 9) Las Perlas, Gulf of Panama; 10) Bahía Honda, Cayo Nancy, Bocas del Toro; 11) North of Bastimentos Islands, Bocas del Toro.

**Table 1.** Number of sponges sampled as a function of taxonomy, ocean (Caribbean, Pacific), locality (sites and regions; see Fig. 1), and season (R = rainy, D= dry)

| | | | | Caribbean | | | | | | | Pacific | | | | | | | Overall |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Site 10 | | Site 11 | | Other | | | Region1 | | Region2 | | Region 3 | | | |
| Order | Family | Genus | species | R | D | R | D | R | D | Total | R | D | R | D | R | D | Total | Total |
| Chondrosida | Chondrillidae | *Chondrilla* | *c.f.nucula* | 3 | 0 | 0 | 1 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| Chondrosida | Chondrillidae | *Chondrilla* | *sp* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 1 | 0 | 3 | 3 |
| Chondrosida | Chondrillidae | N.I. | N.I. | 1 | 0 | 0 | 2 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 4 |
| Dendroceratida | Darwinellidae | *Chelonaplysilla* | *erecta* | 2 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| Dictyoceratida | Irciniidae | *Ircinia* | *campana* | 1 | 2 | 0 | 0 | 0 | 1 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| Dictyoceratida | Irciniidae | *Ircinia* | *strobilina* | 1 | 6 | 1 | 1 | 0 | 0 | 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 9 |
| Dictyoceratida | Irciniidae | *Iricina* | *sp* | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Dictyoceratida | Spongiidae | *Hyatella* | *cf intestinalis* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 0 | 0 | 0 | 0 | 8 | 8 |
| Dictyoceratida | Spongiidae | *Spirastrella* | *sp* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 |
| Dictyoceratida | Spongiidae | *Spongia* | *pertusa* | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Dictyoceratida | Spongiidae | *Spongia* | *tubulifera* | 0 | 1 | 1 | 1 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 |
| Hadromerida | Chalinidae | *Haliciona* | *caerula* | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Hadromerida | Chalinidae | *Haliciona* | *sp* | 0 | 0 | 0 | 4 | 1 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 |
| Hadromerida | Chalinidae | N.I. | N.I. | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Hadromerida | Clionaidae | *Cliona* | *delitrix* | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Hadromerida | Clionaidae | *Cliona* | *sp* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| Hadromerida | Hadromeridae | N.I. | N.I. | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Hadromerida | N.I. | N.I. | N.I. | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 2 |
| Hadromerida | Placospongiidae | *Placospongia* | *intermedia* | 0 | 2 | 0 | 3 | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 |
| Hadromerida | Placospongiidae | *Spirastrella* | *sp* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| Hadromerida | Spirastrellidae | *Spirastrella* | *sp* | 2 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 3 |
| Hadromerida | Suberitidae | *Laxosuberites* | *sp* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 2 | 7 | 0 | 0 | 1 | 14 | 14 |
| Hadromerida | Suberitidae | N.I. | N.I. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| Hadromerida | Tethyidae | *Tethya* | *cf taboga* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 3 | 4 | 4 |
| Halichondrida | Axinellidae | *Axinella* | *sp1* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 1 | 0 | 5 | 5 |
| Halichondrida | Axinellidae | *Axinella* | *sp2* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 |
| Halichondrida | Axinellidae | *Dragmacidon* | *reticulata* | 0 | 0 | 0 | 3 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 |
| Halichondrida | Dictyonellidae | *Scopalina* | *sp* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 3 | 3 |
| Halichondrida | Halichondriidae | *Axinyssa* | *isabela* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 3 | 3 |
| Halichondrida | Halichondriidae | *Halichondria* | *sp* | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Halichondrida | Tethyidae | *Tethya* | *sp* | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Haplosclerida | Chalinidae | *Haliclona* | *caerulea* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 | 2 | 4 | 0 | 3 | 0 | 15 | 15 |
| Haplosclerida | Chalinidae | *Haliclona* | *sp1* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 1 | 0 | 3 | 3 |
| Haplosclerida | Chalinidae | *Haliclona* | *sp2* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 6 | 0 | 4 | 4 | 15 | 15 |
| Haplosclerida | Chalinidae | *Haliclona* | *sp3* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 2 | 3 | 3 |
| Haplosclerida | Chalinidae | *Haliclona* | *sp4* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 2 | 2 |
| Haplosclerida | Niphatidae | *Amphimedon* | *compressa* | 3 | 0 | 1 | 0 | 1 | 1 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 |
| Haplosclerida | Niphatidae | *Amphimedon* | *viridis* | 1 | 0 | 1 | 0 | 0 | 2 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| Haplosclerida | Niphatidae | N.I. | N.I. | 1 | 0 | 2 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 |

| | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Haplosclerida | Niphatidae | *Niphates* | *erecta* | 7 | 1 | 3 | 0 | 0 | 2 | 13 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 13 |
| Haplosclerida | Niphatidae | *Niphates* | *sp* | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Haplosclerida | Petrosiidae | N.I. | N.I. | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Haplosclerida | Petrosiidae | *Xestospongia* | *muta* | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Haplosclerida | Petrosiidae | *Xestospongia* | *rosariensis* | 0 | 5 | 0 | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 |
| Haplosclerida | Petrosiidae | *Xestospongia* | *sp* | 2 | 2 | 1 | 0 | 0 | 1 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 |
| Haplosclerida | Phloeodictyidae | *Aka* | *coralliphagum* | 0 | 0 | 1 | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| Haplosclerida | Phloeodictyidae | *Aka* | *sp* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 |
| Homosclerophorida | Plakinidae | N.I. | N.I. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| Homosclerophorida | Plakinidae | *Plakinastrella* | *onkodes* | 1 | 0 | 0 | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| Homosclerophorida | Plakinidae | *Plakortis* | *angulospiculatus* | 0 | 2 | 0 | 6 | 0 | 0 | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8 |
| Homosclerophorida | Plakinidae | *Plakortis* | *albicans* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 |
| Lithistida | Desmanthidae | *Desmanthus* | *sp* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 2 | 2 |
| N.I. | N.I. | N.I. | N.I. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 |
| Poecilosclerida | Coelosphaeridae | *Lissodendoryx* | *colombiensis* | 0 | 2 | 0 | 2 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| Poecilosclerida | Iotrochotidae | *Iotrochota* | *birotulata* | 0 | 1 | 0 | 2 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 |
| Poecilosclerida | Microcionidae | *Artemisina* | *melana* | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Poecilosclerida | Microcionidae | *Chlatria* | *microchela* | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Poecilosclerida | Microcionidae | *Chlatria* | *sp* | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Poecilosclerida | Microcionidae | *Chlatria* | *echinata* | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Poecilosclerida | Microcionidae | *Desmapsana* | *anchorata* | 2 | 1 | 1 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| Poecilosclerida | Mycalidae | *Mycale* | *citrina* | 3 | 0 | 0 | 2 | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 |
| Poecilosclerida | Mycalidae | *Mycale* | *sp* | 7 | 0 | 2 | 2 | 0 | 0 | 11 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 11 |
| Poecilosclerida | Raspaillidae | N.I. | *sp3* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 1 | 0 | 4 | 0 | 8 | 8 |
| Poecilosclerida | Raspaillidae | N.I. | *sp1* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 3 | 0 | 4 | 4 |
| Poecilosclerida | Raspaillidae | N.I. | *sp2* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 |
| Poecilosclerida | Raspaillidae | N.I. | *sp4* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 |
| Poecilosclerida | Tedaniidae | *Tedania* | *ignis* | 4 | 1 | 0 | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 |
| Spirophorida | Tetillidae | *Cynachirella* | *alloclada* | 0 | 2 | 0 | 2 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| Verongida | Aplysinidae | *Aplysina* | *sp6* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 9 | 3 | 12 | 12 |
| Verongida | Aplysinidae | *Aplysina* | *cauliformis* | 0 | 2 | 1 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 |
| Verongida | Aplysinidae | *Aplysina* | *fulva* | 2 | 2 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| Verongida | Aplysinidae | *Aplysina* | *sp* | 2 | 2 | 0 | 1 | 0 | 1 | 6 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 7 |
| Verongida | Aplysinidae | *Aplysina* | *gerardogreeni* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 18 | 12 | 5 | 0 | 7 | 5 | 47 | 47 |
| Verongida | Aplysinidae | *Aplysina* | *chiriquensis* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 11 | 13 | 0 | 0 | 0 | 0 | 24 | 24 |
| Verongida | Aplysinidae | *Aplysina* | *sp1* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 3 | 0 | 0 | 0 | 0 | 5 | 5 |
| Verongida | Aplysinidae | *Aplysina* | *sp2* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 1 | 3 | 3 |
| Verongida | Aplysinidae | *Aplysina* | *sp3* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| Verongida | Aplysinidae | *Aplysina* | *sp4* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| Verongida | Aplysinidae | *Aplysina* | *sp5* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 4 | 0 | 0 | 0 | 0 | 9 | 9 |
| Verongida | Aplysinidae | *Aplysina* | *sp7* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 4 | 4 |
| Verongida | Aplysinidae | *Verongula* | *reiswigi* | 0 | 1 | 0 | 1 | 0 | 0 | 2 | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 2 |

**Table 2.** Isolation frequency for fungi (mean and standard deviation) on five media (PD 15, PD30, EM, PD, and SNA) from marine sponges. Total = overall isolation frequency (#isolates obtained/number of isolates plated on all media); N=number of sponge collections (sec Table 1) evaluated per order, occan and season.

| | | | | | P 15 | | P 30 | | EM | | PD | | SNA | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Order | Ocean | Season | N | Total | Men | SD | Men | SD | Men | Sd | Men | Sd | Men | SD |
| Chondrosida | Caribbean | dry | 3 | 12.0 | 36.7 | 55.1 | 26.7 | 37.9 | 46.7 | 28.9 | 33.3 | 41.6 | 36.7 | 28.9 |
| | | rainy | 4 | 30.0 | 35.0 | 45.1 | 20.0 | 40.0 | 47.5 | 45.0 | 42.5 | 37.7 | 32.5 | 25.0 |
| | Pacific | dry | 3 | 84.0 | 56.7 | 55.1 | 60.0 | 30.0 | 63.3 | 40.4 | 43.3 | 58.6 | 43.3 | 45.1 |
| | | rainy | 1 | 2.0 | 0.0 | N/A | 0.0 | N/A | 10.0 | N/A | 0.0 | N/A | 0.0 | N/A |
| Dendroceratida | Caribbean | rainy | 2 | 16.0 | 5.0 | 7.1 | 0.0 | 0.0 | 20.0 | 14.1 | 0.0 | 0.0 | 20.0 | 28.3 |
| Dictyoceratida | Caribbean | dry | 12 | 0.0 | 53.3 | 28.4 | 37.5 | 31.4 | 53.3 | 23.1 | 40.8 | 35.5 | 34.2 | 26.4 |
| | | rainy | 6 | 30.0 | 33.3 | 28.0 | 43.3 | 32.7 | 48.3 | 27.9 | 35.0 | 35.1 | 34.3 | 27.9 |
| | Pacific | dry | 6 | 22.0 | 21.7 | 38.7 | 15.0 | 32.1 | 36.7 | 39.8 | 25.0 | 37.3 | 21.7 | 25.6 |
| | | rainy | 3 | 2.0 | 0.0 | 0.0 | 0.0 | 0.0 | 10.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Hadromerida | Caribbean | dry | 5 | 38.0 | 50.0 | 40.6 | 40.0 | 35.4 | 62.0 | 29.5 | 40.0 | 43.0 | 36.0 | 33.6 |
| | | rainy | 6 | 0.0 | 26.7 | 37.8 | 20.0 | 33.5 | 33.3 | 41.3 | 30.0 | 35.2 | 26.7 | 23.4 |
| | Pacific | dry | 11 | 68.0 | 23.6 | 34.4 | 19.1 | 32.4 | 33.6 | 35.6 | 21.8 | 29.9 | 22.7 | 23.3 |
| | | rainy | 12 | 72.0 | 54.2 | 30.9 | 30.0 | 33.3 | 51.7 | 19.5 | 40.0 | 36.9 | 35.0 | 30.3 |
| Halichondrida | Caribbean | dry | 3 | 84.0 | 76.7 | 51.3 | 26.7 | 25.2 | 73.3 | 25.2 | 53.3 | 47.3 | 53.3 | 46.2 |
| | | rainy | 1 | 20.0 | 0.0 | N/A | 20.0 | N/A | 50.0 | N/A | 20.0 | N/A | 10.0 | N/A |
| | Pacific | rainy | 12 | 36.0 | 28.3 | 32.4 | 30.8 | 34.2 | 40.8 | 34.5 | 31.7 | 34.1 | 26.7 | 26.1 |
| Haplosclerida | Caribbean | dry | 24 | 16.0 | 39.2 | 33.0 | 29.6 | 32.1 | 45.8 | 29.2 | 31.3 | 33.0 | 26.7 | 25.1 |
| | | rainy | 25 | 0.0 | 24.5 | 34.3 | 25.0 | 30.0 | 38.5 | 32.5 | 22.0 | 29.3 | 22.0 | 23.5 |
| | Pacific | dry | 9 | 2.0 | 0.0 | 0.0 | 6.7 | 11.5 | 23.3 | 23.1 | 6.7 | 11.5 | 3.3 | 5.8 |
| | | rainy | 30 | 22.0 | 20.0 | 28.5 | 23.2 | 29.6 | 35.3 | 29.9 | 23.2 | 29.6 | 23.2 | 23.6 |
| Homosclerophorida | Caribbean | dry | 10 | 50.0 | 49.0 | 32.8 | 44.0 | 33.1 | 62.0 | 22.5 | 46.0 | 36.9 | 38.0 | 28.2 |
| | Pacific | dry | 2 | 14.0 | 10.0 | 0.0 | 0.0 | 0.0 | 40.0 | 14.1 | 5.0 | 7.1 | 20.0 | 28.3 |
| Lithistida | Pacific | dry | 2 | 2.0 | 5.0 | 7.1 | 5.0 | 7.1 | 25.0 | 35.4 | 5.0 | 7.1 | 0.0 | 0.0 |
| N.I. | Pacific | rainy | 1 | 0.0 | 20.0 | N/A | 50.0 | N/A | 50.0 | N/A | 0.0 | N/A | 0.0 | N/A |
| Poecilosclerida | Caribbean | dry | 12 | 40.0 | 50.8 | 28.7 | 44.6 | 29.9 | 56.2 | 30.7 | 41.5 | 34.1 | 35.4 | 25.7 |
| | | rainy | 23 | 36.0 | 34.3 | 34.0 | 29.6 | 32.8 | 43.5 | 31.4 | 29.6 | 34.0 | 27.0 | 25.7 |
| | Pacific | dry | 4 | 2.0 | 27.5 | 48.6 | 22.5 | 38.6 | 35.0 | 50.7 | 35.0 | 43.6 | 22.5 | 28.7 |
| | | rainy | 10 | 80.0 | 56.0 | 26.3 | 30.0 | 35.6 | 52.0 | 19.3 | 41.0 | 37.3 | 32.0 | 29.4 |
| Spirophorida | Caribbean | dry | 2 | 14.0 | 50.0 | 42.4 | 0.0 | 0.0 | 45.0 | 7.1 | 0.0 | 0.0 | 0.0 | 0.0 |
| | | rainy | 2 | 22.0 | 25.0 | 7.1 | 20.0 | 28.3 | 30.0 | 28.3 | 20.0 | 14.1 | 35.0 | 7.1 |
| Verongida | Caribbean | dry | 12 | 0.0 | 50.8 | 32.3 | 22.5 | 28.3 | 50.0 | 18.6 | 30.8 | 31.2 | 27.5 | 26.3 |
| | | rainy | 3 | 40.0 | 60.0 | 10.0 | 23.3 | 40.4 | 63.3 | 11.5 | 43.3 | 30.6 | 23.3 | 15.3 |
| | Pacific | dry | 45 | 88.0 | 15.0 | 34.6 | 13.8 | 27.7 | 33.8 | 36.2 | 15.0 | 31.2 | 13.8 | 23.3 |
| | | rainy | 62 | 72.0 | 20.6 | 30.7 | 22.5 | 32.4 | 35.0 | 32.2 | 21.9 | 31.7 | 20.6 | 24.9 |

(Table 2). Isolation frequency was higher for Caribbean vs. Pacific sponges on all five media (Table 2). Isolation frequency was similar in the dry- (26.4% ± 2.1; N = 173 collections) and wet seasons overall (28.3% ± 1.6; N = 195) (P = 0.3395), but different effects of season were observed in each ocean (see below).

When effects of ocean and season were taken into account, orders of sponges differed significantly in isolation frequency (overall effect of order in multiple regression, P = 0.0297). Mean isolation frequency for sponges of each order are shown in Table 2. overall four orders had significantly higher isolation frequencies in the Caribbean than in the Pacific ($p<0.005$; Dictyoceratida, Halichondrida, Haplosclerida, Verongida), and the others not differing significantly as a function of ocean (Chondrosida, Hadromerida, Homosclerophorida, Poecilo-

sclerida) or sampled too infrequently for comparisons (e.g., Dendroceratida, Spirophorida, and an unidentified lineage).

One to seven families of sponges were sampled per order. In orders with sufficient sampling for comparison, we found no evidence for differences among co-ordinal families in isolation frequency as a function of family (for example: P>0.05 for family effects in multiple regression assessing effects of family, ocean, and season on isolation frequecy from sponges in the Chalinidae vs. Niphatidae, within the Haplosclerida).

After combining data from proximate sites into three regions in the Pacific (Figure 1) we assessed regional, seasonal, and taxonomic variation in isolation frequency. In samples from that ocean, isolation frequency was more than twofold greater in the rainy season (28.2% ± 24.8; N = 122 samples) than in the dry season (10.8% ± 18.7; N = 90; $c^2$ = 54.7, df = 1, P<0.0001), and isolation frequency differed among orders (Table 2; $c^2$ = 35.0, df = 8, P<0.0001). When these effects of season and order were taken into account, we found no evidence for regional differences: isolation frequency from the Coiba region, Azuero region, and Gulf of Panama were similar ($c^2$ assessing effects of region on residuals following test of season and order = 4.1, df = 2, P = 0.1268).

In the Caribbean, we compared sites 10 and 11, which were physically proximate but differed in aspect (10, seaward; 11, landward). In this ocean, isolation frequency was nearly twofold higher in the dry season (45.0% ± 27.1, N = 73 samples) than the rainy season (25.2% ± 15.6, N = 68; $c^2$ = 17.7, df = 1, P<0.0001), and isolation frequency differed among orders (Table 2; $c^2$ = 18.2, df = 9, P = 0.0328). When these effects were taken into account, we found significantly higher isolation frequency in site 10 (38.2% ± 23.4, N = 90) than in site 11 (30.5% ± 25.4, N = 51) ($c^2$ assessing effects of site on residuals following test of season and order = 10.5, df = 1, P = 0.0012). Further evaluations are needed to assess the mechanisms driving such patterns, including seasonal inputs of terrestrial runoff in both oceans, nutrient upwelling in the Pacific, and water quality and pollution status.

***Bioassay results :*** Sixty-two isolates were selected for bioassays on the basis of morphological distinctiveness and for their ability to change the color of the culture media, suggesting exudation of notable metabolites. Extraction of liquid media from shaken and stationary liquid cultures yielded 95 ethyl acetate extracts that were evaluated using radioligand-binding assays to assess inhibition of specific binding of [$^3$H] BQ123 to the endothelin $ET_A$ receptor and [$^3$H] NPY to the $Y_1$ receptor. Results of the biological activities for the ethyl acetate fractions of their liquid cultures are sumarized in Table 3. The remaining aqueous fraction of thirty-four samples were also tested and found inactive, suggesting that either no active compounds were present on this fraction or that the presence of high quantities of salt could mask their effect on the binding assay (data not shown).

No ethyl acetate extract from any of the stationary cultures was active, but active extracts were obtained from isolates cultivated on the orbital shaker (Table 3). Strains M20335P30, M194IP15, M20301E, M1883YYEM and M20112P30 inhibited by > 50% the binding of [$^3$H] BQ-123 to its receptor at a concentration of 100 μg $ml^{-1}$ and showed no activity on the second receptor. At the same concentration, strain M2009X1MSK30 inhibited the binding of [$^3$H] NPY to its receptor, with no effect on the endothelin receptor (Table 3).

The first three strains inhibited the total uptake of radiolabeled thymidine by cells in

**Table 3.** Results of the screening of ethyl acetate extracts from cultured sponge-associated fungi from the Republic of Panama in assays assessing binding inhibition of the endothelin $ET_A$ and neuropeptide Y $Y_1$ receptors. Results are expressed as the percentage of total inhibition of [$^3$H] BQ-123 and [$^3$H] NPY binding to the receptors. Results of the viability test by [$^3$H] thymidine uptake are shown for the most active samples. Values represent the mean ± standard error of the mean of two to three independent experiments performed in duplicate (number of independent experiments in brackets).

| | | | | | $ET_A$ | | $Y_1$ | | Thymidine |
|---|---|---|---|---|---|---|---|---|---|
| SAMPLE | FUNGAL ID | HOST | SITE | CULTURE | 100 mg/ml | 10 mg/ml | 100 mg/ml | 10 mg/ml | 100 mg/ml |
| M1862M | - | *A. compressa* | 10 | O.S. | 15±2 (2) | 4±4 (2) | 3±1 (2) | 0±0 (2) | N.T. |
| M1863E | - | *A. compressa* | 10 | O.S. | 16±4 (2) | 0±0 (2) | 13±4 (2) | 0±0 (2) | N.T. |
| M1864M | - | *A. compressa* | 10 | O.S. | 0±0 (2) | 0±0 (2) | 12±6 (2) | 0±0 (2) | N.T. |
| M1864MSK18 | - | *A. compressa* | 10 | O.S. | 0±0 (2) | 0±0 (2) | 13±1 (2) | 0±0 (2) | N.T. |
| M1864MS18 | - | *A. compressa* | 10 | S. | 0±0 (2) | 0±0 (2) | 3±3 (2) | 0±0 (2) | N.T. |
| M1871E | - | *Xestospongia sp.* | 10 | O.S. | 26±7 (2) | 1±1 (2) | 20±4 (2) | 0±0 (2) | N.T. |
| M1883ZE | - | *Aplysina sp.* | 10 | O.S. | 15±1 (2) | 0±0 (2) | 1±1 (2) | 0±0 (2) | N.T. |
| M1883ZES | - | *Aplysina sp.* | 10 | S. | 11±1 (2) | 0±0 (2) | 0±0 (2) | 0±0 (2) | N.T. |
| M1883YYES | - | *Aplysina sp.* | 10 | S. | 26±5 (2) | 2±2 (2) | 0±0 (2) | 0±0 (2) | N.T. |
| M1883YYEM | - | *Aplysina sp.* | 10 | O.S. | **53±4 (3)** | **6±1 (3)** | 4±2 (2) | 0±0 (2) | N.T. |
| M1941P15 | *"W. tropicalis"* | *Ircinia sp.* | 10 | O.S. | **51±6 (3)** | **2±1 (3)** | 15±3 (2) | 0±0 (2) | 82±1 (3) |
| M2004E | - | *Haliclona sp.* | 10 | O.S. | 3±6 (2) | 0±0 (2) | 5±5 (2) | 0±0 (2) | N.T. |
| M2009X1MSK30 | *"T. inflatumtum"* | *Haliclona sp.* | 10 | O.S. | 20±6 (2) | 2±1 (2) | **88±1 (3)** | **20±4 (3)** | N.T. |
| M2009ZMS30 | *"T. inflatumtum"* | *Haliclona sp.* | 10 | S. | 0±0 (2) | 0±0 (2) | 29±9 (3) | 6±3 (3) | N.T. |
| M2005(2)M | - | *Haliclona sp.* | 10 | O.S. | 25±4 (2) | 0±0 (2) | 20±1 (2) | 0±0 (2) | N.T. |
| M20071P30 | - | Not identified | 7 | O.S. | 15±3 (2) | 2±2 (2) | 8±1 (2) | 0±0 (2) | N.T. |
| M20071P30SK18 | - | Not identified | 7 | O.S. | 0±0 (2) | 0±0 (2) | 5±3 (2) | 0±0 (2) | N.T. |
| M20071P30S | - | Not identified | 7 | S. | 19±4 (2) | 1±0 (2) | 7±1 (2) | 0±0 (2) | N.T. |
| M20071E | - | Not identified | 7 | O.S. | 0±0 (2) | 0±0 (2) | 0±0 (2) | 0±0 (2) | N.T. |
| M20111P30 | - | Not identified | 7 | O.S. | 26±6 (2) | 7±6 (2) | 12±2 (2) | 0±0 (2) | N.T. |
| M20111P30SK18 | - | Not identified | 7 | O.S. | 12±0 (2) | 5±4 (2) | 5±5 (2) | 0±0 (2) | N.T. |
| M20111P30S18 | - | Not identified | 7 | S. | 32±1 (2) | 4±4 (2) | 28±3 (2) | 2±2 (2) | N.T. |
| M20112P30 | - | Not identified | 7 | O.S. | **81±6 (3)** | **13±2 (3)** | **93±3 (3)** | **0±0 (3)** | 100±0 (3) |
| M20121E | - | *Laxosuberites sp.* | 7 | O.S. | 0±0 (2) | 0±0 (2) | 10±5 (2) | 1±1 (2) | N.T. |
| M20171P30 | - | *Laxosuberites sp.* | 7 | O.S. | 26±9 (2) | 0±0 (2) | 25±4 (2) | 0±0 (2) | N.T. |
| M20171E | - | *Laxosuberites sp.* | 7 | O.S. | 15±0 (2) | 3±1 (2) | 13±5 (2) | 8±8 (2) | N.T. |
| M20172E | - | *Laxosuberites sp.* | 7 | O.S. | 25±0 (2) | 6±3 (2) | 23±2 (2) | 0±0 (2) | N.T. |
| M20173P15 | - | *Laxosuberites sp.* | 7 | O.S. | 14±0 (2) | 5±2 (2) | 10±2 (2) | 0±0 (2) | N.T. |
| M20173P15S | - | *Laxosuberites sp.* | 7 | S. | 18±4 (2) | 3±1 (2) | 0±0 (2) | 0±0 (2) | N.T. |
| M20173P15S18 | - | *Laxosuberites sp.* | 7 | S. | 17±1 (2) | 0±0 (2) | 3±3 (2) | 0±0 (2) | N.T. |
| M20173P15SK18 | - | *Laxosuberites sp.* | 7 | O.S. | 17±2 (2) | 7±2 (2) | 0±0 (2) | 0±0 (2) | N.T. |
| M20173P30SK18 | - | *Laxosuberites sp.* | 7 | O.S. | 18±3 (2) | 7±1 (2) | 13±0 (2) | 0±0 (2) | N.T. |
| M20173P30S18 | - | *Laxosuberites sp.* | 7 | S. | 0±0 (2) | 0±0 (2) | 12±0 (2) | 0±0 (2) | N.T. |
| M20173P30 | - | *Laxosuberites sp.* | 7 | O.S. | 13±0 (2) | 1±1 (2) | 5±5 (2) | 0±0 (2) | N.T. |
| M20181P15 | - | *Laxosuberites sp.* | 7 | O.S. | 22±6 (2) | 5±5 (2) | 6±5 (2) | 0±0 (2) | N.T. |
| M20241P15 | *"E. scoparia"* | *A.gerardogreeni* | 7 | O.S. | 23±9 (2) | 0±0 (2) | 31±6 (2) | 12±4 (2) | N.T. |
| M20241P30 | - | *A.gerardogreeni* | 7 | O.S. | 16±9 (2) | 2±2 (2) | 0±0 (2) | 0±0 (2) | N.T. |
| M20251P15 | - | *A. chiriquensis* | 9 | O.S. | 20±0 (2) | 0±0 (2) | 26±2 (2) | 3±3 (2) | N.T. |
| M20251E | - | *A. chiriquensis* | 9 | O.S. | 15±0 (2) | 13±0 (2) | 17±2 (2) | 6±2 (2) | N.T. |
| M20251M | - | *A. chiriquensis* | 9 | O.S. | 19±1 (2) | 6±0 (2) | 3±2 (2) | 0±0 (2) | N.T. |
| M20252P30 | - | *A. chiriquensis* | 9 | O.S. | 8±2 (2) | 2±2 (2) | 16±1 (2) | 3±3 (2) | N.T. |
| M20252E | - | *A. chiriquensis* | 9 | O.S. | 16±4 (2) | 6±6 (2) | 5±1 (2) | 0±0 (2) | N.T. |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| M20252ES | - | *A. chiriquensis* | 9 | S. | 15±6 (2) | 6±7 (2) | 2±2 (2) | 0±0 (2) | N.T. |
| M20253E | - | *A. chiriquensis* | 9 | O.S. | 4±3 (2) | 0±0 (2) | 6±1 (2) | 5±3 (2) | N.T. |
| M20253ES | - | *A. chiriquensis* | 9 | S. | 16±4 (2) | 0±0 (2) | 3±3 (2) | 0±0 (2) | N.T. |
| M20254P30 | - | *A. chiriquensis* | 9 | O.S. | 13±2 (2) | 0±0 (2) | 15±2 (2) | 0±0 (2) | N.T. |
| M20256P30 | - | *A. chiriquensis* | 9 | O.S. | 5±1 (2) | 2±1 (2) | 18±6 (2) | 4±4 (2) | N.T. |
| M20271E | - | *A. gerardogreeni* | 9 | O.S. | 12±4 (2) | 3±4 (2) | 28±3 (2) | 22±5 (2) | N.T. |
| M20271ES | - | *A. gerardogreeni* | 9 | S. | 7±8 (2) | 0±0 (2) | 2±0 (2) | 0±0 (2) | N.T. |
| M20283XP30 | - | *Laxosuberites sp.* | 9 | O.S. | 14±1 (2) | 0±0 (2) | 12±1 (2) | 0±0 (2) | N.T. |
| M20283E | - | *Laxosuberites sp.* | 9 | O.S. | 22±8 (2) | 0±0 (2) | 5±5 (2) | 0±0 (2) | N.T. |
| M20284M | - | *Laxosuberites sp.* | 9 | O.S. | 22±0 (2) | 8±1 (2) | 14±6 (2) | 0±0 (2) | N.T. |
| M20284E | - | *Laxosuberites sp.* | 9 | O.S. | 12±4 (2) | 0±0 (2) | 19±12 (2) | 0±0 (2) | N.T. |
| M20284P15 | - | *Laxosuberites sp.* | 9 | O.S. | 28±8 (2) | 1±1 (2) | 8±5 (2) | 0±0 (2) | N.T. |
| M20284P15S | - | *Laxosuberites sp.* | 9 | S. | 24±8 (2) | 0±0 (2) | 6±6 (2) | 0±0 (2) | N.T. |
| M20286E | - | *Laxosuberites sp.* | 9 | O.S. | 11±4 (2) | 1±1 (2) | 11±1 (2) | 0±0 (2) | N.T. |
| M20286ES | - | *Laxosuberites sp.* | 9 | S. | 13±6 (2) | 0±0 (2) | 6±6 (2) | 0±0 (2) | N.T. |
| M20286P15 | - | *Laxosuberites sp.* | 9 | O.S. | 0±0 (2) | 0±0 (2) | 7±1 (2) | 0±0 (2) | N.T. |
| M20286P15S | - | *Laxosuberites sp.* | 9 | S. | 30±5 (2) | 3±3 (2) | 0±0 (2) | 0±0 (2) | N.T. |
| M20285P15 | - | *Laxosuberites sp.* | 9 | O.S. | 26±1 (2) | 0±0 (2) | 10±1 (2) | 0±0 (2) | N.T. |
| M20285P15S | - | *Laxosuberites sp.* | 9 | S. | 0±0 (2) | 0±0 (2) | 33±1 (2) | 17±7 (2) | N.T. |
| M20285P30 | - | *Laxosuberites sp.* | 9 | O.S. | 0±0 (2) | 0±0 (2) | 8±3 (2) | 0±0 (2) | N.T. |
| M20285P30S | - | *Laxosuberites sp.* | 9 | S. | 3±3 (2) | 0±0 (2) | 13±3 (2) | 0±0 (2) | N.T. |
| M20287P30 | - | *Laxosuberites sp.* | 9 | O.S. | 10±4 (2) | 0±0 (2) | 30±4 (2) | 20±6 (2) | N.T. |
| M20287E | - | *Laxosuberites sp.* | 9 | O.S. | 6±3 (2) | 1±0 (2) | 28±0 (2) | 0±0 (2) | N.T. |
| M20287ES | - | *Laxosuberites sp.* | 9 | S. | 24±4 (2) | 0±0 (2) | 6±0 (2) | 0±0 (2) | N.T. |
| M20301E | *"H. jecorina"* | *H. caerulea* | 10 | O.S. | **50±6 (3)** | **4±0 (3)** | 0±0 (2) | 0±0 (2) | 67±1 (3) |
| M20301YP15 | - | *H. caerulea* | 10 | O.S. | 9±0 (2) | 0±0 (2) | 29±1 (2) | 19±0 (2) | N.T. |
| M20302P15 | - | *H. caerulea* | 10 | O.S. | 21±2 (2) | 0±0 (2) | 6±6 (2) | 0±0 (2) | N.T. |
| M20303P15 | - | *H. caerulea* | 10 | O.S. | 14±6 (2) | 1±1 (2) | 4±4 (2) | 0±0 (2) | N.T. |
| M20303P15S | - | *H. caerulea* | 10 | S. | 20±9 (2) | 0±0 (2) | 2±2 (2) | 0±0 (2) | N.T. |
| M20307E | - | *H. caerulea* | 10 | O.S. | 5±5 (2) | 0±0 (2) | 32±4 (2) | 19±2 (2) | N.T. |
| M20307P30 | - | *H. caerulea* | 10 | O.S. | 16±7 (2) | 0±0 (2) | 37±1 (2) | 0±0 (2) | N.T. |
| M20307P30S | - | *H. caerulea* | 10 | S. | 14±3 (2) | 4±6 (2) | 9±1 (2) | 0±0 (2) | N.T. |
| M20308E | - | *H. caerulea* | 10 | O.S. | 23±0 (2) | 0±0 (2) | 29±6 (2) | 18±3 (2) | N.T. |
| M20312E | - | Not identified | 10 | O.S. | 30±5 (3) | 3±3 (2) | 29±2 (2) | 19±5 (2) | N.T. |
| M20312ES | - | Not identified | 10 | S. | 26±4 (2) | 0±0 (2) | 24±1 (2) | 0±0 (2) | N.T. |
| M20312P15 | - | Not identified | 10 | O.S. | 27±1 (2) | 11±3 (2) | 28±3 (2) | 19±1 (2) | N.T. |
| M20317P30 | - | Not identified | 10 | O.S. | 14±3 (2) | 0±0 (2) | 6±2 (2) | 0±0 (2) | N.T. |
| M20317P30S | - | Not identified | 10 | S. | 30±2 (2) | 0±0 (2) | 4±4 (2) | 0±0 (2) | N.T. |
| M20321M | - | *A. compressa* | 10 | O.S. | 32±3 (2) | 6±5 (2) | 29±1 (2) | 17±1 (2) | N.T. |
| M20321MS | - | *A. compressa* | 10 | S. | 13±4 (2) | 1±13 (2) | 22±4 (2) | 2±1 (2) | N.T. |
| M20322P15 | - | *A. compressa* | 10 | O.S. | 27±0 (2) | 0±0 (2) | 15±1 (2) | 0±0 (2) | N.T. |
| M20322P15S | - | *A. compressa* | 10 | S. | 12±5 (2) | 0±0 (2) | 7±0 (2) | 0±0 (2) | N.T. |
| M20322E | - | *A. compressa* | 10 | O.S. | 0±0 (2) | 0±0 (2) | 0±0 (2) | 0±0 (2) | N.T. |
| M20322ES | - | *A. compressa* | 10 | S. | 21±3 (2) | 9±6 (2) | 0±0 (2) | 0±0 (2) | N.T. |
| M20331bP30 | - | *N. erecta* | 10 | O.S. | 20±2 (2) | 0±0 (2) | 8±4 (2) | 0±0 (2) | N.T. |
| M20334P15S | - | *N. erecta* | 10 | S. | 22±0 (2) | 0±0 (2) | 0±0 (2) | 0±0 (2) | N.T. |
| M20335P30 | *"F. rhodense"* | *N. erecta* | 10 | O.S. | **83±2 (3)** | **32±8 (3)** | 0±0 (2) | 0±0 (2) | 44±3 (3) |
| M20335P30S | *"F. rhodense"* | *N. erecta* | 10 | S. | 4±2 (2) | 0±0 (2) | 6±0 (2) | 0±0 (2) | N.T. |
| M20338YP15 | - | *N. erecta* | 10 | O.S. | 22±2 (2) | 0±0 (2) | 0±0 (2) | 0±0 (2) | N.T. |
| M20338YP15S | - | *N. erecta* | 10 | S. | 15±3 (2) | 2±0 (2) | 2±2 (2) | 0±0 (2) | N.T. |
| M20339P15 | - | *N. erecta* | 10 | O.S. | 4±4 (2) | 0±0 (2) | 11±1 (2) | N.T. | N.T. |
| M20342MM | - | *A. gerardogreeni* | 8 | O.S. | 5±5(2) | 0±0 (2) | N.T. | N.T. | N.T. |
| M20342ME | - | *A. gerardogreeni* | 8 | O.S. | 20±7 (2) | 0±0 (2) | N.T. | N.T. | N.T. |

**N.T.: Not tested; O.S.: Culture on orbital shaker; S.: Cultures on shelves.**

**Appendix 1.** In preliminary trials we assessed isolation of sponge-associated fungi on media M1D and CM (M1D: 0.28 g $l^{-1}$ $Ca(NO_3)_2.4H_2O$, 0.07 g $l^{-1}$ $KNO_3$, 0.065 g $l^{-1}$ KCl, 0.36 g $l^{-1}$ $MgSO_4$, 0.017 g $l^{-1}$ $NaH_2PO_4$, 30 g $l^{-1}$ sucrose, 4.99 g $l^{-1}$ ammonium tartrate, 0.002 g $l^{-1}$ $FeCl_3.6H_2O$, 0.0045 g $l^{-1}$ $MnSO_4$, 0.0014 g $l^{-1}$ $ZnSO_4$, 0.0014 g $l^{-1}$ $H_3BO_3$, 0.00074 g $l^{-1}$ KI, 38 g $l^{-1}$ marine salt, 20 g $l^{-1}$ agar. CM: 2 g $l^{-1}$ cornmeal, 38 g $l^{-1}$ marine salt, 18 g $l^{-1}$ agar). Numbers of isolates (per 10 small fragments of sponges; see Methods) are shown.

| Order | Family | Genus | specie | Code | Site | Season | M1D | CM |
|---|---|---|---|---|---|---|---|---|
| Dictyoceratida | Spongiidae | *Spongia* | *pertusa* | M184 | 10 | dry | 7 | 1 |
| Dictyoceratida | Irciniidae | *Ircinia* | *campana* | M189 | 10 | dry | 2 | 0 |
| Dictyoceratida | Irciniidae | *Ircinia* | *sp* | M194 | 10 | dry | 5 | 0 |
| Verongida | Aplysinidae | *Aplysina* | *sp* | M188 | 10 | dry | 4 | 5 |
| Haplosclerida | Petrosiidae | *Xestospongia* | *sp* | M178 | 10 | dry | 5 | 0 |
| Haplosclerida | Niphatidae | *Amphimedon* | *compressa* | M186 | 10 | dry | 2 | 2 |
| Haplosclerida | Chalinidae | *Haliclona* | *sp* | M200 | 10 | dry | 14 | 3 |
| Haplosclerida | Chalinidae | *Haliclona* | *caerulea* | M2030 | 10 | rainy | 3 | N.U. |
| Haplosclerida | Chalinidae | N.I. | N.I. | M2031 | 10 | rainy | 2 | N.U. |
| Haplosclerida | Niphatidae | *Amphimedon* | *compressa* | M2032 | 10 | rainy | 1 | N.U. |
| Haplosclerida | Niphatidae | *Niphates* | *erecta* | M2033 | 10 | rainy | 5 | N.U. |
| Verongida | Aplysinidae | *Aplysina* | *gerardogreeni* | M2034 | 8 | rainy | 2 | N.U. |
| Verongida | Aplysinidae | *Aplysina* | *gerardogreeni* | M2035 | 8 | rainy | 1 | N.U. |
| Verongida | Aplysinidae | *Aplysina* | *sp6* | M2036 | 8 | rainy | 0 | N.U. |
| Verongida | Aplysinidae | *Aplysina* | *sp7* | M2037 | 8 | rainy | 5 | N.U. |
| Verongida | Aplysinidae | *Aplysina* | *sp6* | M2039 | 8 | rainy | 2 | N.U. |
| Poecilosclerida | Raspaillidae | N.I. | sp3 | M2038 | 8 | rainy | 7 | N.U. |
| Verongida | Aplysinidae | *Aplysina* | *chiriquensis* | M2025 | 9 | rainy | 1 | N.U. |
| Verongida | Aplysinidae | *Aplysina* | *gerardogreeni* | M2026 | 9 | rainy | 0 | N.U. |
| Verongida | Aplysinidae | *Aplysina* | *gerardogreeni* | M2027 | 9 | rainy | 0 | N.U. |
| Hadromerida | Suberitidae | *Laxosuberites* | *sp* | M2028 | 9 | rainy | 7 | N.U. |

N.U. : not used

**Table 4.** One hundred sponge-associated fungi for which ITS-LSU sequence data were obtained. 'Species' indicates the genotype group (based on 95% sequence similarity) into which each isolate was placed. Top BLAST hits estimate taxonomy, but are not meant to indicate precise species identification

| Isolation Code | Isolate ID | Species | Host | Site | Season | Accession# of Top BLAST Match | Blast Score | E Value | Top BLAST Match |
|---|---|---|---|---|---|---|---|---|---|
| M2024-1-P15 | CC1 | 5 | Aplysina gerardogreeni | 7 | rainy | FJ172283.1 | 1034 | 0 | Eutypella scoparia MUT 485 |
| M200T-9Z-M1D | CC2 | 6 | Haliclona sp. | 10 | dry | AB044645.1 | 1698 | 0 | Tolypocladium inflatum |
| M200T-9X-M1D | CC3 | 6 | Haliclona sp. | 10 | dry | AB044645.1 | 1698 | 0 | Tolypocladium inflatum |
| M2030-1-EM1 | CC6 | 21 | Haliclona caerulea | 10 | rainy | AF510497.1 | 1341 | 0 | Hypocrea jecorina |
| M2033-5-P30 | CC7 | 1 | Niphates erecta | 10 | rainy | EU035440.1 | 1039 | 0 | Fusicladium rhodense strain CPC 13156 |
| M194-1-P15 | CC8 | 11 | Ircinia sp. | 10 | dry | AF506490.1 | 1269 | 0 | Wrightoporia tropicalis strain LR40352 5 |
| B1a009-7-PD2 | HM1 | 16 | Niphates erecta | 10 | rainy | EU687008 | 1205.94 | 0 | Fungal endophyte isolate 1988 |
| B1a039-2-P152 | HM10 | 2 | Placospongia intermedia | 10 | rainy | EU686943 | 1923.68 | 0 | Fungal endophyte isolate 1701 |
| B1a019-1-PD2 | HM100 | 18 | Lissodendoryx colombiensis | 10 | rainy | EF094551 | 1640.55 | 0 | Microsphaeropsis arundinis strain 2190/04 |
| B1a009-5-P302 | HM102 | 14 | Niphates erecta | 10 | rainy | EU686990 | 1142.82 | 0 | Fungal endophyte isolate 1954 |
| B1a027-1-PD2 | HM103 | 4 | Not identified | 10 | rainy | EU686780 | 1739.73 | 0 | Uncultured fungus clone C47 |
| X0016R-1-EM1 | HM107 | 4 | Not identified | 9 | dry | EU490070 | 1440.37 | 0 | Uncultured ascomycete clone C31_E08 |
| B1a003-1-P302 | HM109 | 7 | Aplysina fulva | 10 | rainy | FJ434202 | 1802.85 | 0 | Hypocrea sp. LY 30.1 |
| B1a028-8-PD2 | HM11 | 16 | Aplysina sp. | 10 | rainy | EU686961 | 1184.3 | 0 | Fungal endophyte isolate 1830 |
| C4009-3-PD1 | HM110 | 4 | Aplysina sp5 | 5 | dry | EU490070 | 1871.38 | 0 | Uncultured ascomycete clone C31_E08 |
| C4B-2-SNA1 | HM112 | 17 | Aplysina sp1 | 5 | dry | EF417482 | 1424.14 | 0 | Trichoderma atroviride |
| B1a035-2b-PD2 | HM12 | 28 | Tedania ignis | 10 | rainy | EU552122 | 1543.17 | 0 | Diaporthe cynaroidis culture-collection CBS:122676 |
| C4025-2-P301 | HM121 | 8 | Axinella sp1. | 5 | dry | EU687125 | 1658.58 | 0 | Fungal endophyte isolate 487 |
| C4064-2-P151 | HM122 | 15 | Aplysina gerardogreeni | 5 | dry | EU687182 | 1701.86 | 0 | Fungal endophyte isolate 887B |
| C3053-2-EM1 | HM123 | 37 | Not identified | 4 | dry | EU118623 | 1418.73 | 0 | Cystostereum murrayi voucher KHL 12496 (GB) |
| B1a008-1-P152 | HM124 | 22 | Niphates erecta | 10 | rainy | EU687071 | 1415.13 | 0 | Fungal endophyte isolate 2218 |
| C4064-1-P151 | HM127 | 3 | Aplysina gerardogreeni | 5 | dry | EF157664 | 1582.84 | 0 | Xylaria sp. NRRL 40192 |
| B1a019-3-PD2 | HM128 | 3 | Lissodendoryx colombiensis | 10 | rainy | EU686955 | 1701.86 | 0 | Fungal endophyte isolate 178 |
| B1a041-1-P152 | HM129 | 9 | Desmapsana anchorata | 10 | rainy | AF310977 | 1416.93 | 0 | Fusarium sp. BBA 65925 |
| B1a009-3b-PD2 | HM13 | 9 | Niphates erecta | 10 | rainy | AF310977 | 1415.13 | 0 | Fusarium sp. BBA 65925 |
| C4064-1-EM1 | HM131 | 11 | Aplysina gerardogreeni | 5 | dry | AF506490 | 1624.32 | 0 | Wrightoporia tropicalis strain LR40352 |
| X0016-3-P303 | HM132 | 19 | Not identified | 9 | dry | EU687016 | 1326.76 | 0 | Fungal endophyte isolate 2057 |
| B1a020-3-PD2 | HM136 | 4 | Mycale sp. | 10 | rainy | EU686780 | 1651.37 | 0 | Uncultured fungus clone C47 |
| B1aA-2a-PD2 | HM137 | 22 | Spirastrella sp. | 10 | rainy | EU686797 | 1512.51 | 0 | Fungal endophyte isolate 1007 |
| B1a019-1a-PD2 | HM138 | 12 | Lissodendoryx colombiensis | 10 | rainy | EU687131 | 1779.41 | 0 | Fungal endophyte isolate 544 |
| B1a003-7-EM2 | HM139 | 7 | Aplysina fulva | 10 | rainy | FJ434202 | 1743.34 | 0 | Hypocrea sp. LY 30.1 |
| B1a047-4b-P152 | HM14 | 3 | Mycale sp. | 10 | rainy | EU687008 | 1599.07 | 0 | Fungal endophyte isolate 1988 |
| C4007-1-PD1 | HM141 | 8 | Aplysina chiriquensis | 5 | dry | EU687125 | 1294.3 | 0 | Fungal endophyte isolate 487 |
| C4005-2-PD1 | HM142 | 38 | Aplysina chiriquensis | 5 | dry | EU686996 | 1745.14 | 0 | Fungal endophyte isolate 1961 |

| Isolation Code | Isolate ID | Species | Host | Site | Season | Accession# of Top BLAST Match | Blast Score | E Value | Top BLAST Match |
|---|---|---|---|---|---|---|---|---|---|
| B1a019-5-PD2 | HM143 | 14 | Lissodendoryx colombiensis | 10 | rainy | EU687060 | 1137.41 | 0 | Fungal endophyte isolate 2204 |
| B1a003-1-EM2 | HM144 | 7 | Aplysina fulva | 10 | rainy | FJ434202 | 1759.57 | 0 | Hypocrea sp. LY 30.1 |
| B1a050-2-P302 | HM146 | 16 | Aplysina fulva | 10 | rainy | EU687008 | 1196.92 | 0 | Fungal endophyte isolate 1988 |
| C4066-1-P151 | HM147 | 39 | Aplysina gerardogreeni | 5 | dry | EU552126 | 1377.26 | 0 | Eutypa consobrina culture-collection CBS:122677 |
| C3060B-2-SNA1 | HM148 | 40 | Not identified | 4 | dry | AF210671 | 1629.73 | 0 | Stephanonectria keithii strain CBS 100005 |
| C4008-2-PD1 | HM149 | 2 | Aplysina gerardogreeni | 5 | dry | EU686855 | 1768.59 | 0 | Fungal endophyte isolate 1417 |
| B1a018-3-P152 | HM15 | 5 | Xestospongia sp. | 10 | rainy | EU552125 | 1251.02 | 0 | Eutypa consobrina culture-collection CBS:122678 |
| B1a003-2a-P302 | HM150 | 7 | Aplysina fulva | 10 | rainy | FJ434202 | 1682.03 | 0 | Hypocrea sp. LY 30.1 |
| B1a040-1-PD2 | HM151 | 41 | Cynachirella alloclada | 10 | rainy | DQ384571 | 1582.84 | 0 | Leptosphaerulina chartarum |
| C4004-1-SNA1 | HM155 | 8 | Axinella sp1. | 5 | dry | EU687125 | 1723.5 | 0 | Fungal endophyte isolate 487 |
| B1aA-2b-PD2 | HM156 | 42 | Spirastrella sp. | 10 | rainy | EU686933 | 1413.32 | 0 | Fungal endophyte isolate 1679 |
| B1aA-2-P152 | HM16 | 18 | Spirastrella sp. | 10 | rainy | EF094556 | 1755.96 | 0 | Microsphaeropsis arundinis strain AMMRL 159.03 |
| B1a018-8-PD2 | HM169 | 5 | Xestospongia sp. | 10 | rainy | EU552125 | 1296.11 | 0 | Eutypa consobrina culture-collection CBS:122678 |
| B1a018-4-P152 | HM17 | 29 | Xestospongia sp. | 10 | rainy | AF354095 | 1604.48 | 0 | Ceratobasidium sp. AGQ |
| C3060B-3-P151 | HM18 | 24 | Not identified | 4 | dry | EU214559 | 1833.51 | 0 | Fusarium solani NBAIM:350 |
| B1a027-1-PD2 | HM19 | 15 | Not identified | 10 | rainy | EU687175 | 1930.89 | 0 | Fungal endophyte isolate 86 |
| B1a027-8-P152 | HM190 | 43 | Not identified | 10 | rainy | EU552126 | 1178.89 | 0 | Eutypa consobrina culture-collection CBS:122677 |
| C4091-6-EM1 | HM193 | 4 | Chondrilla sp. | 5 | dry | EU686780 | 1590.05 | 0 | Uncultured fungus clone C47 |
| B1b003-2-PD2 | HM199 | 44 | Mycale sp. | 11 | rainy | EU552110 | 1862.36 | 0 | Bionectria cf. ochroleuca CBS 113336 culture- collection CBS:113336 |
| B1a020-6-P302 | HM2 | 10 | Mycale sp. | 10 | rainy | EF094556 | 1613.5 | 0 | Microsphaeropsis arundinis strain AMMRL 159.03 |
| B1a040-2-PD2 | HM20 | 18 | Cynachirella alloclada | 10 | rainy | EU552105 | 1694.65 | 0 | Camarosporium brabeji culture-collection CBS:123026 |
| B1a040-4-PD2+AB | HM211 | 22 | Cynachirella alloclada | 10 | rainy | EU686797 | 1443.98 | 0 | Fungal endophyte isolate 1007 |
| B1b011-3b-PD2 | HM212 | 10 | Not identified | 11 | rainy | EF094556 | 1651.37 | 0 | Microsphaeropsis arundinis strain AMMRL 159.03 |
| B1b011-8a-PD2 | HM213 | 45 | Not identified | 11 | rainy | EU552162 | 1633.34 | 0 | Trichothecium roseum culture-collection CBS:113334 |
| M2024-1-P15 | HM214 | 5 | Aplysina gerardogreeni | 7 | rainy | EU552125 | 1301.52 | 0 | Eutypa consobrina culture-collection CBS:122678 |
| P5012-1-PD1 | HM215 | 46 | Aplysina gerardogreeni | 9 | dry | EU552101 | 1267.25 | 0 | Anthostomella proteae culture-collection CBS:110127 |
| C3003-1-PD1 | HM216 | 12 | Aplysina gerardogreeni | 4 | dry | EU687179 | 1793.83 | 0 | Fungal endophyte isolate 880 |
| C4008-1a-PD1 | HM217 | 8 | Aplysina gerardogreeni | 5 | dry | EU687125 | 1550.38 | 0 | Fungal endophyte isolate 487 |
| B1a047-4a-P152 | HM220 | 3 | Mycale sp. | 10 | rainy | EU687185 | 1651.37 | 0 | Fungal endophyte isolate 902 |
| M2025-1-P30 | HM222 | 4 | Aplysina chiriquensis | 1 | rainy | EU686780 | 1579.23 | 0 | Uncultured fungus clone C47 |
| C3003-1a-PD1 | HM223 | 12 | Aplysina gerardogreeni | 4 | dry | EU687179 | 1763.18 | 0 | Fungal endophyte isolate 880 |

| Isolation Code | Isolate ID | Species | Host | Site | Season | Accession# of Top BLAST Match | Blast Score | E Value | Top BLAST Match |
|---|---|---|---|---|---|---|---|---|---|
| B1a050-2-P52 | HM224 | 9 | Aplysina fulva | 10 | rainy | AF310977 | 1463.82 | 0 | Fusarium sp. BBA 65925 |
| B1a027A-2-EM2 | HM226 | 4 | Spirastrella sp. | 10 | rainy | EU686780 | 1663.99 | 0 | Unculturedfungus clone C47 |
| B1a020A-3-P152 | HM227 | 47 | Not identified | 10 | rainy | EU687079 | 1631.53 | 0 | Fungal endophyte isolate 231 |
| B1a020-5a-PD2 | HM23 | 30 | Mycale sp. | 10 | rainy | FJ240316 | 1880.4 | 0 | Fusarium sp. NRRL 45833 |
| C4025-1-EM1 | HM232 | 25 | Axinella sp1. | 5 | dry | EU490103 | 1591.86 | 0 | Uncultured ascomycete clone C32_B06 |
| B1a018-2-P152 | HM233 | 16 | Xestospongia sp. | 10 | rainy | EU687079 | 1184.3 | 0 | Fungal endophyte isolate 231 |
| B1a006-1-PD2 | HM24 | 24 | Not identified | 10 | rainy | EU029589 | 1766.78 | 0 | Fusarium solani isolate S-0900 |
| B1a009-8-PD2 | HM25 | 10 | Niphates erecta | 10 | rainy | EF094556 | 1606.28 | 0 | Microsphaeropsis arundinis strain AMMRL 159.03 |
| B1a050-1-PD2 | HM26 | 13 | Aplysina fulva | 10 | rainy | EU605882 | 1878.59 | 0 | Pestalotiopsis sp. AJH26 |
| B1a009-11-P302 | HM27 | 31 | Niphates erecta | 10 | rainy | EU686817 | 2295.17 | 0 | Fungal endophyte isolate 1186 |
| B1a020-5a-P152 | HM28 | 20 | Mycale sp. | 10 | rainy | EU687094 | 1546.77 | 0 | Fungal endophyte isolate 328 |
| C3060-1-P301 | HM29 | 25 | Hyatella cf intestinalis | 4 | dry | EU490088 | 1674.81 | 0 | Uncultured ascomycete clone C31_H02 |
| B1a020-3-EM2 | HM3 | 21 | Mycale sp. | 10 | rainy | AB027384 | 1649.57 | 0 | Hypocrea lutea |
| B1a027A-7-EM2 | HM30 | 4 | Spirastrella sp. | 10 | rainy | EU686780 | 1813.67 | 0 | Unculturedfungus clone C47 |
| B1a020-6b-P152 | HM32 | 32 | Mycale sp. | 10 | rainy | EU552125 | 1480.05 | 0 | Eutypa consobrina culture-collection CBS:122678 |
| X0016R-1-PD1 | HM34 | 25 | Not identified | 9 | dry | EU489946 | 1750.55 | 0 | Uncultured ascomycete clone 4M4_A12 |
| B1a018-3-P301 | HM36 | 16 | Xestospongia sp. | 10 | rainy | EU687008 | 1225.77 | 0 | Fungal endophyte isolate 1988 |
| B1a020-2-P152 | HM4 | 26 | Mycale sp. | 10 | rainy | DQ389684 | 1775.8 | 0 | Psathyrella microrrhiza voucher LO185-02 |
| B1a050-1-P302 | HM41 | 14 | Aplysina fulva | 10 | rainy | EU687060 | 1160.85 | 0 | Fungal endophyte isolate 2204 |
| B1a020-4-P302 | HM43 | 7 | Mycale sp. | 10 | rainy | FJ434202 | 1723.5 | 0 | Hypocrea sp. LY 30.1 |
| X0016R-4-SNA1 | HM44 | 19 | Not identified | 9 | dry | EU687016 | 1400.7 | 0 | Fungal endophyte isolate 2057 |
| B1a001-1-P152 | HM45 | 17 | Niphates erecta | 10 | rainy | EF417482 | 1952.53 | 0 | Trichoderma atroviride |
| C3009-2-P151 | HM47 | 23 | Aplysina sp1 | 4 | dry | EU687084 | 1923.68 | 0 | Fungal endophyte isolate 269 |
| B1a028-3-PD2 | HM48 | 10 | Aplysina sp. | 10 | rainy | EF094556 | 1674.81 | 0 | Microsphaeropsis arundinis strain AMMRL 159.03 |
| B1a020-1-PD2 | HM5 | 17 | Mycale sp. | 10 | rainy | FJ430784 | 1905.64 | 0 | Trichoderma koningiopsis strain CCF3813 |
| B1a009-7-P152 | HM7 | 27 | Niphates erecta | 10 | rainy | AB044636 | 1851.54 | 0 | Cordyceps sp. 97005 |
| B1a020-7-P302 | HM79 | 33 | Mycale sp. | 10 | rainy | EU686953 | 1400.7 | 0 | Fungal endophyte isolate 177 |
| B1a020-2-P302 | HM8 | 13 | Mycale sp. | 10 | rainy | EU605882 | 1568.41 | 0 | Pestalotiopsis sp. AJH26 |
| B1a009-8-P152 | HM84 | 34 | Niphates erecta | 10 | rainy | FJ821507 | 1544.97 | 0 | Bionectriaceae sp. NRRL 54009 |
| B1a041-2-P152 | HM89 | 3 | Desmapsana anchorata | 10 | rainy | EU687191 | 1635.14 | 0 | Fungal endophyte isolate 933 |
| B1a028-3-SNA2 | HM9 | 23 | Aplysina sp. | 10 | rainy | EU687084 | 1570.22 | 0 | Fungal endophyte isolate 269 |
| B1a027A-2-P302 | HM90 | 20 | Spirastrella sp. | 10 | rainy | EU687185 | 1442.18 | 0 | Fungal endophyte isolate 902 |
| B1a031-3-EM2 | HM97 | 35 | Tedania ignis | 10 | rainy | EU686953 | 1404.31 | 0 | Fungal endophyte isolate 177 |
| C4008-1-SNA1 | HM98 | 8 | Aplysina gerardogreeni | 5 | dry | EU687125 | 1611.69 | 0 | Fungal endophyte isolate 487 |
| C4030-5-EM1 | HM99 | 36 | Axinella sp2. | 5 | dry | EU687052 | 1568.41 | 0 | Fungal endophyte isolate 2189 |

viability assays (Table 3). These results suggest at first that toxic substances might be present in the extracts. However, the same samples showed no effect on [$^3$H] NPY binding, suggesting that some compounds present in the samples could interfere with the incorporation of thymidine with no toxic effect. Strain M20112P30 showed almost a complete inhibition of binding to both receptors and prevented the thymidine uptake (Table 3).

***Fungal diversity :*** Fungi that yielded active extracts were sequenced and compared with the sequences obtained from GenBank. Results are shown in Table 4. As broader preliminary step to evaluating the diversity, geographic distributions, and host specificity of sponge-associated fungi, we sequenced ITS-LSU for a total of100 representative strains (Table 4). These strains represented 95 distinct genotypes. When grouped according to 95% sequence similarity, a proxy for estimating species (31), these strains clustered into approximately 47 distinct species. Of these, 24 species (51%) were represented more than once in the sample (nonsingletons) (Table 4).

Although further sampling is needed, these data provide a preliminary perspective on five main ecological aspects of sponge-associated microfungal communities which may be of use in designing sampling protocols to capture their diversity. First, the majority of species recovered (>90%) here represent Ascomycota, with a minority representing the Basidiomycota. Within the Ascomycota, fungi represented several major lineages of the diverse subphylum Pezizomycotina, including Sordariomycetes, Dothideomycetes, and Eurotiomycetes. This taxonomic distribution is similar to that of plant-symbiotic fungi in Panama (28); in that case, symbiosis is thought to have evolved multiple times (34), and the same may be true for fungi that associate with sponges. Second, although BLAST hits are insufficient for identifying fungi conclusively at the species level or matching them precisely to known strains, we found that the top BLAST match for 40 strains was to fungal endophyte sequences from Panamanian forest plants. This suggests continuity with terrestrial symbiotic fungi that may have remarkably wide host ranges and ecological niches, or that closely relate fungi have evolved in parallel in each habitat. Third, the vast majority of nonsingleton species (23 of 24 species, 95.8%) were found in sponges representing more than one genus, consistent with strong host-generalism. Fourth, only a minority (10 species, 41.7%) were found in both the Caribbean and Pacific, consistent with strong partitioning of fungal communities as a function of geography. Geographic partitioning also was observed within oceans: 12 of 14 species found in only one ocean (85.7%) were found in only one study site (Caribbean) or region (Pacific). Last, only 8 of 24 species (30%) were found in more than one season: the majority of species were found in the rainy season or dry season, but not in both.

## Conclusions

Coupled with our isolation data, these data suggest the following recommendations: (a) a diversity of cultivation media should be used to capture a large number of isolates; (b) many different sites should be sampled, rather than extensive sampling of a large number of sponge species in a single site; and (c) sampling should be conducted in different seasons in strongly seasonal tropical regions such as Panama. In turn, further work will determine the relative importance of depth, water quality, and other local characteristics on fungal diversity from sponges.

To our knowledge, this is the first report of the effect of the extracts of sponge-associated fungi on G-protein coupled receptors like $ET_A$ and $Y_1$ receptors. The assays used to screen these fungi will also help us to carry out bioassay-guided

isolation of the active components, unravel the corresponding mechanism of action, and evaluate their potential as new leads to treat cardiovascular disorders.

**Acknowledgments**

We thank the National Secretariat of Science and Technology of Panamá for financial support by grants of international collaboration numbers COL06-006 and COL08-014, the College of Agriculture and Life Sciences at The University of Arizona for technical and logistical support for molecular analyses, Malkanthi Gunatilaka and members of the Arnold research group for research assistance, and the Smithsonian Tropical Research Institute for laboratory facilities, boats and technical support. The authors also want to thank the panamanian Authority of the Environment (ANAM) for their collaboration.

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Current Trends in Biotechnology and Pharmacy
Vol. 4 (4) 900-907 October 2010. ISSN 0973-8916


# Genetic Polymorphism of CD18 gene in Karan Fries Young bull Calves using PCR-RFLP Analysis

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**Abstract**

CD18 gene encodes the common subunit of $b_2$ integrin molecules that are responsible for the leukocytes to migrate into the site of inflammation. Point mutation from adenine to guanine at 383 position of CD18 gene causes the loss of CD18 & CD11 subunits aggregation ability and which leads to the Bovine Leukocyte Adhesion Deficiency (BLAD) syndrome. BLAD is an autosomal recessive congenital disease of Holstein Friesian (HF) cattle breed and is characterized by recurrent bacterial infections, delayed wound healing, stunted growth and persistent marked neutrophilia. BLAD syndrome was spread throughout the world mainly by the use of semen from carrier animals in artificial insemination (AI) as they have viability. Use of HF bulls or their semen extensively for crossbreeding programmes during the last five decades made the screening of farm born HF and its crossbreds mandatory before their use in breeding programmes. In the present study, isolated genomic DNA from young Karan Fries bull calves was amplified and PCR products were subjected to RFLP analysis using *Taq I* restriction enzymes. Result indicated that out of 55 examined calves, 2 (3.64%) were BLAD carriers (BL/TL) and 1 (1.82%) was BLAD affected (BL/BL). This study has recommended the nation to screen all HF and its crosses across the country for BLAD.

**Key Words:** CD18, BLAD, Karan Fries, Congenital Disease, PCR-RFLP.

## Introduction

The Neutrophils (leukocytes) are first line of defense system especially in new born calves against pathogenic infections. These cells smell or recognize chemicals produced by pathogens, and migrate towards the site of infection to kill the invading pathogens (phagocytosis). These neutrophils require surface protein molecules that help to migrate to the site of inflammation. The common subunit of $b_2$ integrin molecules on the leucocytes is encoded by CD18 gene (Cluster of differentiation). These integrin molecules play a critical role in adhesion of leukocytes to intercellular adhesion molecule I (ICAM I) expressed on vascular endothelium, to other surface molecules of leukocyte and to bacteria. Leukocyte adhesion is crucial in the ability of leukocytes to travel, communicate, inflame, & fight infection. Impairment in these pathways leads to poor inflammation because of meager recruitment of neutrophils into the infected site. Mutation in CD18 gene prevents the expression of all $b_2$-integrins like lymphocyte function associated antigen (LFA-1), macrophage antigen (MAC-1) and p150, 95a (Leukocyte Adhesion Glycoproteins) on the leukocytes (1) and in turn causes the imperfection in leukocyte migration. Two point mutations were identified in CD18 gene

in HF cattle affected with BLAD (2). The mutation form cytosine (C) to thymine (T) at nucleotide position 775 is silent, as it causes no alteration in the normal amino acid sequence and has no phenotypic expression (2, 3). The other mutation from adenine (A) to guanine (G) at nucleotide position 383 causes an aspartic acid to glycine substitution at amino acid 128 (D128G) (2, 4, 5, 6, 7). This mutation abolished the *Taq I* restriction site and creates a *HaeIII* site, which allows the identification of normal, carrier and affected animals (8). Dysfunction of CD18 gene due to D128G mutation is expressed by the loss of CD18 and CD11 subunit aggregation ability and leads to the Bovine Leukocyte Adhesion Deficiency (BLAD) syndrome. BLAD is an autosomal-recessive congenital disease in HF cattle (1). Affected animals from homozygous form of BLAD are characterized by severe and recurrent bacterial infections such as pneumonia, gingivitis, ulcerative and granulomatous stomatitis, enteritis with bacterial overgrowth, periodontitis, loss of teeth, impaired pus formation, delayed wound healing, stunted growth, persistent marked neutrophilia & early mortality prior to sexual maturity (5, 9, 10, 11, 12, 13). In some cases, the BLAD affected animals survive past two to three years of age (14, 15). Also, the surviving animals demonstrate retarded growth and weakness (16, 17). India being a country where cross breeding is practiced on a large scale, the information relating to the genetic disorders which mainly concern HF cattle and its crosses becomes essential. At present, the information on occurrence of genetic disorders in KF is very scanty. Keeping this in view, our investigation was carried out to screen the young KF bull calves for polymorphism of CD18 gene, which causes missense mutation i. e. BLAD.

## Materials and Methods

All experimental procedures were approved by Institute's Animal Ethical Committee. Ten milliliters (ml) of blood samples were collected from 55 KF young bull calves maintained at Cattle Yard and Artificial Breeding Research Complex (ABRC) of National Dairy Research Institute (NDRI), Karnal using EDTA coated vacutainers. Genomic DNA was extracted from blood using *OMEGA* Midiprep Kit as manufacturer instruction. The quality and purity of isolated DNA was examined by 0.6% agarose gel electrophoresis and UV Spectrophotometer, respectively.

Polymerase chain reaction (PCR) was performed using primers (Table 1) consisting of 21 bases each (5'- AGG TCA GGC AGT TGC CTT CAA -3' and 5'-GGG GAG CAC CGT CTT GTC CAC -3') as propounded by Czarnik & Kaminski, 1997(18). Targeted region of size 367bp in CD18 gene was amplified. PCR was carried out in a total volume of 15 µl containing 7.4 µl milli Q water, 3 µl of 1X PCR buffer (Sigma), 0.2 µl of 200 µM dNTPs (Sigma), 1.0 µl of each primer (5 pmol/ µl), 0.2 µl of Taq polymerase (5U/ µl), 1.2 ml magnesium chloride (2.0 mM) and1.0 ml of the template DNA was directly added into the cocktail in each lane of the PCR plate.

The PCR was carried out in Bio-Rad thermocycler (Table 2). The PCR protocol involved an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation (94°C for 45 sec), annealing (56°C for 45 sec) and extension (72°C for 60 sec) proceeded by one cycle of final extension (72°C for 10 min) (Table 3).

Amplification of the targeted region in CD18 gene was validated through 0.6% agarose gel electrophoresis. 8.0 µl of the PCR product was subjected to restriction digestion with 4 units of *Taq I* (Sib Enzyme, Russia) restriction enzyme in incubator for 5 hours at 65°C (Table 4). The digested products were evaluated in 2% agarose gel electrophoresis and ethidium bromide staining, and were classified as BLAD-free (homozygous

for the normal allele), BLAD-carrier (heterozygous) and BLAD-affected (homozygous, D128G mutation) respectively.

PCR products were sequenced directly by dye terminator cycle sequence procedure on an ABI PRISM DNA sequencer. The gene frequency of the normal animals, carriers and affected for BLAD were calculated based on the Hardy-Weinberg principle.

**Results and Discussion**

The size of PCR product was 367 bp and it was subjected to RFLP analysis using *Taq I* restriction enzyme. In normal calves, size of the PCR product yielded two fragments of size 313 bp and 54 bp (Figure 1, Lane 1-5, 7-13, 15-33, 35-54), whereas, in carrier and affected calves (recessive homozygous), three fragments of size 367 bp, 313 bp, 54 bp (Figure 1, Lane 14 and 34) and only one fragment of 367 bp (Figure 1, Lane 6), respectively.

PCR products of size 367 bp were used for DNA sequencing to insure the point mutation (A to G) in affected and carrier animals. The point mutation had detected in all BLAD affected and carrier animals (Figure 2, 3 and 4).


**Fig.1.** Electrophoretic pattern of PCR products and the three genotypes of bovine CD18 gene. Lane UC is the Un-Cut PCR product. Lane M is the 100 bp marker. Lane 6 is homozygous recessive genotype. Lane 14 and 34 are heterozygous genotype. Remaining lines are homozygous wild genotypes.

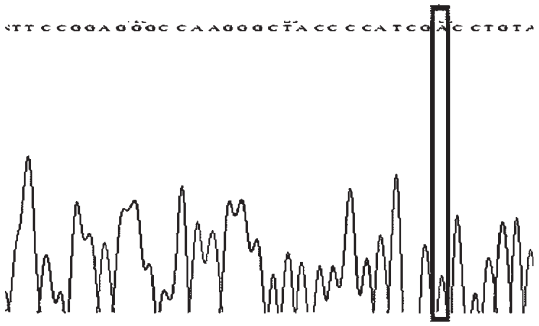

**Fig. 2.** PCR product sequence of animal normal for BLAD. At nucleotide position 1200, 'A' is present in normal animals

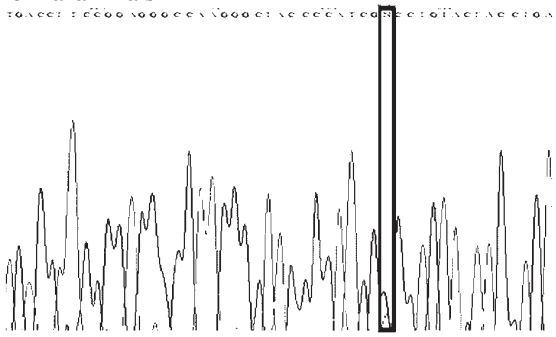

**Fig. 3**. PCR product sequence of animal carrier for BLAD. Point mutation from A to G at nucleotide position 1200 is recognized in one sequence.

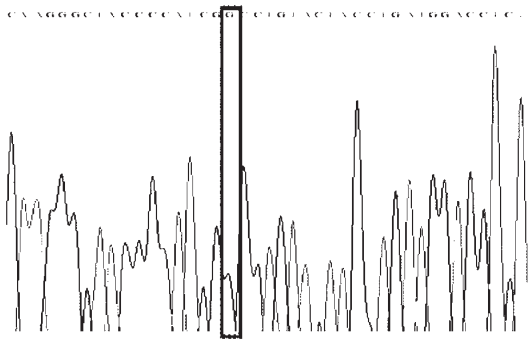

**Fig.4.** PCR product sequence of animal affected with BLAD. Point mutation from A to G at nucleotide position 1200 is recognized in both sequences.

In our investigation, out of 55 bull calves, one animal was found homozygous recessive and two animals heterozygous for BLAD. The frequency of carrier and homozygous recessive animals were 3.64% and 1.82% respectively. The estimated frequencies of BLAD dominant and recessive alleles in the KF young bull calves population that we screened were 0.964 and 0.036 respectively (Table 5).

Development of artificial insemination enabled the advent of modern breeding practices in the universe. These practices involve importation of HF bulls or their semen, intense selection of bulls based on their daughters lactation performance and the widespread use of these few genetically superior bulls. During the last three decades, these practices have not only augmented the milk production potential of HF cows but also the within breed genetic relationship among individuals. This increased relatedness is conducive for the expression of recessive genetic diseases. This has made the screening a mandatory practice for autosomal recessive disorders in farm-born HF and its crossbreds prior to their introduction into the breeding programs. Even though the initial incidence of BLAD is low, number of carriers could be substantially higher in coming days if the animals are not screened routinely for BLAD. Spread of undesirable genetic disorder is hastened by the artificial insemination program. Therefore, vigilance is required to diminish the risk of dissemination of recessive defects resulting from increased selection pressure within the dairy industry, which is presently dominated by HF breed worldwide.

In India, Muraleedharan *et al*. (19), Patel *et al*. (20), Mahdi *et al*. (21) and Kumar V. (22) have reported the carrier animal's frequency of 1.33%, 3.23%, 7.31% and 21.82% in Holstein animals and its crosses respectively. The incidence of BLAD carriers among top sires was found to be 23 % in USA (2), 10% in France (23), 13.5 % in Germany (24), 2.88 % in Argentina (25), 16 % in Japan (26), 2.8 % in Brazil (27) and 3.33% in Iran (28). In our present investigation the frequency of carrier and homozygous recessive animals were found as 3.636% and 1.818% respectively.

This research on recessively inherited genetic disorder will bolster an attempt to select animals using molecular markers. Routine and obligatory screening of HF animals contained the rapid spread of BLAD in several countries. Restricted breeding and long-term investigations have enabled a great reduction of this threat to the population. The result of our study has signified the considerable decrease in the number of identified D128G carriers in the population. An integration of international and national associations of Holstein cattle breeders with the genetic defect discovery programs is necessary to decrease the number of carriers and affected animals at a faster rate. Such actions will instill the confidence in international exchange of superior bull semen and in containing the local cattle population from being affected with the genetic defects. These actions will make the animal husbandry an economical venture as the losses incurred are reduced.

## Conclusion

Research on recessively inherited genetic disorder will definitely endorse the selection of animals using molecular markers. Severity of hereditary disorders like BLAD on the productivity of herd could be mitigated by stringent adoption of the following actions. (i) An accurate maintenance of the pedigree of all cows for sire and maternal grandsire. (ii) Data for the status of BLAD in the sire and maternal grandsire of each cow in the herd should be maintained. (iii) Selection index should be employed to identify the sires that are likely to be used for the next three months in the herd. (iv) Use of semen from BLAD carrier animals should be avoided on any

cow whose sire or maternal grandsire is a carrier. (v) Modern genetic tools such as molecular markers would be used to identify undesirable genes causing BLAD and to eliminate them in a rapid and efficient manner. The present findings and eradication of the BLAD carriers are of great significance in India for animal improvement programs in making the animal and dairy industry as an economical endeavor. This also calls for screening of other population of HF and its crosses in the country.

**Acknowledgement**

The authors are thankful to all the supportive scientists and colleagues at DCB Division, NDRI and National Bureau of Animal Genetic Resources (NBAGR), Karnal, India for their invaluable inputs towards this research.

**Table 1.** Primers for amplification of CD18 gene

| Primer Name | Primer Sequence (5' – 3') | Tm | GC Content | Primer Length (bp) | Nucleotide Covered (bp) | Product Length (bp) |
|---|---|---|---|---|---|---|
| CD1 F | AGGTCAGGCAGTTGCCTTCAA | 59.3ºC | 52.3% | 21 | 1144 to | 367 |
| CDI R | GGGGAGCACCGTCTTGTCCAC | 62.6ºC | 66.6% | 21 | 1510 | |

**Table 2.** The cocktail for one reaction of PCR for amplification of CD18 gene

| Component | *Stock* | *Volume/reaction* | *Conc. /Reaction* |
|---|---|---|---|
| 10X PCR Buffer | 5X | 3 ml | 1X |
| dNTPs | 100 mM | 0.2 ml | 200mM |
| Primer (Forward) | 50 pmol/ml | 1.0 ml | 5 pmol/ml |
| Primer (Reverse) | 50 pmol/ml | 1.0 ml | 5 pmol/ml |
| Taq Polymerase | 5 U/ml | 0.2 ml | 1U/reaction |
| Magnesium chloride | 25mM | 1.2ml | 2.0mM |
| Milli Q water | | 7.4ml | |
| Total | | 14.0ml | |

**Table 3**. PCR Program used for amplification of CD18 gene

| Serial Number | Steps | Temperature | Time | Number of cycles |
|---|---|---|---|---|
| I | Initial Denaturation | 95 ºC | 5 min | 1 |
| II | Final Denaturation | 94 ºC | 45 sec | 35 |
| | Annealing | 56 ºC | 45 sec | |
| | Extension | 72 ºC | 1 min | |
| III | Final Extension | 72 ºC | 10 min | 1 |
| IV | Hold | 10 ºC | 8 | 1 |

**Table 4.** Reaction Mixture for Restriction Digestion of PCR Product (CD18 gene)

| Component | Stock | Volume/reaction | *Conc. /Reaction* |
|---|---|---|---|
| PCR Product | 8.0ml | | |
| Digestion buffer | 10 X | 1.0ml | 1 X |
| BSA | 100 X | 0.02ml | 0.2 X |
| Restriction endonuclease | 20U/ml | 0.2ml | 4U/Reaction |
| Milli Q water | 0.78ml | | |
| Total | 10ml | | |

**Table 5.** Gene and Genotypic frequency for BLAD

| Animals Screened Carrier | Animals Affected | Carrier Animals | Gene Freq. (p) | Gene Freq. (q) | Carrier Genotypic Freq. | Affected Genotypic Freq. |
|---|---|---|---|---|---|---|
| 55 | 1 | 2 | 0.964 | 0.0364 | 3.636% | 1.818% |

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Current Trends in Biotechnology and Pharmacy
Vol. 4 (4) 908-916 October 2010. ISSN 0973-8916


# Metabolism and Glucose Tolerance Factor Activity of Synthetic Amino acid- Chromium Complexes in Yeast

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**Abstract**

The Glucose Tolerance Factor (GTF), a chromium containing complex, has been isolated from yeast by Schwartz & Mertz. This was instrumental in the recognition of the role of trivalent chromium, complexed to amino acids, in regulation of glucose metabolism and of active GTF as a potential antidiabetic agent. We report herein on newly devised simple, synthetic procedures for soluble binary AA-Cr (1:1) complexes of known structure(s) and on their activities in *S.cerevisiae*. Lys.Cr permits post exponential growth of yeast and prevents apoptosis, unlike free lysine which inhibits post exponential growth and cell viability. Phe.Cr is a better sole carbon source for yeast than the free amino acid and complexation, in this case, enhances the rate of metabolism of its carbon skeleton. In addition, Phe.Cr as well as Gln.Cr, but not Lys.Cr, exhibit considerable GTF-like activity in yeast. The results obtained suggest that soluble AA.Cr are good model compounds for exploring structure activity correlations that determine the role of trivalent chromium in cellular metabolism.

**Keywords:** GTF (Glucose Tolerance Factor), Amino acid-Chromium complexes (AA.Cr), ligands, Metabolism, *Saccharomyces cerevisiae.*

## Introduction

The Glucose Tolerance Factor (GTF) was isolated from Brewer's yeast (1) as a water soluble complex containing ~ 5-6% trivalent chromium and glycine, glutamic acid and cysteine as well as nicotinic acid as putative ligands (2). A material claimed to be similar to the isolate in many aspects was also synthesized by Toepfer et al. (2) by refluxing chromium acetate with these amino acids as well as nicotinic acid. However, the exact nature of the complexes remains unknown in both cases. Several researchers like Galuszka et al., (3) have synthesized different $AA_n$.Cr (where n=2 or 3 and AA=Amino acid) by classical organic synthetic procedures, devised by Ley and Ficken in 1912 (4). The products, although non toxic, were poorly soluble, and their biological activity is unknown.

Davis and Vincent (5) isolated, from animal tissues, a GTF-active oligopeptide (Low Molecular weight Chromium Binding material) or LMWCr (6), which contained aspartic acid in place of the glutamic acid in GTF and no nicotinic acid. The first synthetic Amino acid-Chromium complex with GTF-activity $(D\text{-}Phe)_3$.Cr, was prepared by Yang et al (7). However, this poorly soluble complex was rather unusual in that the Amino acid is the D-isomer and phenylalanine has not been considered earlier as a putative ligand in either GTF or LMWCr.

More recently, we (8) had synthesized a soluble Lys.Cr by reacting chromium sulphate with an equimolar amount of L-Lysine under mild,

near physiological conditions akin to those that yeast utilizes to elaborate GTF. This Lys.Cr is a binary(1:1) complex and has been characterized by physicochemical techniques. *S.cerevisiae* was found to metabolize Lys.Cr very differently from the manner in which free lysine was utilized, but Lys.Cr added to metabolizing yeast cells had no effect on their glucose metabolism.

The synthetic procedure used by us for Lys.Cr (8), has now been developed into a rapid, mild and general preparative procedure for binary AA.Cr complexes. Metabolic effects of Gln.Cr, Phe.Cr and Lys-Cr in yeast have been studied. The observed strong GTF like activity of Gln.Cr and Phe.Cr in *S.cerevisiae* is emphasized in view of the utility of such AA.Cr as potential auxiliary therapeutic agents. Structural characteristics of different AA.Cr complexes that determine biological activity of chromium are discussed.

## Material and Methods

***Synthesis of binary (1:1) Amino acid-Chromium complexes (AA.Cr) :*** AA.Cr complexes were synthesized by reacting AA (Phe. Gln, or Lys) with chromium sulfate hexahydrate $[Cr_2(SO_4)_3].6H_2O$. All chemicals were of AR grade and products of Merck.

Amino acid and $Cr_2(SO_4)_3.6H_2O$, (500 µmoles each in total volume of 6.0 ml)were reacted in a thermostated water bath at 45±1$^0$C, for a period of upto 60 min. at a pH 4.0±0.1. Routinely, complex formation was monitored in a Klett-Type Elico colorimeter with a 57 filter (550-600nm) and designated as "$A^{570}$".

***Kinetics of AA.Cr formation :*** Kinetics of AA.Cr formation was monitored by following increase in $ÄA^{570}$ ($=A^{570}_{AA.Cr} - A^{570}_{Cr}$) with time under the reaction conditions. From the graphical plots obtained, $t_{½}$ for formation was obtained.

***Stoicheiometry of formation of AA.Cr complexes: Job's plot :*** The continuous variation method of Job (9) was modified herein by using a constant concentration of $Cr^{3+}$ (500 µmoles) and varying the amino acid concentration (0-1200 µmoles), in a total volume of 6.0 ml. Following completion of complexation, $ÄA^{570}$ was plotted against increasing amino acid concentration. The mole ratio of AA:Cr in the complex was derived from the graphical plots.

***Spectral features of AA.Cr complexes (UV/Vis,-IR) :*** UV/Vis spectra of the AA.Cr complexes were measured in a U-2800 Beckman spectrophotometer over the wavelength range 220-800nm. For IR spectra, recrystallized amino acid-chromium complexes were dried to constant weight over phosphorous pentoxide in a vacuum dessicator. The sample (1.0-1.5mg) was mixed thoroughly with KBr in a (1:100) ratio, pelleted in a KBr press under 6 tonnes pressure and the pellet scanned in a Fourier Transform Thermo Nicollet Nexus 670 Spectrophotometer (resolution 4$cm^{-1}$) over the range of 4000$cm^{-1}$ to 400$cm^{-1}$.

***Thin Layer Chromatography (TLC) of AA.Cr complexes :*** TLC plates (15cm x 9cm) were prepared by suspending silica gel (40 g) in sodium carbonate (1mM, 90ml) and spreading the mix on thin layer plates as per standard techniques. The plates were air dried and activated at 110ºC for 1h just prior to a chromatographic run. Recrystallized complexes in aqueous solution (50 µg) were spotted in ~ 20-40 µl on to the TLC plates along with amino acid standards as well as $Cr^{3+}$ complexes separately. The solvent system used was butanol: pyridine: water (1:1:1) with running time of ~ 2h. Visualization of AA.Cr and free AA was by ninhydrin spraying.

***Yeast growth experiments :*** *Saccharomyces cerevisiae* (NCIM 3558) was routinely maintained on a medium containing yeast extract

(1%), peptone (2%),and dextrose(2%) in Agar slants (2%) by weekly transfers.

In growth experiments, yeast was grown generally on a complete basal minimal salts medium (CBM) described by Watson (10).This also contained ammonium sulphate (2mM) and glucose(1%).

For studying post exponential growth (11) for which a suboptimal level of nitrogen in the medium is mandatory, the growth medium contained 0.5mM $NH_4^+$.

When metabolism of AA-Cr was examined by using them as either Nitrogen N, Carbon C or (N+C) sources for yeast ,based on preliminary experiments, each AA-Cr was used at a level of 10mM in place of $NH_4^+$ or glucose or both, respectively in the CBM mentioned above; invariably AA-Cr was separately sterilized in such cases and added aseptically to the rest of the medium which had also been presterilized.

When glucose utilization was to be determined during growth, at the required time, culture flasks were gently shaken so that a uniform cell suspension was achieved, following which aliquots (1.0ml) were withdrawn and their absorbance($A^{660}$) measured. Cells were pelleted out by centrifugation at 0ºC.From the clear supernatant obtained, glucose present was estimated (12).

***Post exponential growth :*** In experiments involving post exponential (PE) growth (11), the following procedures were employed. Yeast was grown for 24 h (20 ml CBM in 100 ml conical flasks) to the exponential phase. Growth was measured and the entire culture was centrifuged under sterile conditions and the supernatant medium was discarded.

The yeast cells were then re-suspended in a known volume of growth medium limiting in nitrogen (0.5 mM $NH_4^+$) and transferred to fresh culture flasks containing the rest of the volume, other media constituents (to make to 20 ml) also containing lysine or Lys-Cr as required. Growth was thereafter followed up to 144h. All operations were performed aseptically, in a laminar flow chamber.

## Results

***Synthesis of binary (1:1) AA.Cr complexes :*** The rather insoluble $(AA)_n$-Cr (n=2 or 3) complexes, made by organic synthetic procedures based on those first employed by Ley and Ficken (4) are quite different from the water soluble, bioactive chromium complexes such as GTF and LMWCr. The procedures developed herein are much milder and designed to be analogous to conditions for binary AA.Cr complex formation first devised for Lys.Cr (8) (These have been examined by varying time, temperature, pH etc. and the conditions finalized herein are the minimal optimal ones). AA.Cr with AA being phenylalanine, lysine, glutamine, aspartic acid, asparagine or glycine have been successfully made accordingly. The data for Phe.Cr, Gly.Cr, and Gln.Cr have been presented herein as typical examples. Fig 1 shows the kinetics of formation of Phe.Cr (Phe:$Cr^{3+}$ =1:1) and its $t_{1/2}$ is seen to be around 6-7 min. $t_{1/2}$ was found to vary from 6-40 min for different amino acids.

During Phe.Cr formation it was found, for the first time herein, that concomitant with complexation, the aromatic absorption ( $ë_{max}$=257 nm ) of this amino acid was lost leaving only end absorption. Consequently the time course of this loss of absorption has been examined and is shown in Fig. 2. In conformity with the data of Fig 1, $t_{1/2}$ derived from abolitition of UV absorbance is also 6 min. The UV/Vis spectrum of Phe.Cr is displayed in Fig 3.The $ë_{max}$ of Phe.Cr is 540nm.

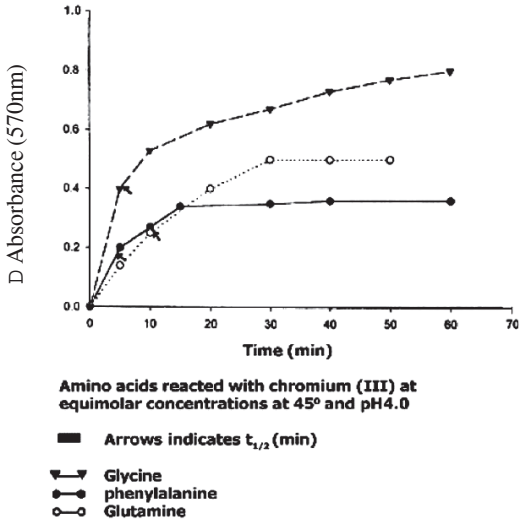



**Fig. 1.** Kinetics of formation of binary aminoacid chromium (III) complexes.

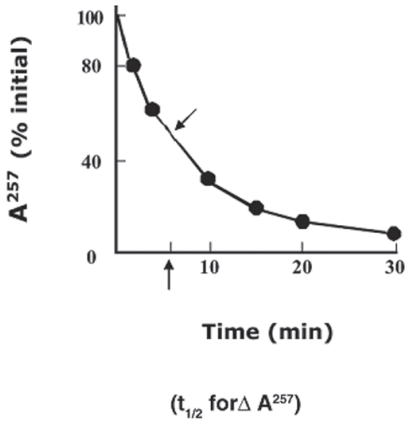



**Fig. 2** Kinetics of Loss of UV absorbance During formation of Phe.Cr

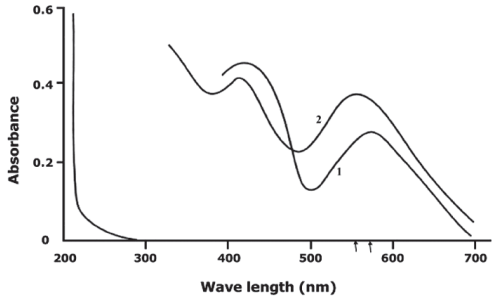



**Fig. 3.** Absorption Spectrum of Phenylalanine-$Cr^{3+}$ binary Complex (Phe.Cr)

To determine the stoicheiometry of AA.Cr formation, Job's plot (9) has been modified by keeping the concentration of the chromophoric $Cr^{3+}$ constant and observing increase in $_{D}A^{570}=(A^{570}_{AArCr} - A^{570}_{Cr})$ as a function of AA concentration. When such data are plotted for Phe.Cr (Fig 4), the stoicheiometry can be deduced to correspond to that for a binary (1:1) complex. Similar plots for synthesis of other AA.Cr, wherein the amino acid were, for example, glycine, aspartic acid, aspargine or glutamine also confirmed that

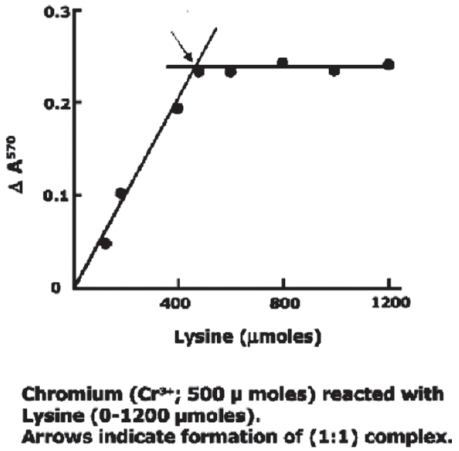



**Fig. 4.** Formation of Lysine-Cr (1:1) Complex (LysCr) : Job's Plot

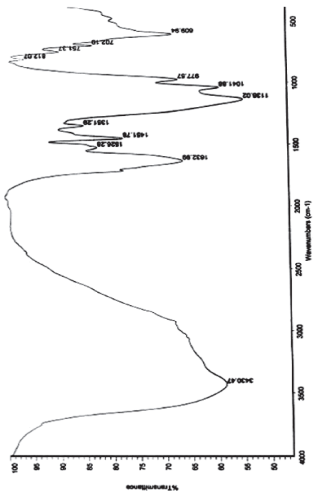


**Fig. 5.** IR Spectrum of Phe.Cr (Phenylalanine-Cr3+, 1:1 binary complex)

the procedure devised herein invariably leads to binary complexes.

An examination of the IR spectra revealed the nature of the bonding involved. In Fig 5,6 and 7 are presented the

IR spectra of Phe.Cr, and Gln.Cr. The relevant ë$_{max}$ in the visible region and characteristic IR peak frequencies are summarized in Table1. It can be seen that the õ$_{as}$(COO$^-$) vibrational peak, due to –O-CO-O-Cr is dominant in all complexes. The changes in the õ (S=O) vibrational frequencies for chromium sulphate to a single peak at ~1129cm$^{-1}$ for Lys.Cr and Gln.Cr (but not for Phe.Cr) indicate that in some AA.Cr. the sulphate is more strongly to bound to Cr$^{3+}$ than in others. The structures derived for AA.Cr are shown in Fig 7.

***GTF activity of AA-Cr in Yeast:*** The rate of glucose metabolism in *S.cerevisiae* (NCIM 3558) was first shown to be enhanced by 10mM Gln.Cr

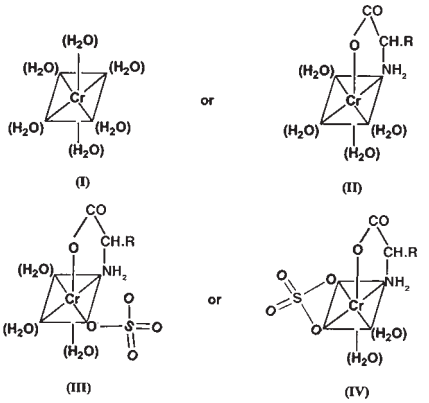



**Fig. 7.** Possible structures of Amino acid-Cr3+ complexes with co-ordination via carboxyl as well as sulfate (R. COOH = AA) (Based on IR Spectra)
I - Hexaaquo - Cr
In II, AA = Phenylalanine
In III & IV, AA = Lysine or Glutamine

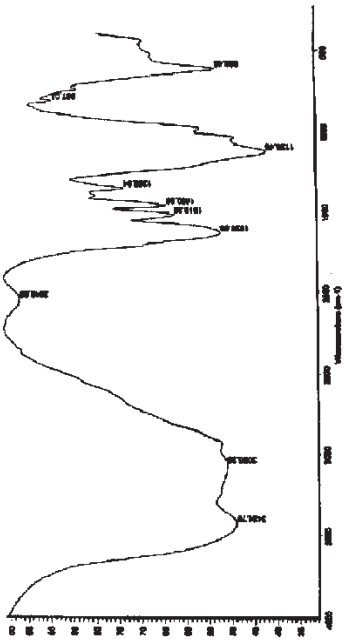


**Fig. 6.** IR spectrum of Lysine-Cr$^{3+}$ (1:1) binary complex

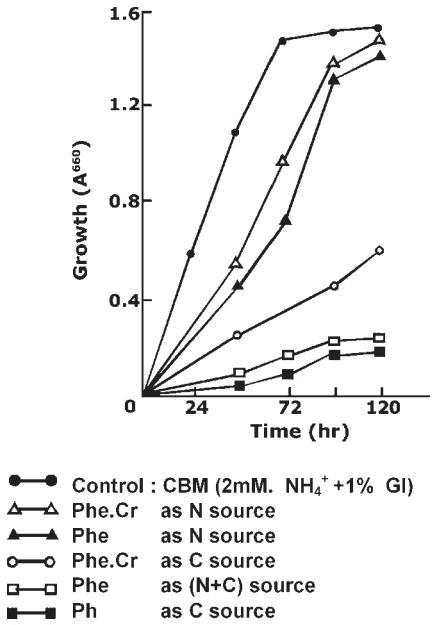
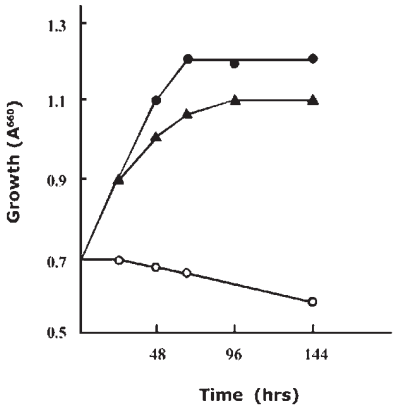



**Fig. 8.** Phenylalanine-Cr3+ utilization by *Saccharomyces cerevisiae* NCIM 3558.




**Pregrown, log phase cells of *S.cerevisiae* (24 hrs; $A^{660}$ = 0.70) grown on CBM, washed and resuspended in CBM (●—●) Lysine, 10mM (○—○) or Lys.Cr, 10mM (▲—▲) and growth followed for 144 hrs thereafter.**

**Fig.9.** Effects of Lysine and LysCr on Post exponential growth of *S.cerevisiae* NCIM 3558

or Phe.Cr, in our earlier studies (13 ). We therefore examined the minimal concentration of AA.Cr for affecting glucose metabolism in yeast and found that 1mM was adequate. When AA.Cr was also included, along with glucose (1%) in the medium, the time for metabolizing (disappearance) 50% of the glucose was as follows: Control (CBM), 100h; Lys.Cr 91 h; Phe.Cr 50h.and Gln.Cr, 34h.Variations in $t_{50}$ in replicates was no greater than 5-7% of the given values. Thus some binary AA.Cr possess remarkable GTF activity in Yeast

## Metabolism of AA.Cr in Yeast

The metabolism of Phe.Cr has also been examined by employing it as a N,C as well as (N+C) source for yeast. As the results in Fig.8 show, phenylalanine as well as Phe.Cr are equally good nitrogen sources. In contrast, phenylalanine is poorly metabolized as a sole "carbon" source and this becomes rate limiting as evident from growth on phenylalanine as a sole (N+C) source. With Phe.Cr, growth is much enhanced over that on phenylalanine alone.

A special feature of the metabolism of lysine in yeast is that lysine strongly inhibits post exponential (P.E.) growth of Yeast. The effect of Lys.Cr on P.E. growth has been studied. Fig. 9 presents the results obtained.

In S.cerevisiae (NCIM 3558), lysine is seen to powerfully inhibit P.E.growth and also lead to an actual decrease in turbidity of the culture, a consequence of cellular apoptosis. In contrast, Lys.Cr permits good P.E. growth and the toxic

**Table 1 :** Major characteristic vibrational IR frequencies of Chromium complexes and ligands

| Complex | 3500-2000* [uOH & $uNH_2$]... | 1650-1500 [$u_{as}$ (Coo)] | 1500-1400 | 1130-1000 $u_3$ [$SO_4$] | 600 [u (Cr-O)] | 500-400 u[Cr-N] |
|---|---|---|---|---|---|---|
| Phenylalanine-Cr | $3430^{S}$; $2929^{S}$ | $1633^{m}$ | $1451^{w}$ | $1136^{S}$; $1041^{S}$ | $609^{m}$ | $498^{w}$; $462^{w}$ |
| Phenylalanine | $3449^{W}$; $3087^{m}$; $2939^{m}$ | $1626^{w}$; $1561^{S}$ | - | - | - | - |
| Lysine-Cr | $3424^{S}$; $3065^{S}$; $2939^{m}$ | $1629^{m}$; $1518^{mw}$ | $1460^{w}$ | $1129^{S}$ | $609^{m}$ | $407^{w}$ |
| Lysine | $2929^{S}$ | $1585^{S}$; $1505^{S}$ | $1406^{w}$ | - | - | - |
| Glutamine-Cr | $3434^{S}$ | $1669^{S}$ | $1440^{m}$ | $1121^{S}$ | $617^{m}$ | $453^{w}$ |
| Glutamine | $3409^{S}$; $3052^{m}$ | $1672^{S}$; $1585^{S}$ | $1406^{m}$ | - | $615^{m}$ | - |

* Infra red frequencies in $Cm^{-1}$. Regions within which vibrational frequencies of groupings indicated in brackets (†) are found in the complexes examined.
[Strengths of vibrational bands : s-Strong; m-Medium; w-Weak]

effect of the free amino acid is offset by binding to $Cr^{3+}$.

## Discussion

The present investigation addresses three major aspects of the role of chromium in cellular metabolism:

(i) Synthesis and structure of water soluble, binary AA.Cr and their importance as model compounds: (ii) the GTF activity of AA.Cr and (iii) the metabolism of AA.Cr in Yeast.

***Synthesis and structure of AA-Cr :*** GTF as well as LMWCr are thought to contain $Cr^{3+}$ bound to amino acids but the structural features of the complexes present therein are unknown. To understand this aspect, it is necessary to synthesize complexes of known structure. In the synthetic procedure of Ley and Ficken (4 ), which has been generally followed ever since, glycine was reacted with chromium chloride in a 3:1 ratio, for 48h at $60^0$C. They obtained a red $(Gly)_3$ Cr. On prolonged boiling in water, this complex lost two amino acid residues to become a binary Gly.Cr. This indicates the greater stability of AA-Cr Over $(AA)_3$. Cr. Over the years, many researchers, likewise, generally reacted trivalent chromium with amino acids and ended up with rather insoluble $(AA)_n$.Cr.(n=2or 3). Under such conditions, the reactive chromium species would be an olated form with the structure

or polymers thereof. Such polynuclear forms predominate at pH > 6.0 with multiple linked chromium ions. It has been stressed by Galuszka et al., (3) that the nature of amino acid chromium complexes formed is dependent on the conditions employed and since bioactive chromium complexes are water soluble and stable, a completely different procedure has been developed herein. This is essentially based on the findings of Shuttleworth et al (14)., who reacted á, â and ã amino butyric acids with chromium sulphate under mild conditions and showed that AA.Cr type binary complexes do form; such formation was accompanied by a shift of the $ë_{max}$ from that of the $Cr^{3+}$ as well as an increase in $å_{max}$ (in the region of 540-570nm).

We have now utilized this absorbance increase for determining stoicheiometry by using a modified Job's plot and optimal conditions for binary (1:1) AA.Cr synthesis. The formation of Phe.Cr is accompanied by a parallel and progressive loss of absorbance of its aromatic peak ($ë_{max}$=257nm); from this also $t_{1/2}$ for formation was derived. to be 6-7', a value that is identical to that got from ?$A^{570}$, validating the procedure.

The use of chromium sulphate, in place of $CrCl_3$ used by earlier workers, as the source of $Cr^{3+}$ to form AA.Cr has been useful. Chromium sulphate has two õ (s=o) vibrational peaks at 1137 $cm^{-1}$ and at 1040 $cm^{-1.}$ These are found in Phe.Cr as well. In Lys.Cr and Gln.Cr, on the other hand, they are replaced by a single peak at 1129 $cm^{-1}$; clearly sulphate is structurally part of these two complexes but not of Phe.Cr. We may therefore designate them as "sulphato" complexes. However, whether the sulphate bonding is monodentate or bidentate is not clear since IR spectra cannot resolve this issue. (15, 16).

The reactive chromium species obtaining in these studies is hexaaquo chromium. Accordingly and from the physiochemical characteristics of these complexes, the structures of AA.Cr would be those shown in (Fig7). Galuszka et al., (3) have also suggested similar unidentate carboxyl binding for their monomeric complexes.

***GTF activity of AA.Cr :*** The first report of a synthetic amino acid chromium complex with GTF activity is that of $(D\text{-}Phe)_3.Cr$ synthesized by Yang et al., (7). However, this is not a binary complex of the AA-Cr (1:1) type which would be the simplest model compound. Our report (13) that 10mM .Gln.Cr shows powerful GTF-like activity in *S. cerevisiae* (NCIM 3558) is the very first report of this kind. We have now found that the minimal effective level is 1mM and Gln.Cr and Phe.Cr but not Lys.Cr considerably enhance the glucose utilization rate, with Gln.Cr. > Phe.Cr. Preliminary experiments have shown recently that Cys.Cr and Gly.Cr also enhance glucose metabolic rates by this Yeast strain and that all four AA.Cr are also active in rats in the critical test of GTF activity, namely the Oral Glucose Tolerance Test (OGTT). (Karthikeyan et al, unpublished) This leads to the hypothesis that the critical determinants of GTF activity are binary AA.Cr complexes.

***Metabolism of AA.Cr in Yeast:*** The first indication that AA.Cr can have hitherto unrecognized roles in metabolism has been the finding that lysine, which cannot be utilized as a nitrogen source by yeast is excellently metabolized, if complexed to trivalent chromium (8).

Present work reveals that such complexation also abolishes the inhibitory effect of lysine on P.E.growth of Yeast. The mechanism involved is unclear. However P.E. growth is known to be due to increase in cell mass due to synthesis of structural polysaccharides but without further cell division(11) and it is clear that Lys.Cr, although ineffective in GTF-like enhancement of rate of the rate of glycolysis, does stimulate the synthetic processes of P.E. growth. As regards Phe.Cr, results of the present study show that complexation of phenylalanine leads to GTF-like activity as well as improved rate of metabolism of its carbon skeleton although not of the amino-N of this amino acid.

Since little is known of the metabolism of binary AA.Cr complexes on yeast, these diverse effects brought to light by the studies herein emphasize that trivalent chromium profoundly modifies the metabolic properties and effects of amino acids to which it binds. In some cases this leads to GTF-active complexes. The finding of highly effective AA.Cr or combinations thereof would be of great importance in developing useful, auxiliary therapeutic agents for diabetes.

## Conclusion

A new, general procedure has been devised for the synthesis of binary Amino acid – Chromium complexes and their structures have been defined. The metabolic effects of the complexation of lysine are profound changes in the metabolism of the amino acid and its amino group. With phenylalanine, complex formation positively affects metabolism of its carbon skeleton. Chromium complexes of phenylalanine as well as glutamine exhibit GTF activity and enhance rate of glucose metabolism. The synthetic procedure developed will be useful for making chromium complexes that could be clinically important in regulating glucose metabolism in diabetes.

## Acknowledgement

The authors would like to thank the Dept. of Biotechnology, Govt. of India for financial support and for grant of Research Associateship to the first author. Dr. K.S. Karthikeyan would also like to thank his mother Mrs. K. Rajalaxmi Sastry for all her moral support & encouragement.

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Current Trends in Biotechnology and Pharmacy
Vol. 4 (4) 917-921 October 2010. ISSN 0973-8916


# Formulation and Evaluation of Transdermal Patches of Ondansetron Hydrochloride Using Various Polymers in Different Ratios

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**Abstract**

This paper describes the formulation and evaluation of transdermal patches of Ondansetron Hydrochloride. The films were formulated using different hydrophilic and lipophilic polymers combination of hydrophilic-lipophlic polymers, ethyl cellulose : poly vinyl pyrrolidine. The polymeric films of Ondansetron Hydrochloride were prepared using film casting technique on mercury substrate. The study is also extended to investigate the effect of plasticizer such as dibutyl phthalate and propylene glycol and effect of penetration enhancer oleic acid by using keshary-chein diffusion cell. The films were evaluated for physico-chemical properties. In vitro drug diffusion study through rat skin indicates hydrophilic polymer showed higher release than the liphophilic and hydrophilic-lipophilic combination. The release rate was found to follow first order kinetics. Also permeation enhancer was found to give favourable permeation enhancement.

**Key words:** Transdermal film, Ondansetron Hydrochloride, polymers, In vitro drug release, permeation enhancer.

## Introduction

Ondansetron is an antinauseant and antiemetic agent indicated for the prevention of nausea and vomiting associated with moderately-emetogenic cancer chemotherapy and for the prevention of postoperative nausea and vomiting.

The chemotherapeutic agents produce nausea and vomiting by releasing serotonin from the enterochromaffin cells of the small intestine, and that the released serotonin then activates 5-$HT_3$ receptors located on vagal efferents to initiate the vomiting reflex. Therefore Ondansetron HCl works by blocking the reception of serotonin at these 5-$HT_3$ receptors. Ondansetron HCl has the half-life of 5-6 hours. It's total bioavailability in the body is 60% due to first pass metabolism (1).

## Materials and Methods

Ondansetron HCl was procured as a gift sample from Sun Rise pharma Ltd., ethyl cellulose pharm grade (colorcon Goa), eudragit LR (Evonik pharma Germany), Dibutyl phthalate LR (Ranbaxy fine chemicals Ltd., New Delhi.), oleic acid (Ranbaxy fine chemicals Ltd., New Delhi), Chloroform LR ( Merck Ltd., Mumbai), Methanol LR (Rankem, fine chemicals limited, Mumbai), poly vinyl pyrrolidine, poly vinyl alcohol (Hi-media), backing membrane gift sample from Hi-media.

***Preparation of Matrix- Type Transdermal Films containing Ondansetron HCl :*** The transdermal films were prepared by solvent evaporation method using different ratio of polymers like PVA, PVP, EC, and EUDRAGIT RS and RL . The polymers in different ratio were dissolved in different solvents such as methanol, water and chloroform respectively. Solutions were prepared at room temperature using plasticizer PG(10%) for PVP:PVA combination, DBP(5%) for Eudragit RS:RL and EC:PVP combination respectively. The drug was added to polymeric solution and stirred on the magnetic stirrer to obtain uniform solution (Table. 1). Oleic acid (10%) was used as penetration enhancer. The total volume of the solution was 20 ml. Then solution was poured with help of volumetric pipette in a petri dish having surface area of 78.5 $cm^2$ . The rate of evaporation of solvent was controlled by inverting cup funnel. The dried films were stored in desiccators. Since the drug is slightly soluble in chloroform, drug was added in small portions to the solvent with continuous and vigorous stirring (2, 3).

***Characterization of Transdermal films :*** The films were evaluated for physical appearance, moisture content, thickness, folding endurance, tensile strength and drug content.

***Thickness :*** The thickness of films was measured by digital Vernier caliper with least count 0.001mm (Table. 2). The thickness uniformity was measured at five different sites and average of five readings was taken with standard deviation (4).

***Tensile strength :*** The drug matrix film was fixed to assembly the weights required to break the film was noticed, and simultaneously film elongation was measured with help of a pointer mounted on assembly and calculated tensile strength of drug reservoir film using following formula (5).

$$TS = \text{break force} / a.b(1+L / I)$$

Where a, b and L are the width, thickness, and length of the film and I is the elongation of film at breaking point (Table. 3).

***Folding endurance :*** This was determined by repeatedly folding on film at the same place till it broke (Table. 2). The number of times the film could be folded at same place without breaking/ cracking (6).

***Weight variation :*** The three disks of 2X2 $cm^2$ was cut and weighed on electronic balance for weight variation test (Table. 2). The test was done to check the uniformity of weight and thus check the batch- to- batch variation (7).

***Moisture uptake :*** The percent moisture absorption test was carried out to check the physical stability and integrity of the films at high humid conditions (2).

The films were placed in the dessicator containing saturated solution of aluminium chloride, keeping the humidity inside the dessicator at 79.5 % R.H. After 3 days the films were taken and weighed the percentage moisture absorption of the films was found (Table. 3).

***Drug content :*** The patch of area 2X2 $cm^2$ was cut and dissolved in the mixture of methanol and 0.1N HCl and then the distilled water was added to make the volume upto 100 ml. Then 1 ml was withdrawn from the solution and diluted to 10 ml(Table. 2). The absorbance of the solution was taken at 303.5nm and concentration was calculated. By correcting dilution factor, the drug content was calculated (8).

***In Vitro Skin Permeation Studies :*** *In vitro* skin permeation studies were performed by using a Keshary - Chein diffusion cell with a receptor

Current Trends in Biotechnology and Pharmacy
Vol. 4 (4) 917-921 October 2010. ISSN 0973-8916



compartment capacity of 20 ml. The excised rat abdominal skin was mounted between the donor and receptor compartment of the diffusion cell (9). The formulated patches were placed over the skin and covered with paraffin film. The receptor compartment of the diffusion cell was filled with phosphate buffer pH 7.4. The whole assembly was fixed on a magnetic stirrer, and the solution in the receptor compartment was constantly and continuously stirred using magnetic beads at 50 rpm; the temperature was maintained at 32 ± 0.5°C. The samples were withdrawn at different time intervals and analyzed for drug content by U V spectrophotometer. The receptor compartment volume was replenished with an equal volume of phosphate buffer at each sample withdrawal. The cumulative amounts of drug permeated per square centimeter of patches were plotted against time (7).

**Results and Discussion**

In the view of low permeability of Ondansetron HCl, monolithic device of drug has been attempted. The films were studied for the thickness, weight variation, tensile strength, drug content, in-vitro drug diffusion (Table. 2).

Study shows that for PVA: PVP, Eudragit RS: RL and EC : PVP along with the plasticizer PG 10% w/w, DBP 5% w/w of polymer weight was suitable for good flexibility, clarity and elasticity. The weight of all the six formulations was found to be in the range of 65.24 to 67.05 mg. Thickness variation was found to be 0.025 to 0.48 mm, tensile strength was found to be between 0.58 - 0.75 (Table. 3).

The formulation F1 comprising of PVA:PVP (5:5), oleic acid 10% and propylene glycol 10% showed tensile strength of 0.75 and % elongation upto 15.25%. The moisture absorption for formulation F1 was 4.5%, whereas for the formulation F5 it was 3.5%. The % drug content for all the formulations was found to be in the range of 92.41 ± 0.1% – 95.9± 0.4 %.

On studying the in-vitro diffusion of drug through the rat skin using keshary chein cell, the drug diffused for formulation F1 was maximum. The drug diffused was 76.69%. The permeability coefficient for the formulations F1 to F6 was 50.75, 43.85, 29.84, 29.17, 32.48, 31.97 μg/cm$^2$/h respectively (Table. 4).

In order to understand mechanism of release, in vitro release data were treated to the models and linearity was observed with respect to Higuchi equation. The correlation coefficient obtained from Higuchi plot was found to be 0.960 to 0.984 (Fig. 2). This indicates that mechanism of drug release was diffusion type (Fig. 1). The

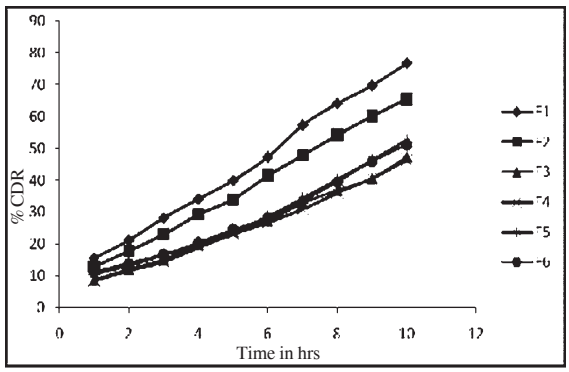


**Fig. 1.** Plot of time v/s % CDR for all six formulations

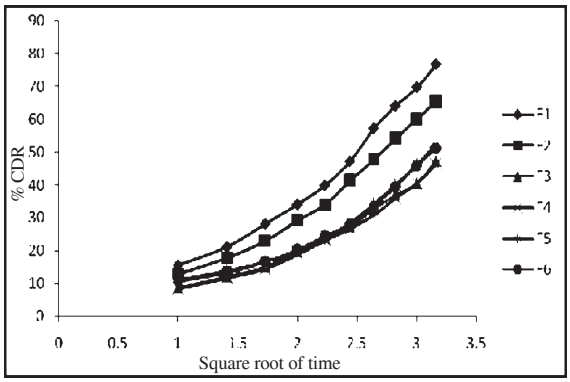


**Fig. 2.** Higuchi plot for all six formulations

**Table 1.** Formulation table

| Formulation | Polymer PVA:PVP | Polymer RLPM:RSPM | Polymer EC:PVP | Plasticizer | Oleic acid | Solvent |
|---|---|---|---|---|---|---|
| F1 | 5:5 | - | - | PG (10%) | 10% | Water |
| F2 | 3:7 | - | - | PG (10%) | 10% | Water |
| F3 | - | 5:5 | - | DBP (5%) | 10% | Acetone |
| F4 | - | 7:3 | - | DBP (5%) | 10% | Acetone |
| F5 | - | - | 8:2 | DBP (5%) | 10% | Chloroform |
| F6 | - | - | 5:5 | DBP (5%) | 10% | Chloroform |

**Table 2.** Thickness, Weight Variation, Drug Content, Folding endurance values

| **Formulation code ± SD** | **Thickness (mm) (mg)** | **Weight variation ± SD** | **% Drug content ± SD** | **Folding endurance ± SD** |
|---|---|---|---|---|
| OND 1 | 0.036 ± 1.2 | 65.24 ± 1.2 | 92.41 ± 0.1 | 78 ± 2 |
| OND 2 | 0.032 ± 1.5 | 62.50 ± 1.8 | 94.28 ± 0.5 | 76 ±1 |
| OND 3 | 0.045 ± 1.8 | 67.05 ± 1.8 | 95.03 ± 0.2 | 79 ± 2 |
| OND 4 | 0.048 ± 1.3 | 66.55 ±1.8 | 95.9 ± 0.4 | 77 ± 1 |
| OND 5 | 0.025 ± 1.4 | 66.89 ±1.9 | 93.66 ± 0.5 | 72 ± 1 |
| OND 6 | 0.029 ± 1.6 | 65.05 ±1.6 | 94.16 ± 0.6 | 71 ± 0.9 |

**Table 3.** Tensile Strength, % elongation, %moisture absorption values

| **Formulation** | **Tensile Strength** | **% Elongation** | **% moisture absorption** |
|---|---|---|---|
| **F1** | 0.75 | 15.25 % | 4.5 % |
| **F2** | 0.73 | 20.54 % | 4.8 % |
| **F3** | 0.68 | 22.89 % | 5.07 % |
| **F4** | 0.70 | 23.86 % | 5.18 % |
| **F5** | 0.61 | 30.5 % | 3.5 % |
| **F6** | 0.58 | 29.56 % | 3.9 % |

drug diffusion was non-fickian. On plotting square root of time v/s % CDR the regression co-efficient value obtained for the formulation F1 was 0.983.

## Conclusion

The Ondansetron HCl transdermal patches developed in this study have great utility and are viable option for effective and controlled

**Table 4.** Cumulative % Drug release

| Time in hrs | Formulations | | | | | |
|---|---|---|---|---|---|---|
| | F1 | F2 | F3 | F4 | F5 | F6 |
| 1 | 15.46 | 12.70 | 8.74 | 8.40 | 10.53 | 11.20 |
| 2 | 21.10 | 17.74 | 12.11 | 11.76 | 13.24 | 13.91 |
| 3 | 28.10 | 22.88 | 15.07 | 14.55 | 16.46 | 16.71 |
| 4 | 34.02 | 29.18 | 19.88 | 18.92 | 19.88 | 20.34 |
| 5 | 39.85 | 33.99 | 23.96 | 23.17 | 23.89 | 24.50 |
| 6 | 47.21 | 41.40 | 27.24 | 26.78 | 28.67 | 28.17 |
| 7 | 57.23 | 47.78 | 32.74 | 30.95 | 34.35 | 33.37 |
| 8 | 64.04 | 54.20 | 36.90 | 36.13 | 40.24 | 39.46 |
| 9 | 69.71 | 60.21 | 40.57 | 40.65 | 46.34 | 45.85 |
| 10 | 76.69 | 65.52 | 47.40 | 46.46 | 52.69 | 51.13 |

management nausea and ometing. However, pharmacodynamic and pharmacokinetic evaluation of these systems in human volunteers is necessary to confirm these findings.

**Acknowledgements**

Thanks to Sun rise Pharma for providing gift sample of Ondansetron HCl.

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Current Trends in Biotechnology and Pharmacy
Vol. 4 (4) 922-929 October 2010. ISSN 0973-8916


# An efficient protocol for shoot bud induction and regeneration of *Tylophora indica*, an endangered medicinal plant

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## Abstract

The efficiency of Marashige and Skoog (MS), Linsmaier and Skoog (LS), B5 and L6 media on shoot bud induction was evaluated with explants such as leaf, shoot and root of *Tylophora indica* in the presence of different concentrations (0.1-10 mg/l) of 6- benzylaminopurine (BAP) or kinetin (KN) administered individually or in combination with auxins. Among the four media and three explants employed, MS medium followed by LS, B5 and L6 (MS>LS>B5>L6) and leaf followed by shoot and root (leaf > shoot > root) resulted in higher frequency (90%) of shoot bud formation and maximum number of mean shoot buds (16.2). Among the auxins supplemented either individually or in combinations 2, 4-dichlorophenoxyaceticacid (2,4-D, 0.1 mg/l) in combination with BAP (2.0 mg/l) produced maximum number of shoot buds with leaf explants. The shoot buds were sub-cultured on MS medium supplemented with low levels (0.01-0.05 mg/l) of BAP which resulted in shoot elongation. When shoots were grown on MS basal medium supplemented with IAA (0.5 mg/l) resulted in rhizogenesis and root growth. Whole plants were transferred to pots successfully with 90 % survival rate

**Keywords**: Explants, plant growth regulators, shoot bud induction

## Introduction

*Tylophora indica*, a twining perennial herb is distributed in forests and hilly places of eastern and southern parts of India. Different parts of this indigenous medicinal plant have been traditionally used as folk medicine to treat asthma, bronchitis, whooping cough, dysentery and diarrhea (1, 2). Roots possess stimulant, emetic, cathartic, expectorant, stomachic, diaphoretic, bacteriostatic and antifeedant properties (3, 4). Roots and leaves are reported as good natural preservatives of food and used in the treatment of rheumatic and gouty pains (5-7). Leaves and roots have been employed as a substitute for ipecacuanha. *Tylophora* extracts are noted for significant anti-inflammatory and anticancer activity (8). The dried leaves are reported to be more uniform and certain in their action than roots. Owing to unscrupulous destruction of plant habitats, illegal and indiscriminate collection of plants from natural habitats and lack of proper awareness and cultivation practices, many medicinal plants including *T. indica* are listed among endangered species. Efficient biotechnological methods of culturing plant cells and tissues should provide new means of conserving and rapidly propagating valuable, vulnerable and endangered plant species. Although

micropropagation of *T.indica* through callus regeneration, axillary bud proliferation and somatic embryogenesis from leaf explants have been previously reported, these methods demonstrated either low number of shoots or low frequency of regeneration (9, 10).

In view of the paramount importance of *Tylophora indica* as one of the principle sources of drugs used in various alternate systems of medicine in addition to being included in the list of endangered plants, and considering the fact that there is paucity of information on efficient means of plant regeneration of *Tylophora* through shoot bud induction, the present study was conceived. Shoot bud induction potential of three explants and four media supplemented with an array of concentrations of auxins (2,4-D, IAA, NAA) and cytokinins (BAP, KN) in individual as well as in combination of treatments was evaluated. Our work demonstrated that leaf explants on MS medium supplemented with BAP (1.0-2.0 mg $L^{-1}$) supported high frequency of regeneration and 90 % survival upon transfer to field conditions.

## Materials and Methods

***Preparation of media:*** Four different media, viz., Marashige and Skoog, (11), Linsmaier and Skoog, (12), B5 (13) and L6 (14) were employed in the present study. Stock solutions of micronutrients, vitamins and plant growth regulators employed in the study were prepared separately and stored for further use. pH of the medium was adjusted to 5.7-5.8, agar (8 g/l) was dissolved, dispensed into culture tubes, autoclaved at 15 lbs/square inch for 20 min (120 $^{o}$C) and left in a cool chamber for further inoculations of explants.

***Explant selection, sterilization and culture conditions :*** The excised leaf, stem and roots were sterilized with 70% ethanol followed by 0.1% mercuric chloride and washed 4-5 times with sterile distilled water. Explants were cut into small pieces (8-10 mm) and were inoculated onto the specific medium. The incubation room was maintained at a temperature of 25 ± 2$^{o}$C and at a relative humidity of 65-70%. All cultures were kept under light intensity of 2000 lux at the level of culture tubes supplied from cool, white fluorescent lamps with 16/8-h photoperiod.

***Shoot bud induction and elongation :*** MS, LS, B5 and L6 media, fortified with auxins 2,4-dichlorophenoxyacetc acid (2, 4-D), naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA) and cytokinins kinetin (KN) and 6-benzylaminopurine (BAP) in the concentration range of 0.1 to 10 mg/l were used for shoot bud induction. Induced shoot buds were excised and placed on MS medium supplemented with very low concentration of (0.01-0.05 mg/l) BAP for shoot elongation.

***Rhizogenesis :*** For root induction, regenerated shoots of 1.5 cm length were transferred onto MS basal media fortified with NAA or IAA, (0.5 mg/l), individually and in combinations and allowed for two to three-weeks. The frequency of rooting and average number of roots per shoot was determined. Rooted plants were removed from culture, rinsed in water to remove adhered agar and transferred to pots containing garden soil and later on to soil. The plantlets were irrigated regularly with quarter-strength MS medium. The survival rate of transferred plantlets was recorded after 2 months of acclimatization.

## Results and Discussion

***Effect of media, explants and plant growth regulators on shoot bud induction :*** The number of shoot buds induced and the frequency of their formation varied with the composition of media, type of explants and concentrations and combinations of growth regulators. Among the

four media and three explants employed in the present study, MS medium resulted in maximum number of shoot buds with the highest frequency in presence of BAP or KN. The order of different media and explants in terms of their shoot bud forming efficiency is MS>LS>B5>L6 and leaf >shoot >root respectively (Figs: 1-4). On MS medium supplemented with BAP resulted in 90% frequency of shoot bud regeneration at 1 mg/l, but maximum number of shoot buds i.e. 16.2 appeared at 2 mg/l BAP. In comparison, KN showed a frequency of 72% (at 2 mg/l) but 13.4 mean number of shoot buds at 3.0 mg/l. When compared to KN, BAP was found better for shoot bud regeneration and mean number of shoot buds/ explants (Figs: 1-4 and 5A-F). The mean number of shoot buds/explant decreased gradually with increasing concentrations of BAP and KN beyond 3 mg /l (data not shown).

In other treatments (BAP + KN, 2,4-D + BAP, 2,4-D + KN, NAA + BAP, NAA + KN, IAA + BAP, IAA + KN), 2,4-D in combination with BAP or KN did not result in shoot bud differentiation from stem and root explants. However, 0.1 mg/l of 2,4-D in the presence of 0.1-2.0 mg/l of BAP and 0.1-2.0 mg/l of KN resulted in 8.4-12.2 and 7.4-9.3 mean number of shoot buds per leaf explant with a frequency of 56-80% and 40-70% respectively (Table 1).

On the other hand, a combination of NAA and BAP at any given concentration did not result in shoot bud differentiation with stem explants. However, leaf and root explants with 1-2 mg/l of NAA and 2 mg/l of BAP resulted in shoot bud differentiation frequency of 28-36% and 4.4-4.6 mean number of shoot buds per explant. In contrast, NAA and KN combination did not

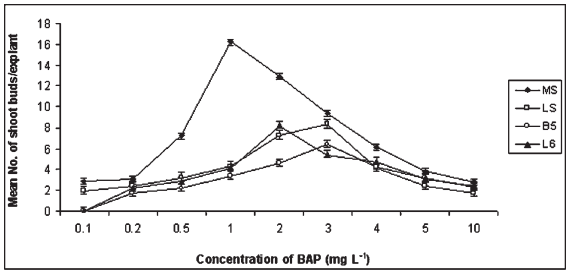


**Fig. 1.** Effect of different media on mean number of shoot buds differentiated from leaf explants of *T.indica* in the presence of BAP

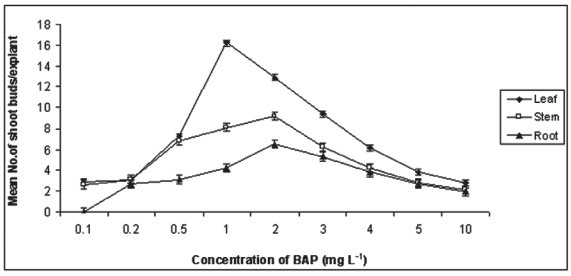


**Fig. 3.** Effect of BAP on mean number of shoot buds formed on MS media from different explants of *T.indica*.

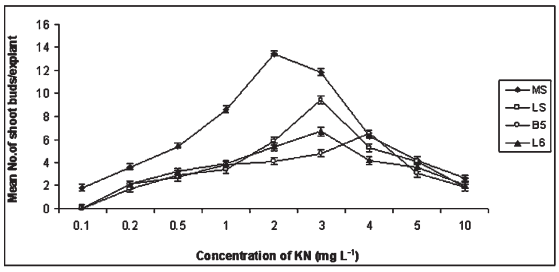


**Fig. 2.** Effect of different media on mean number of shoot buds differentiated from leaf explants of *T.indica* in presence of KN

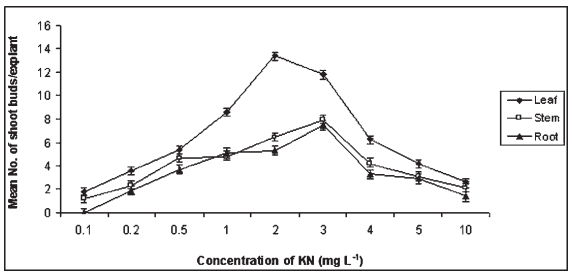


**Fig. 4.** Effect of KN on mean number of shoot buds differentiated on MS media with different explants of *T.indica*.

**Table 1.** Effect of different growth regulators on shoot bud induction from explants *T. indica* on MS medium

| MS + Growth regulator (mg $L^{-1}$) | **Leaf** | | **Stem** | | **Root** | |
|---|---|---|---|---|---|---|
| | F | N | F | N | F | N |
| **2,4D + BAP** | | | | | | |
| 0.1+0.1 | 56 | 12.2 | - | - | - | - |
| 0.1+2.0 | 80 | 8.4 | - | - | - | - |
| 3.0+0.1 | - | - | - | - | - | - |
| 3.0+2.0 | - | - | - | - | - | - |
| **2,4-D + KN** | | | | | | |
| 0.1+0.1 | 40 | 9.3 | - | - | - | - |
| 0.1+2.0 | 70 | 7.4 | - | - | - | - |
| 3.0+0.1 | - | - | - | - | - | - |
| 3.0+3.0 | - | - | - | - | - | - |
| **BAP + KN** | | | | | | |
| 0.1+0.1 | 22 | 9.6 | - | - | - | - |
| 0.1+2.0 | 48 | 6.7 | - | - | - | - |
| 2.0+0.1 | - | - | - | - | - | |
| 2.0+2.0 | 56 | 4.6 | 26 | 6.4 | - | - |
| **BAP + NAA** | | | | | | |
| 2.0+0.1 | 28 | 4.4 | - | - | 25 | 3.6 |
| 2.0+2.0 | - | - | - | - | 36 | 4.6 |
| **BAP + IAA** | | | | | | |
| 0.1+0.1 | 22 | 9.3 | - | - | - | - |
| 0.1+2.0 | - | - | - | - | - | - |
| 2.0+0.1 | - | - | - | - | - | - |
| 2.0+0.1 | - | - | 60 | 9.8 | - | - |
| 2.0+2.0 | 28 | 3.8 | - | - | - | - |
| **KN + NAA** | | | | | | |
| 0.1+0.1 | 28 | 6.0 | - | - | - | - |
| 0.1+2.0 | 68 | 10.2 | - | - | - | - |
| 2.0+0.1 | - | - | 36 | 9.2 | - | - |
| 2.0+2.0 | - | - | 32 | 7.1 | - | - |
| **KN + NAA** | | | | | | |
| 0.1+0.1 | - | - | - | - | - | - |
| 0.1+2.0 | 36 | 5.5 | 48 | 7.5 | - | - |
| 2.0+0.1 | 72 | 8.2 | 44 | 6.3 | - | - |
| 2.0+2.0 | - | - | - | - | - | - |

F= Frequency of shoot bud induction; N= Mean number of shoot buds; **-** No response

induce shoot buds at any given concentration with root explants. However, leaf and stem explants with 0.1-2 mg/l of NAA and 0.1-2mg/l of KN combinations induced shoot buds with 36-68% frequency and 9.2-10.2 mean number of shoot buds per explant. A combination of IAA and BAP could not result in such shoot bud formation with root explants under the concentrations tested. However, with stem and root explants, shoot buds were obtained with a frequency of 60-22% and 9.8-9.3 mean number of shoot buds per explant at 0.1-2 mg/l of (BAP) and 0.1 mg/l of (IAA). IAA in combination with KN did not result in shoot bud formation with root explants in any of the given concentrations. However, a concentration of 2 mg/l of KN and 0.1 mg/l of IAA produced shoot buds with a frequency of 72-48 % and 8.2-7.5 mean number of buds per leaf and stem explants (Table 1). Root explants failed to form shoot buds when BAP and KN combination was used. But, leaf explants could give rise to 9.6 mean number of shoot buds with a frequency of 22% with equal concentration (0.1 mg/l) of BAP and KN and 6.4 mean number of shoot buds with a frequency of 26% with stem explants at a concentration of 2 mg/l of BAP plus KN.

Among different concentrations and combinations of plant growth regulators, the highest frequency and mean number of shoot buds (80% and 12.2 and 70% and 9.3 respectively) was recorded with 0.1-2 mg/l of BAP and 0.1-2 mg/l of KN in combination with 2,4-D (0.1 mg/l) with leaf explants. Our data indicate that the response of a given explant in inducing shoot buds indeed depends on the ratio of auxins to cytokinins supplemented in the medium. Our results support the basic hypothesis proposed by Skoog and Miller (15). The results obtained in this study are broadly in conformity with the trends obtained on direct organogenesis/morphogenesis and plant regeneration in other important medicinal plants (16,17). Multiple shoot bud induction has been reported using various concentrations of auxins and cytokinins in plants like *Catharanthus roseus* (18), *Rauwolfia serpentine* (19), *Cleodendrum colebrookianum* (20), *Dioscorea floribunda* (21), *Holarrhena floribunda* (22) and *Ruta* species etc., (23).

***Effect of plant growth regulators on shoot bud elongation, root formation and plantlet regeneration :*** The excised shoot buds when placed on MS medium supplemented with very low concentrations (0.01-0.05 mg/l) of BAP supported shoot elongation. The average number of shoots elongated varied from 15-20 per culture and average shoot length ranged from 4-10 cm. However, maximum elongation of shoot buds was observed on MS media containing 0.4 mg/l BAP (Figs: 5 G and H). The elongated shoots were rooted on MS basal medium fortified with NAA or IAA individually and in combinations (Figs: 5 I and J). About 90 per cent of the shoots rooted readily within 10-15 days on 0.5 mg/l IAA and 70 per cent with 0.5 mg/l NAA. On an average, 2-5 roots were found with a mean length of 3.6 cm (Figs: K and L). The shoots with well developed roots were transferred to soil: vermiculate (1:1) for hardening and later on shifted to green house for acclimatization. The survival rate of transferred plants was 90 per cent. Induction of roots and regeneration of plantlets from shoot buds was accomplished by many workers in several medicinal plants like *Withania somnifera* (16), *Chlorophytum borivilianum* (24), *Clerodendrum colebrookianjum* (25), *Lavandula latifolia* (26) *Rauwolfia micrantha* (27), *Annona squamosa* (28). The success of IBA for efficient root induction was also reported in *Cunila galoides* (29), and *Rauvolfia tetraphylla* (30).

The present work unambiguously establishes that of the four media employed, MS medium, and of the three explants used, leaf has resulted

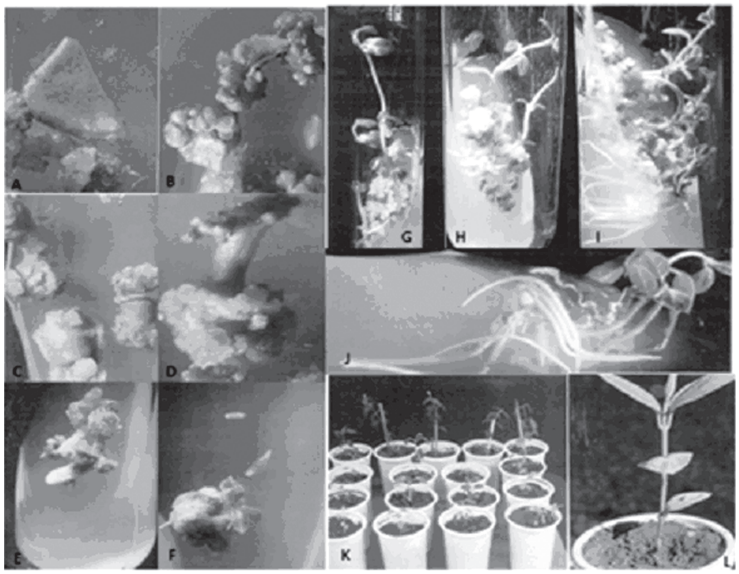

**Fig 5.** Shoot buds induced with leaf explants in the presence of BAP (1-2 mg $L^{-1}$) and KN (2.0-3 mg $L^{-1}$) respectively on MS medium (A and B). Shoot buds induced with stem explants in the presence of BAP (2 mg $L^{-1}$) and KN (3 mg $L^{-1}$) respectively on MS medium (C and D). Shoot buds induced with root explants in the presence of BAP (2 mg $L^{-1}$) and KN (3 mg $L^{-1}$) respectively on MS medium (E and F). Elongation of shoot buds on MS + BAP (0.2 mg $L^{-1}$) +GA3 (0.02 mg $L^{-1}$) showing well developed shoots with leaves (G and H). Elongated shoots showing profuse rooting on MS + 0.5 mg $L^{-1}$ IAA (I and J). Regenerated plantlets transferred in to pots for acclimatization K and L).

in the induction of maximum number of shoot buds with the highest frequency. Among different auxins and cytokinens used individually and in combinations, 1-2 mg/l BAP alone was found as the most efficient for induction of maximum number of shoot buds and also frequency. BAP with a concentration of 0.4 mg/l was observed to be supporting good elongation of shoot buds that were rooted on MS basal medium fortified with 0.5 mg/l IAA and 90% of these plants survived when transferred to soil.

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Current Trends in Biotechnology and Pharmacy
Vol. 4 (4) 930-942 October 2010 ISSN 0973-8916


# Evaluation of Antiangiogenic and Antiproliferative Potential in Ethanolic Extract of *Dioscoria bulbifera* L.

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**Abstract**

Historically, plant products have enjoyed a rich use for their medicinal properties in herbal medicine. Plant compounds with multiple anticancer characteristics are essential to be developed as anticancer drugs. In the same context, we have made an attempt to screen seven crude ethanolic extracts of medicinally vital plants for their antitumor activity using Ehrlich Ascites Tumor (EAT) model. *Dioscoria bulbifera* L. is a plant used as traditional medicine in mainland China, having antitumor effects in its extracts and/or ingredients. And since, our preliminary results indicated that, *D. bulbifera* rhizomes extract (DBRE) had the best antitumor, antiproliferative and antiangiogenic potential amongst other plants; it was chosen for further *in vitro* and *in vivo* studies. The peritoneum of mice treated with DBRE showed significant reduction in peritoneal angiogenesis, which was further confirmed by inhibition of neovascularization in rat cornea and chick chorioallantoic membrane (CAM) *in vivo*. Additionally, we noted attenuated micro vessel density (MVD) count and endothelial cell proliferation in the histological section of DBRE treated mice peritoneum. Quantitation of VEGF in the DBRE treated ascitic fluid of EAT mice showed significant reduction in VEGF secretion when compared to untreated controls. DBRE also exhibited excellent antiproliferative effects against EAT, choriocarcinoma, breast cancer cells, glioblastoma, endothelial cells *in vitro* in a dose dependent manner. Further, antiangiogenic activity of DBRE in the tube formation assay strengthened the presumption that *D. bulbifera* may be a potential supplementary source for cancer therapy.

**Key Words:** Angiogenesis, VEGF, *D.bulbifera*, Anti-proliferation, Matrigel, Rat cornea, CAM

## Introduction

Angiogenesis, or neovascularization, is the process of generating new blood vessels derived as extensions from the existing vasculature (1). It is an elementary step in a variety of physiological and pathological conditions including wound healing, embryonic development, chronic inflammation, and tumor progression and metastasis (2–5). Complex and diverse cellular actions are implicated in angiogenesis, such as extracellular matrix degradation, proliferation and migration of endothelial cells, and morphological differentiation of endothelial cells to form tubes (6). The angiogenic process is strongly controlled by a wide variety of positive or negative regulators, which are composed of growth factors, cytokines, lipid metabolites, and cryptic fragments of hemostatic proteins (6), and many of these

factors are initially characterized in other biological activities. Among these molecules, vascular endothelial growth factor (VEGF), a soluble angiogenic factor produced by many tumor and normal cells, plays a key role in regulating normal and abnormal angiogenesis and inhibition of VEGF expression by tumor cells is known to have an impact on angiogenesis dependent tumor growth and metastasis (7).

Antiangiogenic agents from medicinal plants appear to be suitable for the control of diseases and prolonging the life of the patient. There has been a continuous search for compounds to use in the prevention or treatment of cancer, and especially for agents with reduced toxicity. Several studies have shown that extracts from a number of herbal medicines or mixtures have already displayed their anticancer /antiangiogenic potential in *vitro / in vivo* or both (8, 9, 10, 11, 12, 13, 14, 15). Plant-based compounds, such as, resveratrol, catechins genistein, curcumin, in addition to others, such as diallyl sulfide, S-allyl cysteine, allicin, lycopene, capsaicin, 6-gingerol, ellagic acid, ursolic acid, silymarin, anethol, and eugenol have already proved their anticancer abilities (16). Also, numerous antiangiogenic drugs are in different phases of clinical trials presently (17).

All the above facts led us to pursue the search for novel bioactive lead structures with antiangiogenic activity for which seven medicinal plants were selected for further study which are known medicinal plants having anticancer activity. However, their antiangiogenic and antiproliferative effects have not been investigated before. The plants that have been selected for the study include *Dioscorea bulbifera* L. (Dioscoreaceae), *Acorus calamus* (Araceae), *Annona squamosa* (Annonaceae), *Streblus asper* (Moraceae), *Bauhinia variegata* (Caesalpinaceae), *Thespesia populnia* (Malvaceae) and *Erythrina suberosa* (Fabaceae). The plant *D. bulbifera* L. is found to possess anti bacterial (18) and antitumor activity (19). The rhizome of *Acorus calamus* is reported to possess insecticidal properties (20).It is reported that the fruits of *Annona squamosa* are used in folk medicine as a remedy to treat several microbial diseases (21). *Streblus asper* has been reported to possess anticancer activity (22). It is used traditionally in leprosy, piles, diarrhea, dysentery, elephantiasis (23). *B. variegate* has been shown to have anti-inflammatory (24) and antitumor effect (25). The plant *T .populnia* has been investigated for antibacterial (26), anti-inflammatory (27) and for the treatment of Alzheimer's disease (28). *E. suberosa* has been reported to be used for the treatment of dysentery and ulcer (29).

In the present study, an attempt has been made to screen the ethanolic extracts of these seven medicinal plants using *in vivo* and *in vitro* assays such as peritoneal angiogenesis assay, rat cornea assay, CAM assay, H and E and CD-31 immunostaning, VEGF quantitation (ELISA), tube formation assay and antiproliferation assay. The results of this study are expected to provide better understanding of the antiangiogenic and antiproliferative potential of the therapeutic plant extracts.

## Materials and Methods

The shade dried plant materials (10g each) were extracted exhaustively with 100ml of 50% ethanol at room temperature for a period of seven days. Ethanol was evaporated in order to obtain crude ethanolic extracts of the plants at a concentration of 1mg/ml. The rhizomes of *D. bulbifera* were collected from Western Ghats, Shimoga, India, in March 2008. The shade dried rhizomes of *A. calamus* were purchased from Herb Shop in Mysore, India. The plant *A.squamosa* (except root), the leaves of *S.asper* ,the bark of *B. variegata*, fruits of *T.populnia*

and leaves of *E.suberosa* were collected from the campus of University of Mysore, Manasagangotri, Mysore, India in June 2008.. Identification of the plant material was confirmed by depositing the voucher specimens in the Herbarium of the Department of Studies in Botany, University of Mysore, Mysore.

Swiss albino mice (6-8 weeks old) and Male Wister rats were obtained from the animal house, Department of Zoology, University of Mysore, Mysore, India. EAT (mouse mammary carcinoma) cells are maintained in our laboratory and are routinely used for *in vivo* transplantation. HUVECs were procured from Cambrex Biosciences, Walkersville, USA. BeWo (Choriocarcinoma) cells, MCF-7 (Breast cancer) cells, U-87(Brain tumor) cells and HEK 293 (Un-transformed Human embryonic kidney) cell lines were from the National Center for Cell Science, Pune, India.

Endothelial growth medium (EGM-2) was procured from Cambrex Biosciences, Walkersville, USA [3H] thymidine was from the Baba Atomic Research Center, Mumbai, India. DMEM, FBS and penicillin-streptomycin were from Invitrogen, USA. DMEM/Ham's nutrient mixture F-12 and poly-2- hydroxyl ethylmethacrylate were from Sigma Aldrich, USA. Fertilized eggs were from a government poultry farm in Bangalore, India. Matrigel was from Becton Dickinson Labware, Bedford, MA. All other reagents were of the highest analytical grade.

***In vitro culture of EAT, BeWo, MCF-7, U-87, Endothelial and HEK-293 cells :*** BeWo (choriocarcinoma) cells were cultured in DMEM Ham's F-12 medium with 10% FBS, 1% Penicillin-Streptomycin and Gentamycin. EAT, MCF-7, U-87(Glioblastoma) and HEK 293 cells were maintained in DMEM with 10% FBS and 1% Penicillin-Streptomycin. Endothelial cells were cultured in EGM-2 medium with 2% FBS, 0.04% hydrocortisone, 0.1% long R3-human Insulin like growth factor (IGF-1),0.1%ascorbic acid, 0.4% human fibroblast growth factor (bFGF), 0.1%VEGF, 0.05% gentamycin and 0.05% amphotericin-B according to the manufacturer's protocol. The cells were incubated at $37^0$ C in a humidified atmosphere of 5% $CO_2$. When the cells reached confluency, they were passaged by trypsinization using 0.025% trypsin/0.01% EDTA. For the experiments, cells from passages 2-5 were used.

***In vivo growth of EAT cells and peritoneal angiogenesis assay :*** To understand the mechanism of plant extracts as antiangiogenic compounds, we tested their effect *in vivo* using EAT bearing mice. EAT cells, were maintained by i.p transplantation as described previously (30). In brief, EAT cells or mouse mammary carcinoma cells ($5 \times 10^6$cells) were injected intraperitoneally into mice and growth was recorded every day until the 12th day. These cells grow in the mice peritoneum, forming an ascites tumor with massive abdominal swelling. The animals show a dramatic increase in body weight over the growth period and animals succumbed to the tumor burden 14-16 days after transplantation. To verify whether the plant extracts inhibited tumor growth and angiogenesis mediated by EAT cells *in vivo,* plant extracts (133 mg/kg body weight) were injected into the peritoneum of the EAT-bearing mice every day after the 6th day of transplantation. The animals were sacrificed on the 12th day, 2ml of saline was injected *(i.p*), and a small incision was made in the abdominal region to collect the tumor cells along with ascites fluid. The EAT cells and ascites fluid were harvested into a beaker and centrifuged at 3,000 rpm for 10 min. The ascites volume was measured by subtracting the volume of saline injected while harvesting the EAT cells from the

total ascites volume measured. The pelleted cells were counted by trypan blue dye exclusion method using a hemocytometer. The animals were dissected to observe the effect of the extract on peritoneal angiogenesis. All experiments were conducted according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, India.

***Corneal micro pocket assay / Rat cornea assay :*** This assay was performed in accordance with the method described previously (31). In brief, for the pellet preparation, hydron polymer poly- 2-hydroxyethylmethacrylate was dissolved in ethanol to a final concentration of 12%. An aliquot of the Hydron/EtOH solution was added to VEGF (1 ìg/pellet) with or without 10 ìg/pellet of *Dioscoria bulbifera* rhizome extract (DBRE). Aliquots of 10 ìl of 12% Hydron/EtOH alone (Group 1), with cytokine VEGF (Group 2), and with VEGF and DBRE (Group 3) were placed onto a teflon surface and allowed to air dry for at least 2 h. Male Wister rats weighing 300-350 gms were anesthetized with a combination of xylazine (6 mg/ kg, IM) and ketamine (20 mg/kg, IM). The eyes were topically anesthetized with 0.5% proparacaine and gently proptosed and secured by clamping the upper eyelid with a non-traumatic hemostat. A corneal pocket was made by inserting a 27-gauge needle, with the pocket's base 1 mm from the limbus. A single pellet was advanced into the lamellar pocket to the limbus using corneal forceps. The rats were observed for 24-72 h for the occurrence of nonspecific inflammation and localization of the pellets. On day 7, the rats were anesthetized and the corneas were photographed using a CCD camera (40 ×).

***Chorioallantoic membrane (CAM) assay :*** Original chorioallantoic membrane (CAM) assay has long been a mainstay for the study of embryonic organ development. It was carried out in accordance with the method described previously (32). In brief, fertilized eggs were incubated at 37°C in a humidified and sterile atmosphere for 10 days. Under aseptic conditions, a window was made on the eggshell to check for proper development of the embryo. The window was resealed and the embryo was allowed to develop further. On the 12th day, saline, recombinant cytokine VEGF (10 ng per egg) or DBRE (50 ìg per egg) was air dried on sterile glass cover slips. The window was reopened and the cover slip was inverted over the CAM. The window was closed again, and the eggs were returned to the incubator for another 2 days. The windows were opened on the 14th day and inspected for changes in the vascular density in the area under the cover slip and photographed at 40X magnification.

***H and E staining for microvessel density scoring :*** To determine whether DBRE inhibits microvessel density, its effect on the angiogenic response induced by the cytokine VEGF was verified in EAT-bearing mice. EAT-bearing mice were treated regularly with the extract after the 6th day of transplantation. On the 12th day, the animals were sacrificed and the peritoneum from treated or untreated mice was fixed in 10% formalin. Sections (5 ìm) were made from paraffin embedded peritoneum and stained with hematoxylin and eosin. Microvessel counts were done using a Leitz-DIAPLAN microscope with attached CCD camera and photographs were taken at 40 X magnification (Table.2).

***CD 31 Immunostaining for assessment of proliferating endothelial cells :*** The effect of DBRE on proliferating endothelial cells was determined by staining the paraffin sections of the peritoneum of treated or untreated mice with anti CD 31 antibody. Peritoneum sections were processed as per the protocol supplied by the manufacturer (Santa Cruz Biotechnology, CA,

USA). In brief, sections were dewaxed in xylene thrice for 5 min each. The sections were rehydrated in descending concentrations of ethanol (100% ethanol for 5 min, 95% for 2 min and 80% for 2 min) and washed in distilled water. Antigen retrieval was done by heating the sections at 95° C for 15 min in a humidified atmosphere. The sections were treated with 3% $H_2O_2$ in PBS to block endogenous peroxidase activity. They were blocked in blocking serum for 30 min to reduce the non specific binding and were incubated with anti-CD 31 (PECAM-1) antibodies for 2 hrs. Following PBS washing, slides were incubated with secondary antibody (biotinylated rabbit anti mouse IgG) for 1 hr at room temperature. The slides were washed in PBS for 5 min and incubated with the substrate (100µl/section) followed by ABC reagent for 45 min (2 ml histo buffer + 20µl Avidin solution + 20µl Biotin solution). After incubation, the slides were washed in PBS for 5 min. Antigen and antibody complex was detected using substrate (DAB, 100µl/section) for 5 min. The sections were washed thrice for 2 min in tap water and twice for 2 min in distilled water. Subsequently, the slides were counter stained with 2 % hematoxylin for 5-7 min and washed again in tap water thrice for 5 min each. The slides were washed successfully for 2 min each in 50% ethanol, 80% ethanol and absolute alcohol. After xylene wash, the slides were mounted using Entellan mountant solution and the sections were scored using DIAPLAN light microscope and photographed.

***Quantification of VEGF:*** The quantification of VEGF was carried out by enzyme linked immunosorbent assay (ELISA) and VEGF was estimated in ascitic fluid collected from both untreated and DBRE treated mice as described previously (33). In brief, 100ìl of ascitic fluid from DBRE treated and untreated EAT bearing mice were coated onto 96 well microplates using coating buffer (50mM $Na_2CO_3$, pH. 9.6) and incubated overnight at $4^0$C. Wells were washed and blocked using skimmed milk followed by incubation with anti-VEGF antibodies (1:1000 diluted). The wells were washed and probed with secondary antibody (1:5000 diluted) tagged to alkaline phosphatase. *P*-NPP was used as substrate and absorbance was measured at 405nm with medispec ELISA reader.

***Tube formation assay :*** Tube formation of HUVECs was performed as per manufacturer's instructions (34). Briefly, a 96 well plate was coated with 50 ìl of matrigel which was allowed to solidify at $37^0$C for 1 h. HUVECs (1 x $10^4$cells/well) were seeded on the solidified matrigel and cultured in EGM containing DBRE (5ìg) for 8 h. After 24 h of incubation at 37 $^0$C and 5% $CO_2$, the enclosed networks of complete tubes from five randomly chosen fields were counted and photographed under an Olympus inverted microscope (CK x 40; Olympus, New York, NY) connected to CCD camera at 40x magnification.

***Endothelial and Tumor cell proliferation assay :***[$^3$H] thymidine incorporation assay was carried out as described previously (35) in endothelial and tumor cells. To verify the in vitro effect of DBRE on proliferation of EAT, BeWo, MCF-7, U-87, HUVEC, and HEK 293 cells, 25,000 cells/well were seeded in 12-well plates in their respective media and grown in 5% $CO_2$ at 37°C for 2 days. DBRE was filter sterilized and diluted with cell culture medium (1 ìg/ìl). On the 3rd day, [$^3$H] thymidine (1 ìCi/ ml medium) and DBRE were tested at the concentrations of 25 ìg, 50 ìg, 75 ìg and 100 ìg. After 48 h, the cells were trypsinized and washed with phosphate buffered saline (PBS); high molecular weight DNA was precipitated using 10% ice-cold trichloroacetic acid. Scintillation fluid (5 ml) was added to all of the samples and radioactivity was

determined with a liquid scintillation counter. The concentrations of the samples were then plotted against the percentage cell survival.

## Results

***In vivo treatment of DBRE inhibits growth of EAT cells :*** Our results in Fig.1 indicate that control EAT bearing mice showed a gradual increase in body weight of about 8.5 ± 2.15 gms over 12 days growth period, when $5 \times 10^6$ EAT cells were injected on day zero. In the mice treated with various plant extracts from *Dioscorea bulbifera* L., *Acorus calamus*, *Annona squamosa*, *Streblus asper*, *Bauhinia variegata*, *Thespesia populnia* and *Erythrina suberosa*, a significant decrease in body weight was observed in the groups which were treated with DBRE as compared to that of the other extracts selected for screening, indicating the effect of the DBRE in preventing the growth of the tumor cells ($p < 0.05$). In a fully grown ascites tumor, a volume of 7.5 ± 1.71 ml of ascites was generated during the tumor growth period of 12 days. In DBRE treated mice, the volume of ascites was about 2.5 ± 1.07 ml with $p < 0.01$ (Table 1). The number of viable cells in full-grown EAT-bearing mice was about 805 ± 1.38 $\times 10^6$/mouse, while this number was reduced in DBRE treated mice to 305 ± 2.06 $\times 10^6$/mouse with statistical significance not reaching $p < 0.05$ (Table 1), indicating reduction when compared to the control. These results indicate the antitumor activity of DBRE. In a fully grown ascites tumor *in vivo*, there is extensive peritoneal angiogenesis and in DBRE treated mice, a significant decrease in peritoneal angiogenesis was observed (Figure 2a and 2b).

***Angioinhibitory effect of DBRE :*** The rat cornea assay and CAM assay are commonly used for *in vivo* validation of the angioinhibitory activity of antiangiogenic molecules. Our results indicate that DBRE has a direct effect on inhibition of angiogenesis in an *in vivo* model system. When compared to the extensive angiogenesis seen in VEGF treated rat cornea and CAM, angiogenesis at the site of the application of DBRE was significantly reduced. DBRE at 10ìg /eye concentration, showed decreased angiogenesis in the cornea of the rat induced with VEGF (Figure 2c, 2d and 2e). In the CAM assay model, DBRE induced avascular zone formation in the developing embryos thus by inhibiting capillary development on the CAMs at 50 ìg/egg concentrations (Figure 2f, 2g and 2h).

| | Average volume of ascites (ml) | Average number of cells x $10^6$/mouse |
|---|---|---|
| **EAT bearing(Control)** | 7.5 ± 1.71 | 805 ± 1.38 |
| ***D.bulbifera*** | 2.5 ± 1.07 | 305 ± 2.06 |
| ***A.calamus*** | 6.7 ± 0.95 | 675 ± 1.87 |
| ***A.squamosa*** | 6.9 ± 2.08 | 705 ± 1.09 |
| ***S.asper*** | 7.0 ± 1.09 | 735 ± 0.98 |
| ***T.populnia*** | 6.9 ± 0.99 | 705 ± 2.04 |
| ***B.variegata*** | 6.8 ± 1.54 | 700 ± 1.93 |
| ***E.suberosa*** | 7.0 ± 1.09 | 740 ± 1.33 |

**Table 1.** Average ascites volume and EAT cell number counted using hemocytometer in different plant extracts treated EAT mice *in vivo.* The results are presented as mean ± standard deviation (n=3).

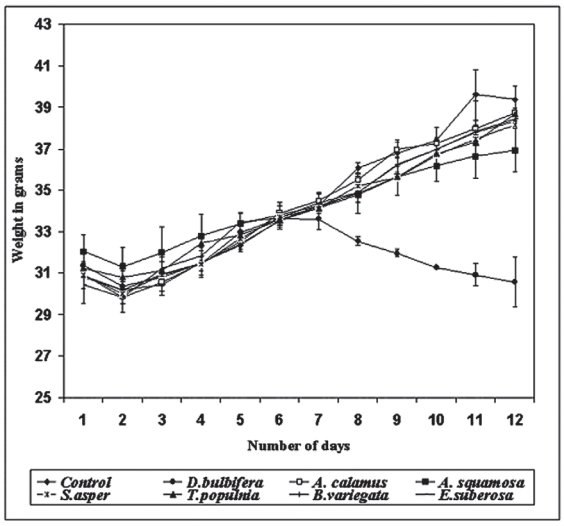


**Fig. 1.** Effect of different plant extracts on *in vivo* growth of EAT cells.
EAT cells ($5x10^6$) were injected i.p into mice and from the sixth day of transplantation, mice were treated with or without the plant extracts (50ìg/ dose) i.p till the $12^{th}$ day of tumor inoculation. Body weight of both the control and treated group was recorded everyday. Values are presented as mean ± standard deviation (n=3).

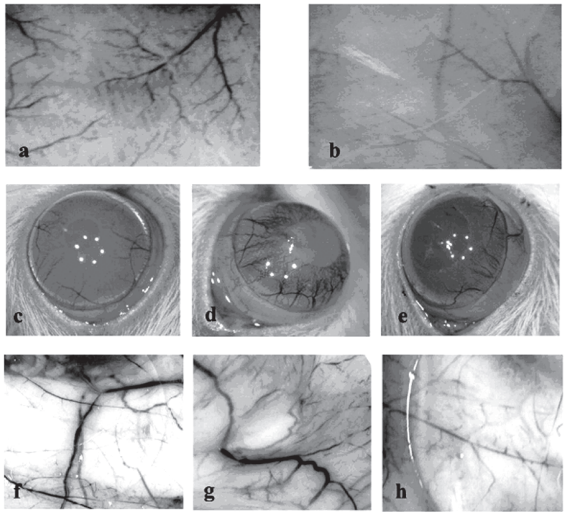


**Fig. 2.** Effect of DBRE on *in vivo* angiogenesis
The peritoneums of EAT bearing mice treated with or without the plant extract (50ìg/ dose) which were sacrificed on the $13^{th}$ day. The extent of angiogenesis in peritoneal wall was observed in a. Untreated, b. *D.bulbifera* treated. In rat cornea assay, DBRE (10ìg) with or without rVEGF (1ìg) was incorporated into sterile pellets. The pellet was implanted at the bottom of each rat cornea. After 7 days, when neovascularization was prominent, the corneal vessels were photographed using a photo slit lamp as shown in c. Without VEGF, d. With VEGF and e. *D.bulbifera* treated. In CAM assay, fertilized chicken eggs were incubated for 12 days in sterile condition. Saline alone, VEGF alone (10ng) and VEGF (10ng) + DBRE (50ìg/ egg) were dried on cover slips and applied onto the CAM of the developing chick embryo through a cut window. The CAM was observed for inhibition of neovascularization on 14th day in f. Without VEGF, g. With VEGF and h. *D.bulbifera* treated.

***H&E and CD 31 immunostaining :*** Comparable reduction in the number of newly formed microvessels in the DBRE treated peritoneum (50 ìg/dose) than that of the control was observed by histological examination of the peritoneal sections of both the group. In this study, the average MVD was significantly higher in control (with an average count of 25) with vascular invasion than in DBRE treated, with an average count of 1 (Figure 3a and 3b). CD 31 is used as a marker for indicating the proliferation of endothelial cells. Our results on CD 31 staining indicated that there was reduction in the number of proliferating endothelial cells in the peritoneum of DBRE treated EAT bearing mice corroborating the results shown in the inhibition of peritoneal angiogenesis *in vivo*. An average count of 1 in the treated while in the control sections an average count of 8 was recorded.

***Inhibition of VEGF production in EAT cells by medicinal plants :*** In control EAT bearing mice, over 0-12 day tumor growth period, quantitation of VEGF indicated that there is a gradual production and secretion of VEGF by

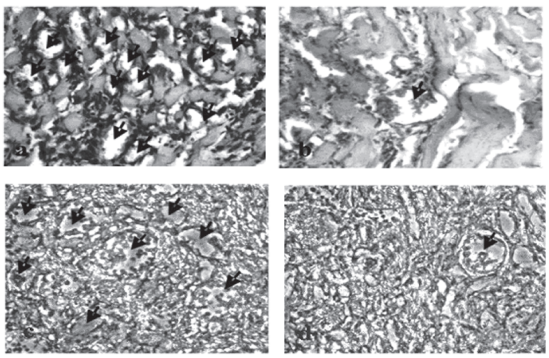

**Fig. 3.** Effect of DBRE on micro vessel density (MVD) and on proliferation of endothelial cells in the mouse peritoneum
EAT bearing mice were treated with or without the plant extract (50 mg/ dose) for 6days. On the 13th day, the animals were sacrificed. The peritoneums of control as well as plant extract treated EAT bearing mice were embedded in paraffin and 5ìm sections were taken using microtome. The sections were stained with hematoxyline and eosine and observed for microvessel density in a. Control and b. *D.bulbifera* treated. Other paraffin sections were also used for immunostaining with anti-CD-31 (PECAM) antibodies as seen in c. Control and d. *D.bulbifera* treated.

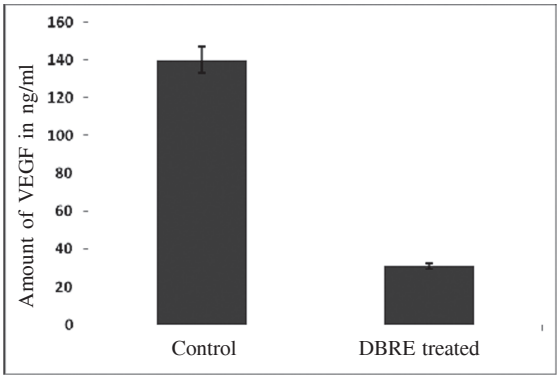


**Fig. 4.** Effect of the DBRE on secretion of VEGF *in vivo*
EAT bearing mice were treated with or without the plant extract (50 mg/ dose) for 6days. On the 13th day, the animals were sacrificed and ascites was collected. ELISA was carried out using the ascites to quantitate VEGF using anti-VEGF 165 antibodies. Values are presented as mean ± standard deviation (n=3).

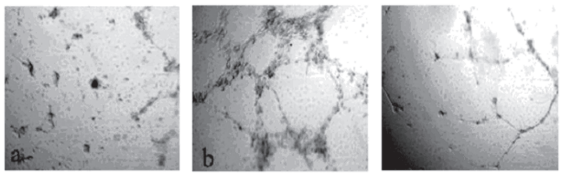

**Fig. 5.** Effect of DBRE on the tube formation in HUVECs
HUVECs (5 x $10^3$ cells/well) were seeded on a Matrigel coated 96 well plate and cultured in EGM containing VEGF, in the presence and absence of 1µg/ well of DBRE. Formation of tubes was observed under phase contrast microscope.

EAT cells. Our results indicate that 140ng of VEGF/ml with $p < 0.01$ to be present in the ascites of a fully grown tumor whereas in DBRE treated mice, reduction in the amount of VEGF was noted (31 ng/ml denoting $p < 0.01$) suggesting the inhibition of VEGF secretion (Fig. 4).

***DBRE inhibits tube formation of HUVECs induced by VEGF :*** One of the most specific tests for angiogenesis is the measurement of the ability of endothelial cells to form three-dimensional *in vitro* assay was performed to verify the effect of DBRE on the formation blood vessels by HUVECs. HUVECs in basal media could not form tubes and VEGF was used to induce tube formation. In the positive control group stimulated with VEGF (10ng), HUVECs rapidly aligned with one another and formed tube like structures resembling a capillary plexus within 8 hours. However DBRE treatment (1ìg/well) prevented VEGF stimulated tube formation of HUVECs. Thus DBRE was shown to interfere with the ability of HUVECs to form *in vitro* vessel like tubes, one of the important traits of endothelial cells (Fig.5).

***DBRE inhibits in vitro proliferation of tumor cells :*** There are numerous well-established

assays for measuring cell proliferation. The most frequently used measure, the thymidine incorporation assay, will serve to introduce several of the key problems of validating *in vitro* angiogenesis assays. Inhibition of proliferation of endothelial cells and tumor cells by DBRE further supported its antiproliferative effect (Fig. 6). HUVECs and different tumor cells like EAT, BeWo, MCF-7, U-87 and untransformed HEK-293 cells were used to verify if DBRE inhibit the proliferation of tumor or normal cells *in vitro*. DBRE efficiently inhibited proliferation of endothelial cells and different tumor cell lines at a concentration range of 25-100 ìg although statistical significance was not reached $p < 0.05$. However, no effect was seen in case of untransformed normal HEK-293 cells.

## Discussion

With the advent of chemo preventive approaches for the treatment of cancer, there is

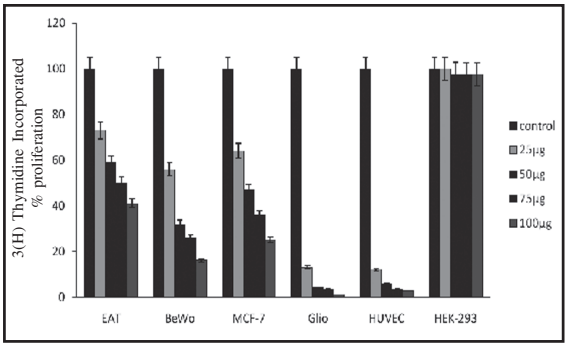


**Fig. 6.** Effect of DBRE on proliferation of endothelial cells and tumor cells *in vitro*
EAT (A), BeWo (B), MCF-7 (C), U-87 (D), HUVEC (E) and HEK-293 (F) were plated in 12 well plates and incubated for 48h. Plant extract in concentrations 25 µg, 50 µg, 75 µg and 100 µg were added to the wells in duplicates prior to the addition of 3[H]thymidine and incubated for another 48 h. The cells were trypsinized after 2 days and processed for scintillation counting. Values are presented as mean ± standard deviation (n=3).

widespread interest in the possibility that this approach may eventually have an effect on, and could improve the quality of life of, human cancer patients. Several natural agents with high anticancer efficacy and no or acceptable toxicity to normal tissues are suggested as possible candidates for use by cancer patients (3, 5, 6, 7, 28). Over the past years, there was a major shift in the development of cancer drugs from screening of cytotoxic drugs to the development of molecular targeted drugs. The conceptual idea is that the knowledge of the mechanism(s) of action of a drug provides a better approach to reach improved clinical results based on patient's molecular characteristics (phytochemistry and pharmacogenomics). This was the starting point of our effort on the screening for natural products derived from plants of traditional medicinal value.

In the present study, with the aim of finding potent antiangiogenic compounds in plants, seven plants (*Dioscorea bulbifera* L., *Acorus calamus*, *Annona squamosa*, *Streblus asper, Bauhinia variegata, Thespesia populnia* and *Erythrina suberosa*) were screened for their effect on proliferation of tumor cells *in vivo* and *in vitro* for the first time. Preliminary results established markedly that DBRE has potent antiproliferative and antiangiogenic effect on Ehrlich ascites tumor (EAT) cells *in vivo*. DBRE treatment in EAT bearing mice brought about a decrease in the body weight (Figure 1), ascites volume and cell number (Table 1) *in vivo*.

The growth of primary tumors and metastases depends on the degree of tumor neovascularization. Our present study provides compelling evidence that suppression of angiogenesis could be at least one of the mechanisms of the antitumor effect of DBRE. By using *ex vivo* and *in vivo* angiogenesis models, the antiangiogenic effects of the DBRE were evaluated. DBRE remarkably inhibited *in*

*vivo* angiogenesis in the peritoneum of the treated EAT bearing mice (Figure 2a and 2b). DBRE inhibited VEGF induced angiogenesis in the cornea of the rat (Figure 2c, 2d and 2e). Further, the antiangiogenic activity of DBRE was confirmed with the results of CAM assay which clearly showed inhibition of capillary development on the CAMs by inhibiting neovascularization (Figure 2f, 2g and 2h) by DBRE.

Vascular invasion and MVD studied by Hematoxylin-eosin staining of peritoneal lining section of EAT bearing mice treated and untreated with DBRE proved that angiogenesis is closely related with microvessel density of tissue and clinical aggressiveness of tumor (Figure 3a and 3b). Further evidence for the antiangiogenic potential of DBRE was seen in the result on inhibition of the extent of proliferating endothelial cells in the peritoneal lining of tumor-bearing mice which was immunostained with anti-CD-31 (PECAM) antibodies (Figure 3c and 3d).

Increased VEGF expression is closely associated with an increase in microvessel density (36). VEGF being a permeability factor plays fundamental role in the fluid accumulation and tumor growth in ascites tumor. By secreting VEGF, ascites tumor enhances the permeability of preexisting microvessel lining of peritoneal cavity to stimulate ascites formation thereby tumor progression. Inhibition of fluid accumulation, tumor growth and microvessel density by neutralization of VEGF has been demonstrated underlying the importance of VEGF in malignant ascites formation (37-39). Our results indicated that there was decrease in the VEGF secretion in DBRE treated EAT bearing mice (Figure 4). Inhibition of VEGF gene expression by DBRE should also be reflected by the levels of VEGF in the ascites secreted by the EAT cells. The current results on quantification of the VEGF in the ascites of EAT bearing mice have clearly indicated that DBRE efficiently decreases the level of VEGF in an *in vivo* model system.

Further, DBRE suppressed human endothelial cell tube formation, which is one of the hallmarks of angiogenesis indicating that DBRE inhibits endothelial cell proliferation Endothelial cells differentiate and form capillary-like structures when seeded on matrigel. This development entails cell-matrix interaction, intercellular communication and cell mobility like *in-vivo* tumor angiogenesis. The effect of DBRE at a concentration of 1µg/well in HUVEC tube formation was studied and total numbers of tubes formed were counted. Scoring of the total number of tubes showed that DBRE caused 90% decrease in total number of tubes as compared to control (Figure 5). In this assay system, DBRE suppressed human endothelial cell tube formation indicating that it inhibits endothelial cell proliferation and consequently angiogenesis *in-vitro*.

The antiproliferative effect of the DBRE was assessed using four different tumor cell lines EAT, BeWo, MCF-7 and U-87 (Figure 6). DBRE showed strong inhibition of proliferation of all the tumor cell lines and also the HUVECs at four different concentrations (25 ìg, 50 ìg, 75 ìg, 100 ìg). Thus DBRE showed that it was the most active species. In order to test the activity of the extract on normal cells, we assessed the effect of the sample on the proliferation of non-transformed HEK-293 cells. The result indicated that the cancer cells were more susceptible to DBRE than non-transformed cells.

The present investigation represents only a preliminary screen for potent antiangiogenic and antitumor activity and points to the necessity of deeper phytochemical and biological investigations because the plant *D.bulbifera* is potentially interesting in yielding biologically active products.

This study provides scientific evidence for the ethnobotanical use of the plant *D.bulbifera* which may help research and development of this plant for cancer. As a continuation of this work, the active compounds will be isolated and the underlying mechanism for antitumor activity will be delineated.

**Acknowledgements**

The authors would like to thank Department of Zoology, University of Mysore for providing animals for this work. This research was supported by Department of Science and Technology, New Delhi and Department of Atomic Energy, Mumbai, India.

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Current Trends in Biotechnology and Pharmacy
Vol. 4 (4) 943-946 October 2010 ISSN 0973-8916


# RP-HPLC Method for the Estimation of Montelukast Sodium in Pharmaceutical Dosage Forms

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**Abstract**

A reverse phase high pressure liquid chromatographic method (HPLC) has been described for the estimation of Montelukast Sodium in its pharmaceutical formulations using an inertsil ODS C-18, 5 µm column having 250×4.6 mm I.D. in gradient mode, with mobile phase A, containing 0.02 M sodium phosphate buffer: methanol (85:15) and mobile phase B, containing acetonitrile: methanol (85:15), at different time intervals. The flow rate was 1.0 ml/min and effluent was monitored at 218 nm and the linearity was found to be in the range of 0.1 to 10 ìg/ml. The method is simple, precise, specific, less time consuming and accurate for the estimation of montelukast Sodium in Pharmaceutical dosage forms.

**Key Words :** HPLC, Montelukast Sodium, Pharmaceutical Dosage Forms, Retention time, Linearity

## Introduction

Montelukast Sodium (1,2) is [R-(E)]-1-[[[1-[3-[2-(7-chloro-2quinolinyl) ethenyl] phenyl]- 3-[2-(1-hydroxy-1-methylethyl)phenyl] propyl] thio] methyl] cyclopropane acetic acid, sodium salt (mono), a leukotriene receptor antagonist used as an alternative to anti-inflammatory medications in the management and chronic treatment of asthma and exercise-induced bronchospasm (EIB) that is marketed under trade names such as SingulairÃ‚Â® and Montair. Only a very few analytical methods have been reported for its estimation in pharmaceutical dosage forms by HPLC (3, 4, 6, 9, 11) Spectrofluorimetry (5) Electrophoresis (10), UV Spectrophotometer (7, 8) and LC-ESI-MS (12) . In the present study a sensitive, specific, precise and accurate HPLC method has been developed for the estimation of Motelukast Sodium in pharmaceutical dosage forms. The structure of the drug with reactive functional groups is given below (Figure 1).

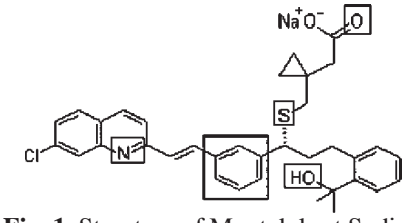



**Fig. 1.** Structure of Montelukast Sodium with Reactive functional groups

## Materials and Methods

Experimental Montelukast Sodium was a gift sample from local pharmaceutical industry. Acetonitrile, Methanol and triple distilled water (TD water) used were of HPLC grade (Qualigens). All other reagents (Sodium phosphate buffer) used in the study were of AR quality (Qualigens).

A gradient high pressure liquid chromatograph (Shimadzu HPLC class VP series) with two LC-20 AT VP pumps, variable wave length programmable UV –Visible detector SPD-20 A VP ,SCL-20A VP system controller (Shimadzu) and a reverse phase C-8 column ( 250x4.6 mm, 5µ) was used for estimation. The HPLC system was equipped with the software "class VP series version 5.03 (Shimadzu)

***Chromatographic Conditions :*** Methanol and buffer (0.02 M sodium phosphate buffer of pH 3.5 adjusted by using 0.01 M Phosphoric acid ) were filtered before use. The flow rate of the mobile phase was maintained at 1 ml/minute in the ratio of 15:85 (Methanol: Buffer). The detection was carried out by UV detector a 218 nm. The data acquired was analyzed with the software class VP series version 5.03 (Shimadzu).

***Procedure :*** About 100 mg of Montelukast Sodium was accurately weighed and dissolved in mobile phase so as to give a 1 mg/ml solution. Subsequent dilution of this solution was made to obtain 100 ìg/ml. The standard solution prepared above was injected five times into the column at a flow rate of 1 ml/minute. The peak area for each of the drug concentrations was calculated. Montelukast Sodium solution containing 20 ìg/ml and 40 ìg/ml were subjected to the proposed HPLC analysis for finding out the intra-and interday variations. The recovery studies were carried out by adding a known amount of Montelukast Sodium to the pre analyzed samples, and subjecting them to proposed HPLC method.

***Estimation of Montelukast Sodium in pharmaceutical dosage forms :*** An accurately weighed portion of the powder equivalent to 100 mg of Montelukast Sodium (bulk sample) was transferred to a 100 ml volumetric flask containing about 50 ml of mobile phase. The contents of the flask were sonicated to dissolve Montelukast Sodium, made up to volume with mobile phase and the resulting mixture was filtered through 0.45 ì filter. One ml of this solution was added to a 100 ml volumetric flask and made upto the volume with mobile phase. This solution 20 ìL was injected five times into the column. The mean value of the peak area was calculated and the drug content in each tablet was quantified using the regression equation. The same procedure was followed for the estimation of Montelukast Sodium in six different brands of tablet dosage forms.

**Results and Discussion**

A typical chromatogram for the proposed method is shown in figure 2. The retention time for Montelukast Sodium was 3.017 minutes. Each of the samples was injected five times and the same retention time was observed in all cases. The peak areas for different concentrations are shown in table-1.

The peak areas for Montelukast Sodium were reproducible as indicated by a low coefficient of variation 2.19. A good linear relationship (r = 0.9998) was observed between the concentrations of Montelukast Sodium (13, 14) and the respective peak areas. When Montelukast Sodium solution containing 20 ìg/ml and 40 ìg/ml were analyzed by the proposed HPLC (4, 6) method for finding out intra and interday variations, a low coefficient of variation was observed (Table-2). This shows that the present HPLC (4, 6) method is highly precise. The amounts of Montelukast Sodium from the pre analyzed samples containing known amounts of the drug are shown in Table-3. About 99.97% of Montelukast Sodium could be recovered from the pre analyzed samples indicating a high accuracy of the proposed HPLC (4, 6) method.

The absence of additional peaks indicates no interference of the excipients used in the

**Table 1.** Standard graph for the estimation of Montelukast Sodium.

| Concentration of Montelukast Sodium in (ìg) | Peak area |
|---|---|
| 20 | 120.216 |
| 40 | 127.423 |
| 60 | 124.621 |
| 80 | 195.939 |
| 100 | 145.764 |

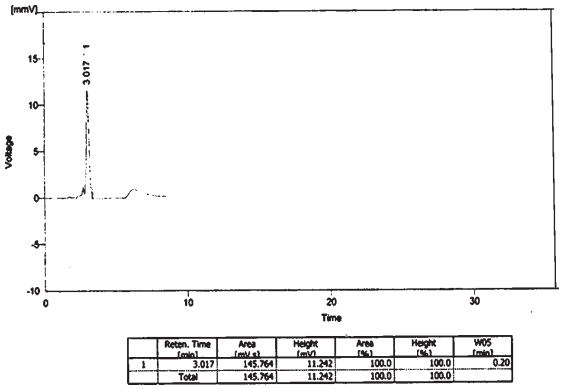
**Table 2.** Precision of the proposal HPLC

| Montelukast Sodium concentration (µg/ml) | Concentration of Montelukast Sodium(µg/ml) found on | | | |
|---|---|---|---|---|
| | Intra-day | | Inter-day | |
| | Mean(n=5) | % CV | Mean(n=5) | % CV |
| 20 | 20.21 | 1.89 | 20.14 | 2.50 |
| 40 | 40.12 | 1.25 | 40.08 | 1.88 |

**Table 3.** Recovery of Montelukast Sodium

| Amount of drug added (mg) | Mean(± s.d) amount (mg) Found (n=5) | Mean(± s.d) % of recovery (n=5) |
|---|---|---|
| 20 | 20.03 ± 0.05 | 100.15 ± 0.30 |
| 40 | 39.9 ± 0.09 | 99.95± 0.40 |

tablets. The tablets were found to contain 99.98 % to 100.1 % of the labeled amount. The low % CV indicates the reproducibility of the assay of Montelukast Sodium in the tablet dosage form. The proposed HPLC method was found to be simple. Precise, highly accurate, specific and less time consuming. Hence it is a preferred method over the reported methods for the estimation of Montelukast sodium in pharmaceutical dosage forms.




| | Retention Time (min) | Area (mV s) | Height (mV) | Area (%) | Height (%) | W05 (min) |
|---|---|---|---|---|---|---|
| 1 | 3.017 | 145.764 | 11.242 | 100.0 | 100.0 | 0.20 |
| | Total | 145.764 | 11.242 | 100.0 | 100.0 | |

**Fig. 2.** RP-HPLC Chromatogram for Montelukast Sodium

The authors are grateful to M/s Vijayasri Chemicals, Hyderabad, for providing pure drug samples, and the management of K L University, Vaddeswaram, Guntur District for their continuous support and encouragement and for providing the necessary infrastructure facilities for executing this work

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Current Trends in Biotechnology and Pharmacy
Vol. 4 (4 ) 947-956 October 2010 ISSN 0973-8916


# Ethanol Tolerant Anaerobic Cellulolytic Ethanologenic Bacteria Isolated from Decomposed Paper

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**Abstract**

Ethanol tolerant anaerobic cellulolytic bacteria were isolated from decomposed paper. The better ethanol tolerant isolate MD2 was characterized and identified as *Clostridium* sp. based on morphological, cultural and biochemical characterization following standard methods. The major fermentation products were ethanol and small amounts of acetic acid and $CO_2$. The optimum fermentation conditions observed for maximum ethanol production and substrate degradation were 40 °C, pH 7.5 and 5% innoculum and the isolate was found to be ethanol tolerant to 3% ethanol. Selected untreated and pre-treated cellulosic agro-wastes supported growth and ethanol production by MD2. Maximum ethanol yield of 0.269 (g/g) was obtained with alkali treated banana leaves and pseudostem and 0.225 (g/g) with avicel. This is also the first report on ethanol tolerant mesophilic *Clostridium* sp. for single step conversion of banana waste to ethanol.

**Key words:** *Clostridium*; Mesophilic; Bioethanol; Cellulolytic; Biomass

## Introduction

Biomass in its variety of forms is a key source of renewable energy for use as solid, liquid and gaseous fuels. The production and utilization of biomass is a primary need for the energization of rural areas in India and for most of the developing countries. Different epochs of history have given preference to different forms of energy. During the industrial revolution, coal was the most favored form, which brought about far-reaching changes in the commercialization of the traditional processes. This was followed by oil, which helped to usher in many significant transformations, particularly in the transportation and industrial sectors. Since the 1970's serious thought began to be given to search for alternative, renewable and non-polluting sources of energy, such as small, mini & micro-hydro, solar and bio-energy. Bio-energy offers very great scope due to a wide spectrum of biomass available under different agro-climatic conditions. Plant biomass is the only foreseeable sustainable source of fuels and materials available to humanity (1, 2). Cellulosic materials are particularly attractive in this context because of their relatively low cost and plentiful supply.

Bio-ethanol is derived from alcoholic fermentation of simple sugars, which are produced from biomass. It is one of the best eco-friendly technologies for biomass conversion in to energy. It is used as a biodegradable fuel additive (3). Ethanol can be produced by either aerobic or anaerobic fermentation. More than

95% of fermentation ethanol produced world wide employs yeast and its related species. Major part of the alcohol produced by yeast is for human consumption. Anaerobic fermentations on the other hand are intriguing for commercial use, because they don't require the expense of large volumes of sterile air or the expense of energy input into the fermentation in the form of vigorous impeller action. For ethanol to be commercially competitive with fossil fuels, reduction in the production cost is necessary. Today, the raw material and the enzyme production are two of the main contributors to the overall cost. Bio-ethanol produced from pretreatment and microbial fermentation of biomass has great potential to become a sustainable transportation fuel in the near future (4, 5).

Energy is obtained from biomass either by direct combustion or by microbial action. Amongst microbial conversion of biomass to fuels, production of ethanol has a great potential and is one of the best eco-friendly technologies for biomass conversion into energy. Energy ranks alongside population growth and food supply as central obstacles to economic growth and social welfare.

The interest on the bioconversion of agricultural waste to liquid fuel has led to extensive studies on cellulolytic, ethanologenic micro organisms. A majority of microbes can however degrade modified cellulose. Fermentation of ethanol produced world wide employs yeast, *Saccharomyces cerevisiae* and its related species (6, 7). However yeasts have a narrow substrate spectrum and can only ferment xylose, glucose, fructose and sucrose. Cellulolytic bacterium such as *Clostridium thermocellum* converts cellulosic biomass to ethanol in single step fermentation (8, 9).

Cellulolytic mesophilic bacteria ferment cellulosic biomass to ethanol at mesophilic temperatures. The bacteria hydrolyze both cellulose and hemicellulose substrates to their oligodextrins and monomeric components. Cellulolytic C*lostridium* species are able to ferment cellulose and cellobiose producing ethanol as the major fermentation product (1, 4, 9) and except for few species the difference seems to be in their ability to assimilate glucose and xylose (10, 11). The major problem in the conversion of biomass to ethanol by *Clostidium* species is the accumulation of xylose, cellobiose and glucose which repress cellulose fermentation resulting in low ethanol yields (12) and their marked intolerance to ethanol (13). These problems might be alleviated by isolating cultures which are more efficient in fermenting sugars to ethanol with good ethanol tolerance (14, 15). Therefore it is helpful to identify species which are more efficient than existing in the bioconversion of cellulose and more adaptable to practical fermentation conditions. In the present study various physical parameters, substrate utilization capability, ethanol produced and soluble sugars formed on cellulose fermentation by *Clostridium* sp. MD2 were studied.

## Materials and Methods

***Microorganisms and culture conditions :*** The bacterial isolates *Clostridium* sp. MD1 and MD2 were isolated from decomposed paper by enrichment culture technique using CMS medium. The isolate were grown in 120 ml serum vials with 20ml of pre-reduced cellulose mineral salt (CMS) medium pH 7.5 containing (g/l): $KH_2PO_4$, 1.5; $KH_2PO_4$, 2.0; Urea, 2.0; $MgSO_4$, 0.8; $CaCl_2$, 0.15; sodium citrate, 3.5; cysteine HCl, 0.15; yeast extract, 4.0; resazurin, 0.002; cellulose, 10.0; in $N_2$ atmosphere. Cellulose substrate was replaced with same concentration of cellulosic agrowaste in CMS medium. The other media used in the study are CM3 medium (g/l):$H2PO_4$,1.5;$K_2HPO_4$ 3 $H_2O$, 2.9; $MgCl_2$ $6H_2O$, 0.2; $CaCl_2$ $2H_2O$, 0.75; Yeast Extract, 2.0; $(NH_4)_2$ $SO_4$, 1.3; $FeSO_4$ 7

$H_2O$,1.25 ; L-Cysteine HCl, 0.5 ; Carbon Source,10 ; Resazurin, 0.001. CM4 medium (g/l): Cellobiose, 6.0; Yeast extract, 5.0; $K_2HPO_4$, 2.9; $KH_2PO_4$, 1.5; $(NH_4)_2$ $SO_4$, 1.3; NaCl, 1.0; Mg $Cl_2$, 0.75; Sodium thioglycolate, 0.5; $CaCl_2$, 0.0132; $FeSO_4$ (1.25% solution), 0.1 ml; Carbon Source,10; Resazurin (1% solution), 0.2 ml. MJ Medium (g/l): NaCl, 30.00; $K_2HPO_4$, 0.14; $CaCl_2$ 2 $H_2O$, 0.14; $MgSO_4$ 7 $H_2O$, 3.40; $MgCl_2$ 6 $H_2O$, 4.18; KCl, 0.33; $NH_4Cl$, 0.25; $NiCl_2$ 6 $H_2O$, 0.50; $Na_2SeO_3$ 5 $H_2O$, 0.50; $Fe(NH_4)_2(SO_4)_2$ 6 $H_2O$, 0.01; Trace element solution, 10.00 ml; $NaHCO_3$, 1.50, $Na_2S_2O_3$ x 5 $H_2O$, 1.50, Carbon Source, 10, Resazurin (1% solution) 0.2 ml. The medium was sterilized by autoclaving at 121 °C for 30 min. The cultures were stored at 4°C in refrigerator and repeatedly sub-cultured once in a month in CMS medium. Ethanol produced, reducing sugars and substrates degraded were estimated by the following mentioned methods.

The cellulolytic and ethanologenic bacterial isolate MD2 was identified by morphology, staining, cultural and biochemical characteristics in comparison to those characteristics of *Clostridium* sps (16, 17, 18).

***Cellulosic substrates :*** Filter paper, avicel, native cotton and CMC (Carboxy methyl cellolse) were used as pure cellulosic substrates; agricultural cellulosic substrates used were *Parthenium* weed, Sunflower stalk, Pongamia shells, Groundnut shell, Banana waste which includes leaves and pseudostem and Pongam shells. The cellulosic agro-wastes were washed thoroughly with water, dried cut into approximately 1cm pieces and these were subjected to treatment.

***Preparation of dried cellulosic agro-waste :*** About 250g of cellulosic agrowaste was taken in a 500ml beaker and dried in a hot air oven at 60 °C to constant weight. Dried material was cut into 1x1 cm size pieces and used as substrate for fermentation.

***Preparation of water treated agro-waste :*** About 250g of agro-waste (dried pieces) was taken in 1 liter conical flasks, containing 500ml of distilled water and boiled for 30 minutes. The supernatant was decanted, and the residue was thoroughly washed with distilled water until the colouring compounds were removed. The residue was then dried at 60°C to constant weight.

***Preparation of alkali or acid treated agro-waste :*** About 250g of the agro-waste (dried pieces) was taken separately in 1 liter conical flasks containing 500ml of 1% of NaOH or 1% $H_2SO_4$ and autoclaved at 121°C for 15minutes. The supernatant was decanted and the residue was neutralized with 1% $H_2SO_4$ or 1% NaOH. The residue was thoroughly washed with distilled water until no colour was imparted to the water and dried at 60°C to constant weight.

***Estimation of ethanol and reducing sugars :*** For the estimation of ethanol 10ml of fermented broth was centrifuged at 10,000 g for 30min at 4 °C. The supernatant was acidified with 1ml of 2N phosphoric acid and 2µl was injected into a chromosorb 101 column, 80-100 mesh in a CIC gas chromatograph equipped with flame ionization detector (86 PRO). The following parameters were chosen for analysis: Oven temperature, 160 °C; injector temperature, 170 °C; carrier gas, $N_2$; and flow rate, 20 µl per min. (19). The reducing sugars were estimated by DNS reagent as described by Miller (20).

***Cellulase enzyme assay :*** 10ml of fermented broth was taken and centrifuged at 10,000g for 15 min and the supernatant was used as crude enzyme. The cellulase enzyme activity was measured as CMCase and filter paperase (FPase) using Carboxy methyl cellulose and Whatman no 1 filter paper as substrates respectively according to the method of Mandels et al., (21).

## Results and Discussion

For the enrichment of bacteria able to convert cellulosic biomass to ethanol (cellulolytes), decomposed paper sample was collected and dilution plating was done in 60ml vials containing CMS medium with 1% cellulose as substrate. Cellulase positive colonies were identified based on zone of hydrolysis. Two bacterial isolates, MD1 and MD2 were found to be positive for cellulose degradation (Table 1). Rate of cellulose degradation for the selected isolates MD1 and MD2 was between 4-7 days with corresponding ethanol tolerance of 1.5 and 2.3% (v/v) respectively at 40 °C. Isolate MD2 with high ethanol tolerance was selected for further study. The cellulolytic, ethanologenic isolate MD2 was identified as *Clostridium* sps by morphology, staining, cultural and biochemical characteristics (Table 2), in comparison to those characteristics reported for *Clostridium* sps. (16, 17, 18). The isolate was Gram positive, obligately anaerobic, rod shaped bacterium and did not grow even under micro aerophilic conditions suggesting that it belongs to the genus *Clostridium* (22). The major metabolic products on cellulose degradation were ethanol and negligible amounts of acetic acid. Fermentation of cellulose to ethanol by *Clostriidum* sp. MD2 was studied in different synthetic media and CMS medium was selected for further studies based on maximum ethanol yield (Table 3). The optimum growth of the organism was observed at 40 °C (Figure 1) and it showed a pH tolerance between 6-9.5, better ethanol yield with maximum cellulose degradation was observed at pH 7.5 (Figure 2). An innoculum size of 5% was found to be best for maximum cellulose degradation and ethanol production (Figure 3). The optimum incubation time for maximum cellulose degradation and ethanol

**Table1.** Rate of cellulose degradation and ethanol tolerance of the cellulolytic isolates from decomposed paper

| Source | Isolate label | Temperature °C | Rate of cellulose degradation (days) | Ethanol tolerance (% v/v) |
|---|---|---|---|---|
| Decomposed paper waste | MD1 | 40 | 7 | 1.5 |
| | MD2 | 40 | 4-6 | 2.3 |

**Table 2**. Identification characteristics of cellulolytic mesophilic bacterial isolate MD2

| Character/test | Result |
|---|---|
| Temperature °C | 35 to 45 °C; optimum 40 °C |
| pH | 7 – 8; optimum 7.5 |
| Differential staining | Gram positive |
| Pigmenttion | +; yellow |
| Final Product | Alcohols; acids; CO2 |
| Structural Morphology | Slightly curved rods |
| Catalase test | + |
| Cellulose | + |
| Glucose | + |
| Mannose | + |
| Arabinose | + |
| Indole Production | - |
| Sorbitol | + |
| Mannitol | + |
| Maltose | + |
| Galactose | + |
| Lactose | + |
| Sucrose | + |
| Fructose | + |
| Cellobiose | + |
| Organism identified as *Clostridium* sp | |

**Table 3.** Fermentation characteristics of C*lostridium* sp. MD2 in different media

| Medium used | Ethanol produced (g/l) | Reducing sugars (g/l) | Substrate degraded (g/l) | Ethanol yield (g/g) |
|---|---|---|---|---|
| MJ medium | 1.11 | 2.71 | 6.0 | 0.185 |
| CM3 medium | 1.20 | 2.41 | 7.0 | 0.171 |
| CM4 medium | 1.10 | 2.56 | 6.79 | 0.162 |
| CMS medium | 1.30 | 2.32 | 6.32 | 0.205 |

**Table 4.**Effect of different concentrations of added ethanol on cellulose fermentation by *Clostridium* sp. MD2

| Ethanol concentration (%v/v) | Acetic acid (g/l) | Reducing sugars (g/l) | Substrate degraded (g/l) |
|---|---|---|---|
| 0 | 0.82 | 2.32 | 6.32 |
| 1 | 0.89 | 2.39 | 6.32 |
| 2 | 1.11 | 2.42 | 6.30 |
| 3 | 1.15 | 2.49 | 6.29 |

production was 120h (Figure 4). The isolate MD2 could tolerate up to 3% (v/v) ethanol in the medium (Table 4).

*Clostridium* sp. MD2 efficiently fermented (> 75% substrate at 10g/l) variety of crystalline, pure substrates such as Avicel, filter paper, native cotton, CMC and crude agricultural cellulosic materials such as parthenium, pongamia shells, groundnut shells, sunflower stalk and banana waste. The cellulase activity of the selected organism was 0.72 units/ml/min (CMCase) and 0.0113 units /ml/min (Filter paperase). The selected isolate MD2 had broad saccharolytic ability and fermented various mono, di and polysaccharide substrates except inulin (Table 5). Cellobiose was found to be the best substrate for ethanol production by MD2 followed by sucrose, glucose and maltose in the decreasing order. *Clostridium* sp MD2 produced 0.13g to 0.19g ethanol per gram substrate (sugar) consumed (Table 5). It fermented all the selected pure cellulosic substrates, but maximum ethanol yield of 0.225 g/g was obtained with avicel (Table 6). Agricultural wastes like pongamia shells, banana waste, parthenium weed (total) sunflower stalk and groundnut shell were used as substrates for ethanol production. The *Clostridium* sp. MD2 was not only able to grow on various agricultural cellulosic substrates but also fermented them to ethanol (Table 7). A maximum ethanol yield of 0.144 g/g was obtained with untreated banana waste followed by 0.136 g/g with untreated pongamia shells. Further, pongamia shells and banana waste were subjected to different pretreatments like water extraction, acid treatment and alkali treatment. Pretreated agricultural cellulosic substrates supported appreciable growth of the selected organism and ethanol production (Table 8 & 9). *Clostridium* sp. MD2 produced ethanol in the range of 1.21-1.42 (g/l) with preteated pongamia shells (Table 8). A maximum ethanol yield of 0.196 (g/g) was obtained with alkali treated pongamia shells. Banana leaves, pseudo stem and total banana waste were subjected to pretreatments and used

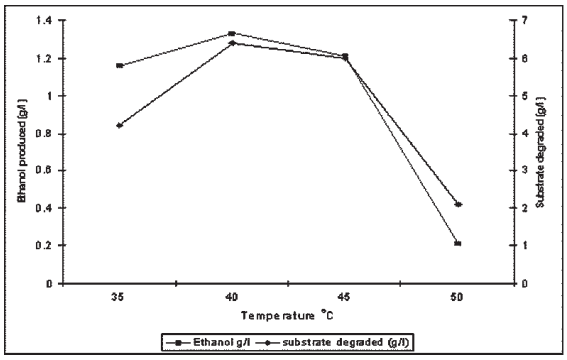


**Fig. 1.** Effect of temperature on fermentation of cellulose (Whatman No 1 filter paper) to ethanol by *Clostridium* sp. MD2

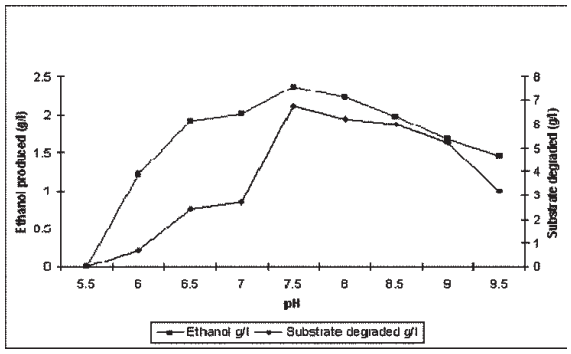


**Fig. 2.** Effect of pH on fermentation of cellulose (Whatman No 1 filter paper) to ethanol by *Clostridium* sp. MD2

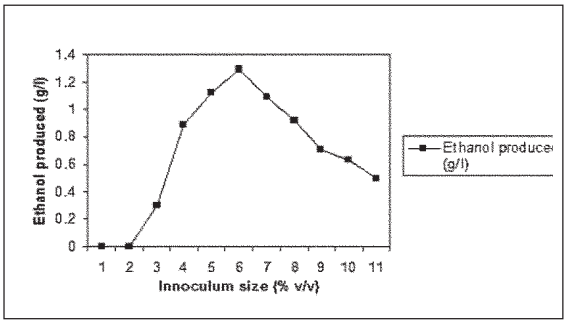


**Fig. 3.** Effect of inoculum size on fermentation of cellulose to ethanol by *Clostridium* sp. MD2

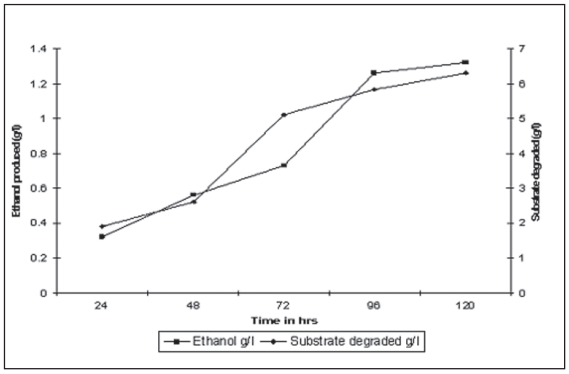


**Fig. 4.** Time Course study on fermentation of cellulose to ethanol by *Clostridium* sp. MD2

**Table 5.** Fermentation of different sugars for ethanol production by *Clostridium* sp. MD2

| Substrate | Ethanol produced (g/l) | Substrate degraded (g/l) | Ethanol yield (g/g) |
|---|---|---|---|
| Glucose | 0.89 | 5.0 | 0.178 |
| Fructose | 0.67 | 3.94 | 0.17 |
| Sucrose | 0.62 | 3.44 | 0.18 |
| Maltose | 0.60 | 3.33 | 0.18 |
| Starch | 0.72 | 4.5 | 0.162 |
| Cellobiose | 1.26 | 6.6 | 0.1 |
| Xylose | 0.52 | 3.8 | 0.136 |
| Inulin | NG | NG | NG |

NG: No growth

**Table 6**. Fermentation of pure cellulosic substrates to Ethanol by *Clostridium* sp. MD2

| Substrate | Ethanol produced (g/l) | Substrate degraded (g/l) | Ethanol yield (g/g) |
|---|---|---|---|
| Whatman no 1 filter paper | 1.30 | 6.32 | 0.206 |
| Avicel | 1.21 | 5.36 | 0.225 |
| Native cotton | 0.95 | 5.29 | 0.179 |
| CMC | 0.22 | 3.1 | 0.07 |

**Table 7.** Fermentation of different untreated agricultural cellulosic substrates to ethanol by *Clostridium* sp. MD2

| Untreated Agricultural Substrate (g/l) | Ethanol produced (g/l) | Reducing sugars (g/l) | Substrate degraded (g/l) | Ethanol yield (g/g) |
|---|---|---|---|---|
| Parthenium weed | 0.3 | 3.56 | 3.00 | 0.100 |
| Pongamia Shells | 1.16 | 2.78 | 8.529 | 0.136 |
| Sunflower Stalk | 1.14 | 2.71 | 9.26 | 0.123 |
| Ground nut Shell | 1.11 | 3.01 | 9.40 | 0.118 |
| Total Banana waste | 1.26 | 2.88 | 8.75 | 0.144 |

**Table 8.** Ethanol production using Pongamia shells by *Clostridium* sp. MD2

| Substrate | Ethanol produced (g/l) | Reducing Sugars (g/l) | Substrate Degraded (g/l) | Ethanol yield (g/g) |
|---|---|---|---|---|
| Pongamia shells-Dry | 1.21 | 2.62 | 7.462 | 0.162 |
| Water extracted pongamia shells | 1.28 | 2.54 | 7.619 | 0.168 |
| Alkali treated pongamia shells | 1.42 | 2.48 | 7.244 | 0.196 |
| Acid treated pongamia shells | 1.36 | 2.43 | 7.195 | 0.189 |

for ethanol production. The ethanol production by MD2 using banana waste was in the range of 1.75-1.86 (g/l) (Table 9). A maximum ethanol yield of 0.269 (g/g) was obtained with alkali treated banana leaves and psuedostem as substrates.

*Clostridium* sp. MD2 in its ability to ferment various sugars resembles *C. thermocellum* M7 reported by Lee and Blackburn (23). It gave maximum ethanol yield of 0.225 (g/g) with avicel and 0.269 (g/g) with banana leaves and psuedostem as substrates. Further it showed a maximum ethanol tolerance of 3% (v/v), which is higher than recent reports on *Clostridium* sps (24 , 25, 26).

**Table 9.** Ethanol production using banana waste by *Clostridium* sp. MD2

| Substrates | Ethanol (g/l) | Reducing sugars (g/l) | Substrate degraded (g/l) | Ethanol yield (g/g) |
|---|---|---|---|---|
| Banana Leaves-Dry | 1.81 | 2.01 | 6.86 | 0.263 |
| Banana Leaves-Water extracted | 1.75 | 2.13 | 6.83 | 0.256 |
| Banana Leaves-Acid treated | 1.80 | 2.03 | 6.76 | 0.266 |
| Banana Leaves-Alkali treated | 1.78 | 1.98 | 6.61 | 0.269 |
| Banana Pseudo stem-Dry | 1.80 | 1.89 | 6.72 | 0.267 |
| Banana Pseudo stem-Water extracted | 1.80 | 1.92 | 6.82 | 0.263 |
| Banana Pseudo stem-Acid treated | 1.81 | 1.90 | 6.79 | 0.266 |
| Banana Pseudo stem-Alkali treated | 1.85 | 1.90 | 6.851 | 0.269 |
| Total Banana waste-Dry | 1.86 | 1.90 | 7.209 | 0.258 |
| Total Banana waste-Water extracted | 1.81 | 2.03 | 7.154 | 0.253 |
| Total Banana waste-Acid treated | 1.83 | 1.98 | 7.038 | 0.260 |
| Total Banana-Alkali treated | 1.85 | 1.9 | 7.186 | 0.263 |

## Conclusion

*Clostridium* sp. MD2 has high ethanol tolerance (3% (v/v)) compared to recent reports. Ethanol yields obtained were also higher than the reported among mesophilic *Clostridium* sps. Because of its high ethanol tolerance and good ethanol yield it can be developed further as a potential strain for single step fermentation of cellulose to ethanol. Further studies on cellulose degradation at higher concentrations and also scaling up studies will give new insights into single step fermentation of cellulosic biomass to ethanol.

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Current Trends in Biotechnology and Pharmacy
Vol. 4 (4 ) 957-970 October 2010 ISSN 0973-8916


# *Agaricus brasiliensis*-enriched functional product promotes in mice increase in HDL levels and immunomodulate to Th1 CD4+T subsets. *A. brasiliensis* functional product and biological benefits.

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**Abstract**

The fruiting bodies and liquid-culture mycelia of *A. brasiliensis* (= *A. blazei*, *A. subrufescens* or Sun mushroom) has been reported to possess immunomodulatory and antitumoral activities and it is used for cure and prevention of diseases, but the effect of solid state culture mycelium have nevertheless been analyzed. This study investigated the effect of consumption of wheat grains cultured with the *Agaricus brasiliensis* mycelium on the metabolism and immunomodulatory alterations in mice. In our data was reported a protein enrichment of 6% on the cultured wheat grains, which present also high levels of ergosterol, and presence of cardiac glycosides. The diet intake by the mice resulted in increase in HDL cholesterol concentration and reduction in triacylglyceromia in plasma. Moreover, there were increases in the ratio of CD4+T to CD8+T cell and in levels of IFN-ɡ and IL-6 in plasma. However, in peritoneal macrophage culture, the levels of IFN-ɡ and IL-6 were unchanged, but the IL-12 concentration was increased; while TNF-a and MCP-1levels were reduced in mice from the *A. brasiliensis* biocultured material group compared to the control. In summary, the solid state culture demonstrated to be a very good technique to develop *A. brasiliensis* mycelium on wheat grains to obtain a protein enriched biocultured material. The long intake of this biocultured material by mice promoted an increase in the "good cholesterol" plasma concentration and probably expansion of the Th1 CD4+T cells.

**Key Words:** Functional product, Sun mushroom, solid state culture, mycelium, immunological effects.

## Introduction

The diet of Western societies has high content of simple sugars and fat, however low in fibers. In addition, they have a sedentary life style where both factors have been associated to the

development of several diseases such as cancer, autoimmune, hypercholesterolemia, among others (1).

Several experimental, clinical and epidemiological studies have shown the incontestable causal relationship between low HDL-cholesterol plasma concentration and coronary heart disease on an atherosclerotic basis (2). On the other hand, HDL-cholesterol exerts atheroprotective effects (2).

Oxidative stress is caused by overproduction of reactive oxygen and/or nitrogen species, such as $H_2O_2$, $NO_3$-, $O_2$·-. Tissue macrophages contribute to sustained oxidant stress events through the activity of the enzymes NADPH oxidase and inducible nitric oxide synthase (3). It has been reported that dietary consumption of nutrients rich in polyphenols and antioxidant molecules protects LDL against lipid peroxidation and inhibits the development of aortic atherosclerotic lesions. Mushrooms fulfill such role and have been used for dietetic prevention due to their elevated content of fibers, protein, microelements and low fat values (4).

*Agaricus brasiliensis* (5) has synonyms as *Agaricus blazei* or *Agaricus subrufescens* (6) and is also known as Sun mushroom and Himematsutake. It has been reported to have antitumoral and immunostimulant activity (7). Many studies pointed to the excellent antioxidant activity of the mushroom *A. brasiliensis*, due to the presence of ascorbic acid, a, b and d-tocoferol, and phenolic compounds which have chelating and scavenging properties and can act as antioxidant and reducing agents (8).

Most studies with this mushroom have been done with animals bearing diseases and the usual experimental approach was the mushroom consumed orally by intake of the fruiting body or of an extracted form, and sometimes the mycelia produced by submerged culture (9). In addition, some studies inoculate intraperitoneally the polysaccharides extracted from these materials (10). Despite all that, interestingly there is no report on the effect of consumption of *A. brasiliensis* mycelia produced in solid-state culture in healthy animal models aiming to study prevention against illness and we are not aware of any investigation in a state of disease. This lack of information lead us to investigate if a solid state *A. brasiliensis* mycelium cultivated on wheat grains and ingested via oral as nutraceutical food has the ability to promote physiological activities. Here, for the first time, it was tested the inclusion of 10% solid state culture *A. brasiliensis* mycelium in the diet of healthy mice during 12 weeks and investigated their effect on plasma cholesterol concentration and immune response *in vivo* and *ex vivo*.

## Materials and Methods

***Chemicals and Medium :***Chemicals and cell culture medium were obtained from Sigma Chemical CO. (St. Louis, MO, USA); Merck (Darmstadt, Germany); Oxoid (Hampshire, England). The wheat grains were bought in the local market. Fluorescent–labeled monoclonal antibodies (phycoerythrin-PE-conjugated anti $CD4^+$, anti $CD8^+$ and anti $CD19^+$) were purchased from BD Biosciences (California, USA) and those directly conjugated (PE) antibody to the various surface proteins were from Pharmingen (California, U.S.A.).

***Microorganism, Pre-inoculum and Inoculum Preparation :*** Strain of *A. brasiliensis*–LPB-03 was cultured in Petri dishes with PDA during 10 days at 30 ºC. The pre-inoculum was prepared with five pieces of mycelium ($1cm^2$) cutted and putted into 50 ml of medium containing ($g.L^{-1}$): glucose (20), yeast extract (3.95), $MgSO_4.7H_2O$ (0.3), $K_2HPO_4.3H_2O$ (0.5), pH 6.0 (±0.2) previously sterilized (121 ºC during 15 min)

(10). The incubation was carried out at 30 ºC, 120 rpm, during 7 days. The mycelium was passed through a filter (mesh of 0.5 $mm^2$) with help of a spatula to break the mycelium and this filtration procedure was improved by washing the biomass on the filter with 50 ml of distilled sterilized water. The inoculum was prepared with this mycelium suspension, it was inoculated in 500 ml of medium ($g.L^{-1}$): glucose (35), yeast extract (2.5), peptone (5), $KH_2PO_4$ (0.88), $MgSO_4.7H_2O$ (0.5), pH 5.5 in the same conditions described for the pre-inoculum. Suspension of the mycelium was obtained by new filtration procedures as described above, with new washing of the biomass with 500 ml of distilled sterilized water and used as inoculum of the wheat grains (11).

***Solid State Cultivation Procedures :*** Wheat grains, previously washed, and wet during 12 hours in clean water, put in polyethylene's tray and sterilized at 121 ºC, 1.2 Torr for 50 min were used as substrate. The inoculation was done using the mycelium suspension obtained as described above, which biomass content analysis (by dry weight) resulted in calculation of the inoculation rate: 0.653g of mycelium per Kg of wheat .The mycelium suspension was mixed to the wheat grains in sterile conditions and the initial humidity of inoculate substrate was adjusted to 40% ± 2 with addition of sterile water. The trays were recovered with filter paper and the incubation was carried out at 30 ºC, 90% relative humidity of air, during 18 days (12).

The cultured wheat grains were dried out (55 ºC), milled (particles<2mm), and analyzed for proximate composition: moisture (content was determined by dry weight to constant mass after 24-36 hour drying at 60° C); crude protein (nitrogen determination by Kjehdahl N x. 6.25); lipids (by Soxhlet method); crude fiber (residue after acid and alkaline digestion); crude ash (muffle furnace) and carbohydrate (by difference) in triplicate by the standard procedure of AOAC (12). The milled cultured wheat grains where supplemented with lipids (soy oil) and proteins (egg albumin) to fill up the nutrition needs (13).

Regular chow was mixed with 10% of this cultured wheat grains and denominated as *A. brasiliensis* biocultured material (treatment). All diets contained the same amounts ($g.Kg^{-1}$) of protein (230), carbohydrate content (660), fat content (40), fiber (60), and vitamins and minerals (10) (Nuvital®CR-1 Curitiba, Pr., Brazil).

***Analysis of the Cultured Wheat Grains with Agaricus brasiliensis :*** Samples of the cultured wheat grains were analyzed by HPLC for ergosterol content (14) and used to calculate the fungi biomass content. Besides this, the presence of flavonoids (UV Shinoda´s reaction); anthraquinones (Bornträger Reaction*);* Alcaloids (RGA´s reactive: Dragendorff, Bertrand, Wagner e Valser-Mayer); Tannins (heavy metals and protein precipitation) and cardiac glycosides (Keller Killiani and Pesez reactions) were analyzed too (15).

***Study Design :*** The University Federal of Paraná Committee of Animal Welfare approved all procedures involving animals. Swiss female mice (*Mus musculus*) aged 30-35 days, weighing 17-24 g were divided into two groups, with 7 mice per group. The control group (C) received normal chow during 12 weeks, while another set of mice was fed with *A. brasiliensis* biocultured material (*Ab*). Water and chow were supplied *ad libidum* and the body weight monitored weekly.

***Blood Metabolite Concentrations :*** At the end of $12^{th}$ week, blood from all mice fed with *A. brasiliensis* biocultured material and control group were withdrawn via heart puncture under

diethyl ether anesthesia. The samples were centrifuged (2054.*g*.10 $min^{-1}$) and plasma glucose concentration, triacylglycerol, HDL-cholesterol and total cholesterol were measured in ADVIA 1650 (Bayer) automated equipment.

***Preparation of B and T cell population :*** Spleens from both groups were removed and pools of lymphocytes were obtained from three or four mice (16). Briefly the organs were immersed in PBS. The spleen cells were obtained by mechanical disruption of the capsule by using a syringe plunger against the Petri dishes. The red blood cells were removed by using a hemolytic solution. Then the solution containing the cells was centrifuged at 900 x *g* for 10 min and the cells were resuspended in PBS buffer containing 2% BSA ($w.v^{-1}$).

***Determination of Lymphocyte Populations by Flow Cytometry :*** Flow cytometry was used to measure and identify $CD4^+$, $CD8^+$ and, $CD19^+$ lymphocytes, helper, cytotoxic and B cells respectively, on the surface of freshly prepared spleen lymphocytes. Pretitered antibodies directly conjugated to PE were added to the cell suspensions ($1.10^6 ml^{-1}$) at 4°C and incubated for 20–30 min in the dark with fluorescent-labeled monoclonal antibodies to PE-$CD4^+$ (clone H129.19), PE-$CD8^+$ (clone 53-6.7) and PE-$CD19^+$ (clone 1D3). After staining, the cells were washed twice with PBS and resuspended in fixative PBS staining buffer with 2% ($wt.vol^{-1}$) paraformaldehyde until analysis for fluorescence by using a Becton Dickinson FACScalibur.

***Peritoneal Macrophage Isolation :*** Immediately after blood harvesting from *A. brasiliensis*-treated and control animals, their resident peritoneal macrophages were obtained by intraperitoneal (i.p.) lavage with 5 ml of sterile phosphate buffer saline (PBS buffer- pH 7.2, NaCl 0.8% and KCl 0.02%). The peritoneal macrophages were collected by centrifugation (290.*g,* 4ºC for 5 min), washed, and ressuspended in PBS after counting in a Neubauer chamber by optical microscopy using a Trypan blue solution (1%); with viability higher than 95%. A macrophages pool was obtained from 3 or 4 animals. Peritoneal macrophages were further purified by incubating peritoneal cells in tissue culture plates for 2 h and then washing three times with PBS to remove the non-adherent cells and used for the *ex-vivo* assays (17).

***Cytokines Determination :*** Cytokines determination was done from peritoneal macrophages culture supernatants and also from the plasma of *A. brasiliensis*-treated and control animals. Concentrations of IL-12p70, IFN-ɡ (Interferon gamma), TNF-a (Tumor Necrosis Factor alpha), IL-6, MCP-1 (Monocytes chemoattractant protein-1) and IL-10 were determined by flow cytometry using a FACSCalibur with CELL Quest software (BD Biosciences). The results are expressed as $pg.mL^{-1}$.

***Peritoneal Macrophages Assays ex vivo***

***Phagocytosis :*** Aliquots ($1x10^5$ cells in a final volume of 0.1 ml) of the peritoneal macrophages suspension were added to the wells of a 96-well flat-bottomed tissue culture plate and left to adhere for 60 min. Then 10 ìl of neutral-red stained zymosan ($1x10^8$ particles.$mL^{-1}$) was added to each well. After incubation for 30 min, the cells were fixed with Baker's formol-calcium (4% formaldehyde, 2% sodium chloride, 1% calcium acetate) to each well. After 30 min, the absorbance at 550 nm of each well was read on a plate reader. Phagocytosis was calculated from a standard curve constructed from known amounts of stained zymosan and results expressed per mg protein (18).

***Lysosomal Volume :*** The uptake of the cationic dye neutral red, which concentrates in cell lysosomes, was used to assess the volume of the macrophage lysosomal system. Twenty microliters of 3% neutral-red in PBS were added to 0.1 ml of peritoneal macrophage suspension per micro plate well for 30 min. The cells were then washed twice with PBS by centrifugation (453g for 5 min). Neutral red was solubilized by a 30-min incubation adding 0.1 ml of 10% acetic acid plus 40% ethanol solution. The absorbance was read at 550 nm and neutral red uptake calculated per mg protein (17).

***Hydrogen Production :*** This assay is based on the HRPO- dependent conversion of phenol red into a colored compound by $H_2O_2$ (19). Peritoneal macrophages (final volume 0.1 ml) were incubated in the presence of glucose (5 mM), phenol red solution (0.56 mM), and HRPO (8.5 $U.ml^{-1}$) in the dark for 1 h at 20 ºC. After this period, the absorbance was measured at 620 nm on a plate reader. The concentration of $H_2O_2$ was determined from a standard curve prepared in parallel. $H_2O_2$ production is expressed as ìmol.$mg^{-1}$ protein.

***Superoxide Production :*** The $O_2^{\cdot -}$ production was estimated by the nitroblue tetrazolium (NBT) reduction assay. Peritoneal macrophages ($4.5x10^5$ cells in a final volume of 0.45 ml) suspended in PBS were incubated for 1 h at 37ºC in the presence of 0.03 ml of phorbol myristyl acetate (10µg/ml) and NBT (0.1%). After adding 0.45 ml of acetic acid (50%) stopped the reaction. Then, the mixture was centrifuged for 30 s at 2500 x *g*. Reduction of NBT results in the formation of blue formazan (proportional to production of $O_2^{\cdot -}$), which was detected spectropho-tometrically (560 nm). The results are expressed as absorbance/mg protein (18).

***Nitric Oxide Production :*** NO was measured as $NO_2^-$. Peritoneal macrophages ($2x10^5$ in a final volume of 0.2 ml) were incubated for 24 h in the absence or presence of LPS (10 ìg.ml final concentration$^{-1}$). Nitrite concentration was measured by the Griess reaction. Equal volumes of cell culture supernatant and Griess reagent were incubated for 10 min at room temperature and the absorbance measured at 550 nm. $NO_2^-$ concentration was determined from a standard curve generated using $NaNO_2$. Nitrite production is expressed per ìmol.$L^{-1}$ (17).

***Protein Determination :*** Protein concentration from peritoneal macrophages preparations were measured by the Bradford method (19), using bovine serum albumin as standard.

***Statistical Analysis :*** The results are expressed as the mean ± SD of at least three independent experiments. Statistically significant differences between groups were determined by one-way ANOVA followed by Tukey test. A *P* value = 0.05 was considered statistically significant.

## Results

***Culture Time and Analysis of the Cultured Wheat Grains with Agaricus brasiliensis :*** The use of wheat grain for the production of *A. brasiliensis* mycelium by solid state culture resulted in a good development without addition of any other nutrient. During the second and third cultivation days we noticed a fast growth from mycelia on all wheat grains, probably due a) the two steps inoculum preparation which resulted in a strong inoculum; b) the filtration procedures provided a broken mycelium, which grows faster than large pieces of mycelium (Data not shown).

Moreover, the *A. brasiliensis* cultured wheat grains presented an increase of crude fiber and ash values, an increase by approximately 6 % of protein and also a presence of cardiac glycosides (Table 1). Furthermore, these grains had an increase in the ergosterol content of 1.95

mg.g$^{-1}$ when compared to 0.002 mg.g$^{-1}$ from wheat grains *in natura* (Table 1). The amount of ergosterol founded in the cultured wheat grains with *A. brasiliensis* (1.95 mg.g$^{-1}$) was used for calculation of the fungi biomass and resulted in 0.29 mg of *A. brasiliensis* mycelium per gram of cultured wheat grains.

***Body Weight :*** All mice fed with wheat grains increased of 30% their body weight, and no difference was observed between the animals fed with *A. brasiliensis* biocultured material and regular chow (*P*=0.05). This suggests that the amount of mushroom consumed was safe and did not cause any apparent undesirable side effect (Fig. 1).

**Table 1.** Analysis of the proximate composition, presence of secondary metabolites and ergosterol content of the cultured wheat grains with *Agaricus brasiliensis* mycelia and wheat grains *in natura* (n=3).

| Analysis | *A. brasiliensis* cultured wheat grains | Wheat grains *in natura* |
|---|---|---|
| Crude Protein (%) | 22.60 ± 0.25 | 15.38 ± 0.10 |
| Lipids (%) | 2.77 ± 0.19 | 2.14 ± 0.83 |
| Crude fiber (%) | 4.08 ± 0.35 | 2.88 ± 0.07 |
| Crude ash (%) | 2.76 ± 0.06 | 1.86 ± 0.03 |
| Flavonoids (presence) | Negative | Negative |
| Anthraquinones(presence) | Negative | Negative |
| Alcaloids (presence) | Negative | Negative |
| Tannins (presence) | Negative | Negative |
| Cardiac Glycosides (presence) | Positive | Negative |
| Ergosterol (mg/g) | 1.95 | 0.002 |

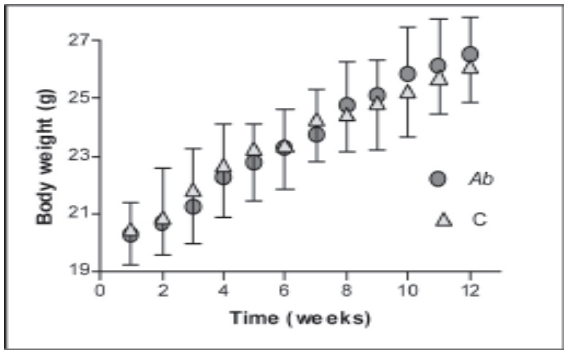


**Fig. 1**. Body weight from mice fed normal chow (C) or *Agaricus brasiliensis* (*Ab*) biocultured material during 12 weeks. (Data are presented as mean ± SD of 7 mice per group).

***Blood metabolite concentrations :*** There was a slight reduction in the plasma glucose concentration in mice fed *A. brasiliensis* biocultured material (*Ab*), but it was not statistically different (*P*>0.05 *vs.* C) (Table 2). Plasma cholesterol and triacylglycerol concentration was not different in the control and *Ab* biocultured material groups. On the other hand, there was a significant increase in the plasma HDL-cholesterol concentration in the *Ab* biocultured material group (*P*=0.05 *vs.* C), but no alteration in the plasma LDL-cholesterol concentration (P>0.05). There was a decrease

in the triacylglycerolemia/HDL-cholesterol ratio of 2.46 in mice fed with *A. brasiliensis* biocultured material when compared to 2.63 of chow fed animals (Table 2).

***Lymphocyte Subsets :*** Mice from Ab group have $CD4^+$:$CD8^+$ ratio increased (P=0.05 vs. C) (Table 3). The population of $CD4^+$ and $CD19^+$ cells in the spleen lymphocytes was not different between Ab and C group (P=0.05), with a tendency to reduce the $CD8^+$ T cells population.

***Production of Cytokines in Blood and Peritoneal Macrophages Culture :*** In the plasma, the levels of IFN-g and IL-6 were significantly higher in mice fed with *A. brasiliensis* biocultured material when compared to control group. However, in the peritoneal macrophage culture, the levels of IFN-gand IL-6 were unchanged, but the IL-12 concentration increased, TNF-a and MCP-1 levels were significantly reduced (*P*=0.05.*vs*. C), in cells obtained from *A. brasiliensis* biocultured material mice when compared to control group (Table 4).

**Table 2.** Blood metabolites concentrations from mice fed a normal chow diet (C) or with *Agaricus brasi-liensis* (*Ab*) biocultured material. (Data are presented as mean ± SD of 7 mice by group, **P*=0.05 *vs*. C)

| Parameters | C | *Ab* |
|---|---|---|
| Glucose | 212.50 ± 13.13 | 203.67 ± 32.48 |
| Total Lipids | 428.17 ± 10.61 | 431.17 ± 2.64 |
| Triacylglycerol (TAG) | 90.25 ± 6.65 | 96.67 ± 5.65 |
| Total Cholesterol | 49.17 ± 4.17 | 50.50 ± 3.15 |
| HDL-Cholesterol | 34.33 ± 3.01 | 39.33 ± 2.25 * |
| VLDL-Cholesterol | 19.00 ± 1.79 | 19.17 ± 1.17 |
| TAG/HDL-Cholesterol | 2.63 ± 0.41 | 2.46 ± 0.28 |

**Table 3.** Proportions of $CD4^+$, $CD8^+$, and $CD19^+$ lymphocytes in the spleen from mice fed a normal chow diet (C) or *Agaricus brasiliensis* (*Ab*) biocultured material. (Data are presented as mean ± SD of 7 mice per group, **P*=0.05 *vs*. C).

| Lymphocytes | C (%) | *Ab* (%) |
|---|---|---|
| $CD3^+$ | 46.91 ± 5.53 | 45.41 ± 7.10 |
| $CD4^+$ | 32.24 ± 2.77 | 33.15 ± 6.08 |
| $CD8^+$ | 17.69 ± 1.19 | 15.01 ± 1.36 |
| $CD4^+/CD8^+$ | 1.84 ± 0.18 | 2.21 ± 0.10* |
| $CD19^+$ | 34.93 ± 4.00 | 38.06 ± 6.50 |

**Table 4.** Cytokines concentration in the plasma and in the supernatant culture of peritoneal macrophages in *ex vivo* assay from mice fed a normal chow diet (C) and mice fed with *Agaricus brasiliensis* (*Ab*) biocultured material. (Data are presented as mean ± SD of 7 mice per group, **P*=0.05 *vs*. C).

| Cytokines | Plasma *in vivo* | | Peritoneal macrophages *ex vivo* | |
|---|---|---|---|---|
| (pg.mL$^{-1}$) | C | *Ab* | C | *Ab* |
| IL 12p70 | 35.88 = 5.27 | 47.50 ± 17.27 | 34.98 ± 1.38 | 43.78 ± 3.05* |
| TNF-α | 25.40 = 1.87 | 24.98 ± 3.19 | 354.33 ± 23.50 | 202.18 ± 20.73* |
| IFN-γ | 7.00 ± 1.37 | 10.43 ± 2.01* | 9.75 ± 1.56 | 10.85 ± 0.66 |
| MCP-1 | 54.63 ± 10.99 | 54.70 ± 1.35 | 2148.08 ±60.81 | 920.50 ± 53.44* |
| IL-10 | 35.33 ± 12.80 | 28.63 ± 5.70 | 76.33 ±3 8.38 | 89.10 ± 42.95 |
| IL-6 | 10.00 = 1.58 | 26.48 ± 10.35* | 7759.29 ± 3073.17 | 4622.78 ± 3266.16 |

***Peritoneal Macrophages in ex vivo Assays :*** Peritoneal macrophages from mice fed *A. brasiliensis* biocultured material did not increase the phagocytosis, lysosomal volume and $H_2O_2$. On the other hand, $O_2^{\cdot -}$ production was 50% lower when compared to control (Table 5 and figure 2, respectively). NO production (Figure 2) by peritoneal macrophages from *A. brasiliensis* biocultured material mice in the absence of LPS stimulus was approximately 5-fold lower when compared to control group (*P*=0.05). Under LPS stimuli peritoneal macrophages from *A. brasiliensis* biocultured material and control mice's increased NO production significantly when compared to the absence; however in the *A. brasiliensis* biocultured material group the response was significantly lower (*P*=0.05 vs. C).

## Discussion

Cereals can be used as fermentable substrates for the production of functional food (20)**.** Wheat grain used for the production of *A. brasiliensis* mycelium by solid state culture

**Table 5.** Macrophages responses in *ex vivo* assays from mice fed a normal chow diet (C) and mice fed with *Agaricus brasiliensis* (*Ab*) biocultured material. (Data are presented as mean ± SD of 7 mice per group, **P*=0.05 *vs*. C).

| **Macrophages responses** | **C** | ***Ab*** |
|---|---|---|
| Phagocytosis (1x10$^8$particles.mL$^{-1}$) | 0.618 ± 0.105 | 0.616 ± 0.117 |
| Lysosomal volume (absorbance mg.protein$^{-1}$) | 0.061 ± 0.018 | 0.053 ± 0.006 |
| Hydrogen peroxide (µmol.mg.protein$^{-1}$) | 0.074 ± 0.016 | 0.073 ± 0.023 |
| $O_2^{\bullet -}$ (µmol.mg.protein$^{-1}$) | 0.124 ± 0.039 | 0.060 ± 0.026* |

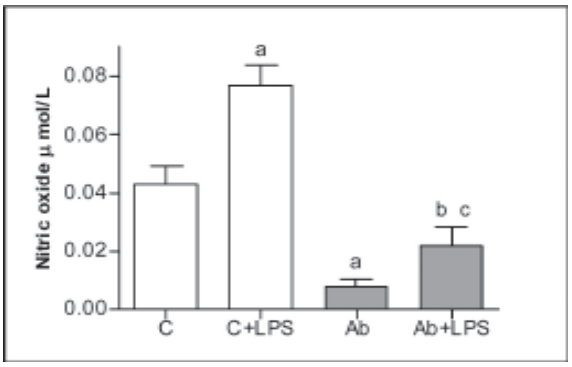


**Fig. 2.** Production of nitric oxide by unstimulated and LPS-stimulated peritoneal macrophages from mice fed a normal chow diet (C) or *Agaricus brasiliensis* (*Ab*) biocultured material. (Data are presented as mean ± SD of 7 mice per group. [a]*P*=0.05 *vs*. C; [b]*P*=0.05 *vs*. *Ab*; [c]*P*=0.05 *vs*. C+LPS).

resulted in a good development without the addition of any other nutrient. Usually the main approach of many researchers is to use fractions or whole fruiting body of the mushroom. Furthermore, mycelia production through solid state cultivation is a simple, mechanized, inexpensive method and do not require isolation/purification of the bioactive molecules present in the cultured substrate (11). Therefore, the use of mycelium suspension instead of the whole mycelium is a good approach which could be used for preparation of inoculum for submerged or solid state culture and also for "spawn" production, resulting in a faster colonization of the substrate and reduction of the culture time, and consequently the probability of contamination.

Protein values in the *A. brasiliensis* biocultured material increased 7% when compared to the wheat grains *in natura*. To our best knowledge, this is the first report of protein values increase in a substrate caused by *A. brasiliensis* mycelium growth. This increase in the protein content might be the result from development of the mushroom mycelium. In fact this is a characteristic of some Basidiomycetes, such occurred with *Pleurotus* sp. (21). This genus has the ability to enrich the protein value after grown on edible substrates, and therefore they can be used for the production of new protein sources, and as functional foods (21). The metabolic route which fungus use to induce the growth and the protein enriched products are not well elucidated, and was not investigated here, remaining an interesting open field for study.

The improved ash contents indicate an increase in mineral contents. In the present work it was observed an increase in ash and crude fibers values in the biocultured wheat grains. Other authors observed a similar increase in crude fiber and ash values after mushroom development in other substrates or wastes, resulting, therefore, in enhances of their nutritional values. Ash contents increased from 8.78% to 9.79% after growth in maize straw of white rot-fungi, and similar results were obtained after the *Volvariella volvacea* development on corn cobs (22, 23).

It has been reported that ergosterol isolated from *A. brasiliensis* (or *A. blazei*) inhibited tumor growth by inhibiting tumor-induced neovascularization (24). In our study we found an increase in the ergosterol amount on the cultured wheat grains and, therefore protein and ergosterol enrichment are indicative that *A. brasiliensis* mycelium growth through solid state culture on wheat grains can be considered a functional product. *A. brasiliensis* mushroom has important nutritional value due to the high presence of proteins, fibers and carbohydrates and low lipid concentration. This mushroom opens new possibilities including their effect on tumor bearing animals and also it could be used as an ingredient in a lot of new nutraceutical products.

Our data also show that *A. brasiliensis* biocultured material presented cardiac glycosides in its composition. These molecules are found as

secondary metabolites in several plants and also in some mushroom such as *Ganoderma lucidum* (25). This is a very interesting finding because some studies have reported that cardiac glycosides have been used in the treatment of congestive heart failure and cardiac arrhythmia (26, 27).

Molecules present in mushrooms are capable of modifying lipid metabolism, such as statins, fibers, and polysaccharides or their hydrolisates (28, 29). These studies and others demonstrated alteration on lipid metabolism in mice after intake of different forms from *Agaricus blazei* (30). On the other hand, the presence of insoluble cellulose, hemicellulose fiber and betaglucans in the wheat grains are known as possible modifier agents of the lipid metabolism, however, the effects of soluble ?ber are responsible for reduction of blood cholesterol, postprandial glucose peaks, and LDL-cholesterol (31, 32). Indeed, total cholesterol and LDL-cholesterol concentrations did not change in our study however; there was an increase in the plasma HDL-cholesterol concentration in the *Ab* mice group. We suggest that this occurred, possibly, due to the presence of mushroom mycelium. The HDL-cholesterol is popular known as the "good cholesterol" because it has the ability to protect against cardiovascular diseases removing cholesterol from peripheral tissues, such as fibroblasts and macrophages and transport it back to the liver for excretion or re-utilization. The risk for cardiovascular disease development is calculated using the ratio triacylglycerolemia TAG/HDL-cholesterol (29). Using such ratio we found a decrease in the atherogenic ratio of TC/HDL of 2.46 in mice fed with *A. brasiliensis* biocultured material *versus* 2.63 in mice fed with normal chow ($P$=0.05). This data corroborate the findings of triacylglycerolemia reduction by the consumption of *A. brasiliensis* in normal and diabetics' animals (29, 30). Regarding to glycemia we found a slight decrease in the plasma glucose concentration in mice fed *A. brasiliensis* biocultured material. Indeed, most people use this mushroom as co-adjuvant for diabetes treatment. It has been also demonstrated that â-glucans and oligosaccharides obtained from the fruiting body of *A. brasiliensis* can cause reduction in the serum glucose concentration in rats and the authors suggested that the mushroom has an anti-diabetic activity by inducing insulin release by the pancreas Langerhans cells (29).

Mushroom extracts have been claimed to enhance immune functions and promote health. Recently, we demonstrate that consumption of *G. lucidum* mycelium by mice provoked an immunomodulatory effect which resulted in an increase of resistance against Sarcoma 180 growth (33, 34). Here we examined changes in the ratio of $CD4^+$T cells to $CD8^+$T cells of spleen from mice. As a matter of fact immune system from *A. brasiliensis* group showed significant increase in the ratio of $CD4^+$T to $CD8^+$T cell populations when compared to control group (Table 3). We think that there was an expansion of the spleen $CD4^+$T cells. Liu et al. (35) reported that the antitumor effect of *A. brasiliensis* is closely related to the increase in spleen $CD4^+$T and NK cells. The naive Th cells produce mainly IL-2 on initial encounter with antigen and may differentiate into a population referred as Th0 cells, which will differentiate further into either Th1 or Th2 cells (36). IL-12, IFN-g, TNF-a are major cytokines associated to Th1 whereas IL-4, IL-5, IL-6 and IL-10 to Th2 subset (37).

Plasma concentrations of IFN-g and IL-6 were increased, but not TNF-a, MCP-1 and IL-10 from *A. brasiliensis* biocultured-fed mice when compared to controled animals and IL-12 increased in the *ex vivo* assay. IL-12 is a critical cytokine that bridges innate and adaptive immune system, and one major role is to induce IFN-g

production in NK and T cells and to enhance their cytolytic activities. IL-12 also drives development of CD4 T cells into Th1 cells and promotes cell-mediated immune response (38). This result suggest that *A. brasiliensis* supplementation in mice might trigger the polarization of the spleen $CD4^+$T cell to Th1-predominant response *in vivo*.

On the other hand, in *ex vivo* assay, the concentrations of TNF-a and MCP-1 were decreased and IFN-g and IL-6 unchanged by peritoneal macrophage in culture. IFN-g is a potent activator of macrophages (39). We found a not change in its levels *in vitro* which could be related with the macrophage activity that was not change also. This data is a strengthening for the IL-6 concentration which was also unchanged (40). Moreover, in the *Ab* group also was detected a reduction by 2.3-fold in the production of MCP-1, meaning that macrophages were not activated. Several studies show that activated macrophages, between other cells, are capable to produce MCP-1 (39, 40).

Other indicative that macrophages were inactive is the phagocytosis and lysosomal volume that were unchanged, and also by the reduced levels of NO (Fig 2) and superoxide anion (Table 5) by peritoneal macrophages in culture obtained from *A. brasiliensis* biocultured-fed mice when compared to control animals. This results suggest that the unchanged IFN-g level could be related with the reduction of 1.8-fold in the TNF-a production concomitantly with the reduction in the NO and superoxide anions levels. In opposite to the presented results, Sorimachi et al. reported that *Agaricus* promoted an increase of TNF and nitric oxide values by macrophages in assay *in vitro* (41). TNF-a is a cytokine that can activate the inducible nitric oxide synthase, which, as consequence, leads to a great quantities of NO (42). It´s important to clarify that Sorimachi et al. (41) used macrophages extracted from mice and put it in contact to the mushroom fractions in assays *in vitro* for cytokines production. In a different way, in our work the mice ingested the mushroom biocultured grains for a long time and the macrophages from these mice were measured for cytokines production in assays *in vitro*. Moreover, the difference between the *in vitro* and *in vivo* assay enables to verify the effect of the biocultured grains only on the macrophages and on the animal organisms. Therefore, the results concerning to the chronic supplementation with *Ab* obtained from experiments *in vitro* must be interpreted with caution and not be totally translated for *in vivo* situation, (In this study),we demonstrate that cytokines are secreted differently when studied *in vivo* and *in vitro.*

The immunomodulatory activity associated with mushroom intake has correlation to isolated â-D-glucans and other polysaccharides fractions. The standard approach has been made to purify the components and then to test them for efficacy (43). It is likely that mushrooms possess multiple immunoregulatory components including the selenium, B vitamins, ergosterol and polysaccharides that, when ingested together, would have effects that differed from the isolated components.

## Conclusion

Solid state culture on wheat grains for development of the *A. brasiliensis* mycelium demonstrated to be a simple, rapid, economic and without phase separation technique. This resulted in a biocultured material with increased nutritional values and also in health benefits in mice. The work is significant in view of improvement in the inoculation and solid state fermentation technique to minimize time and contamination probability for production of biocultured wheat grains, which can be used as ingredient for nutraceutical food.

**Acknowledgements**

To Conselho Nacional de Pesquisa (Process Nº 475798/2003-1); Universidade Federal do Paraná; Paraná Tecnologia and Universidade Estadual do Centro-Oeste - PR, BR for granting the financial support.

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Current Trends in Biotechnology and Pharmacy
Vol. 4 (4) i-v October 2010. ISSN 0973-8916


# NEWS ITEM

## Council for Human resource in health soon - Dr.Manmohan Singh, Prime Minister of India

Stressing on the need to revamp the health education system in the country, Prime Minister Manmohan Singh said that a bill for the formation of a council for human resource in health will be introduced in parliament soon. Highlighting the need to revamp the education system in health, the prime minister said that an inter-disciplinary approach is needed. Addressing the 38th Convocation of All India Institute of Medical Sciences (AIIMS), the country's premier research and referral hospital, he said that the need of the hour is to produce professionals who address health not only from the perspective of the individual patient but as part of a team integrated into the larger health system. Inter-disciplinary and health system connectivity have to be the key coordinates on which medical education has to advance. Dr.Singh said "In the future, it is not biology alone that will drive medical care. Disciplines such as epidemiology, economics, social and behavioral sciences, ethics and human rights will all influence the manner in which health will be promoted and healthcare provided to our nation".

## Manmohan gets World Statesman Award

Indian Prime Minister Manmohan Singh has been honoured with the 2010 World Statesman Award by the Appeal of Conscience Foundation, an interfaith coalition which promotes mutual understanding, tolerance and peace. India's Ambassador to the US, Meera Shankar, accepted the award on behalf of the prime minister. Accepting the award on behalf of the Prime Minister Shankar thanked Rabbi Schneier, President of the Appeal of Conscience and said that his extraordinary mission addresses one of the most important challenges for humanity. In his recorded speech, Manmohan Singh said, "India has been guided since ancient times by the fundamental belief, we are all united by our highest values, ideals and our inherent humanism." Former US Secretary of State Henry Kissinger described Manmohan Singh as a statesman with vision, persistence and integrity, and praised him for his leadership in India.

## US in educational fight with India: Obama

US President, Barack Obama stated that his country is in competition for a well educated future with the countries like India and China. Accusing the Republican leaders in Congress for reducing 20 percent budget in the education field, Obama said that American is in the educational fight with countries. He stated that India, China aren't slashing education by 20 percent right now and that they are in a fight for the future, a fight that depends on education. He also demanded, "Cutting aid for 8 million students, or scaling back our commitment to community colleges, that's like unilaterally disarming our troops right as they head to the frontliners."

## India will soon outpace China's economy

India is one of the fastest growing economy and "much-derided democracy" that will soon outpace China, says The Economist. The surprising India's economy is largely based on private sector with its strong foundation. It is said that the economy is expected to expand by 8.5 percent in 2010. The Economist said though Chinese economy is four times larger than India but the rapid growth of Indian economy could overtake China by 2013. The reason behind this is demography, where China has opted for one-china policy and their workforce will shortly start ageing in few years time. The second reason for optimism is India's much-derided democracy and he said "Indian capitalism is driven by millions of entrepreneurs all furiously doing their own thing." Also he added that since the early 1990s, when India dismantled the "licence raj" and opened up to foreign trade, Indian business has boomed.

Current Trends in Biotechnology and Pharmacy
Vol. 4 (4 ) i-v October 2010 ISSN 0973-8916


# SCIENTIFIC NEWS

## Role of Prolactin and Estrogens in breast cancer

Resistance to chemotherapy is a major complication in the treatment of advanced breast cancer. Estrogens and prolactin (PRL) are implicated in the pathogenesis of breast cancer but their roles in chemo resistance have been ignored. A common feature to the two hormones is activation of their receptors by various compounds, which imitate or antagonize their actions. The PRL receptor is activated by lactogens (PRL, GH, or placental lactogen) originating from the pituitary, breast, adipose tissue, or the placenta. Estrogen receptors exist in multiple membrane-associated and cytoplasmic forms that can be activated by endogenous estrogens, man-made chemicals, and phytoestrogens. Low doses of PRL, estradiol (E2), and bisphenol A (BPA) antagonize multiple anticancer drugs that induce cell death by different mechanisms. Focusing on cisplatin, a DNA-damaging drug which is effective in the treatment of many cancer types but not breast cancer, we compare the abilities of PRL, E2, and BPA to antagonize its cytotoxicity. Whereas PRL acts by activating the glutathione-S-transferase detoxification enzyme, E2 and BPA act by inducing the antiapoptotic protein Bcl-2.

***V.Pavithra***

## Pill to Keep Your DNA Young

Telomeres, repeating DNA sequences at the ends of chromosomes that become shorter with each cell division leading to aging process. When its telomeres become too short, a cell stops dividing and eventually dies. After years of research, the first telomere-targeting pills have hit the market, while other treatments are entering clinical trials. An enzyme called telomerase maintains telomeres in our reproductive and stem cells but not in the rest of the body. In 2001 researchers at the biotech giant Geron Corporation isolated a molecule called TA-65 from the herb astragalus, which has boosted telomerase activity. Physicians began selling TA-65 pills in 2007, and the company says that clients taking it have reported enhanced athletic, visual, and cognitive performance. Sierra Sciences of Reno, Nevada, is also developing possible pharmaceuticals to maintain Telomeres. The Company hopes to have an approved drug within 15 years. Geron, meanwhile, is pursuing a separate telomere therapy aimed at fighting cancer. Although telomerase is not present in most cells, it gets reactivated in cancer cells, allowing them to continue dividing. Blocking the enzyme causes the cancerous cells to die, so the company is working with a telomerase inhibitor, imetelstat that it hopes will kill tumor cells while leaving healthy ones unharmed. The compound is currently in Phase I clinical trials, with Phase II testing slated to begin this year. The unsettling flip side is that telomerase-boosting treatments aimed at slowing aging might also increase the risk of cancer. Some studies in mice have shown that elevated telomerase activity leaves the animals more susceptible to skin tumors and breast cancer.

**G.Swapna**

## Nanobandages for Detection and Treatment of Infection

Protein fragments that self assemble into a web of microscopic fibers. When these fibers are exposing to salt it quickly becomes a gel the gel gently seals a wound in less than 15 seconds, creating a protective cover- inside or outside the body. It promotes healing and prevents scarring. It detects harmful bacteria in a wound and responds by secreting antibiotics. Fifty percent of all people who die as a result of burn injuries as a direct consequence of infection. Harmful bacteria cause infections by attacking cells with toxins that dissolve the cell membrane. Friendly bacteria help the body to function and doesn't carry this toxic arsenal. This simple difference is the big idea behind this nanobadages. If pathogenic bacteria could be made the agents of their own destruction by using their toxins to rupture vesicles containing an antimicrobial agent the vesicles could be attached to bandages that would release antibiotics if a wound became infected. This reduces the risk of the evolution of new antibiotic-resistant superbugs such

as MRSA. The vesicles would also contain a dye, so the dressing would change colour if it came in contact with dangerous bacteria, alerting doctors of an infection.

**V.Leelavathi**

## Designer Babies

Child predetermination by design (CPD) via the technology of pre-implantation genetic diagnosis (PGD) - or what is commonly referred to as creating designer babies according to parental choice - is fast emerging from the realm of science fiction onto the scene of everyday reality. Designer baby has the ability to perfect your child and to prevent diseases. Abortion is observed in India because of their living conditions and habitat, may cause the abortion. Application of Child predetermination by design (CPD) may decrease the rate of people aborting their child. Along with this people would want a male heir and when they do have a girl, they kill the girl. If designer baby were offer to them then they would choose the child they want instead of killing the ones they don't want. Since it has the ability to do that, human life is going to expand because disease killing would decrease.

**V.Asha**

## Magnetic Leaves Indicate Levels of Air Pollution

Air pollution includes particulate matter which on inhaling leads to a number of negative health consequences including breathing troubles and even heart problems. These tiny particles in the polluted air get deep into the lung tissues which may even lead to cancer. Scientists at Western Washington University in Bellingham suggest a new way to monitor the quality of air by using tree leaves as tools. They came to know that leaves along the bus routes and highways were upto 10 times more magnetic than the leaves on quieter streets. That magnetism was due to the tiny particles of pollution such as iron oxides from diesel and petrol that float and stick to the leaves or grow right into them. Measuring the level of magnetism of tree leaves the quality of air of the streets can be monitored and the levels of air pollution can be known. Using leaves is a low-tech, easier and cost effective way to do these studies instead of using fancy particle collectors for measuring pollution levels. A lot of data from a region can be easily collected by using magnetic leaves whose collection is very easy and simple. A variation in the particulate matter can also be studied on a very detailed level and measures to avoid pollution can also be planned accordingly for a better environment. Though magnetic leaves are examples of damage to the nature they can be utilized for saving the nature which is beneficial for the environment.

**G Tapaswini**

# EDUCATION

## PhD Programs

PhD Scholar Program, Institute of Life Sciences, University of Hyderabad Campus, Gachibowli, Hyderabad-500 046. Applications are invited in the areas of Biology, Chemical Biology, Co – Crystal Technology, Computer – Aided Drug Discovery, Drug Discovery, Organic / Medicinal Chemistry. All applicants interested in doing a Ph.D must hold a Master's degree in Organic Chemistry, Physical Chemistry, and Biology related disciplines (Biotechnology, Biochemistry, Pharmacology, Microbiology, Molecular Biology, Genetics, Bioinformatics, etc.,) and have qualified in CSIR / UGC / ICMR / DBT or any other equivalent (private / national / international / industry) fellowship. Interested students can send their applications by email no later than the 1st November, 2010 together with a brief cover letter, Curriculum Vitae, Basic Data Sheet and scanned CSIR certificate or equivalent to PhD@ilsresearch.org. More details about ILS PhD Program could be gathered from the website www.ilsresearch.org. Applications without Curriculum Vitae, filled scanned Basic Data Sheet and CSIR certificate or equivalent will not be processed. Interview Dates: Preliminary Interviews: 24th November, 2010. Final Interviews: 26th November, 2010

### OPPORTUNITIES

Dr. Reddys Laboratories, 7-1-27, Ameerpet, Hyderabad Calls for 3 Cell Biology Professional

Opening(s). Candidate with 2-5 years experience preferably in Pharmaceutical Industry / cell culture techniques / novel cell based and cell free assays development / Good working knowledge of software programs like excel, sigma plot and sigma stat. For More Details Please go through - http:// www.drreddys.com or Contact: Noel T Rajan, Dr. Reddys Laboratories Ltd. Hyderabad, Andhra Pradesh, India. Email Address: talent@drreddys.com. Contact: (D) 040 - 44346837

Indian Institute of Science Education & Research (IISER), Pune. Applications are invited from Indian Nationals to work as Project Assistant – Grade II (PA) for one position in a research project funded by Department of Science & Technology (DST). Title of the project is "Molecular and genetic characterization of Drosophila and mouse Ataxin-2 binding protein 1 in the context of spinocerebellar Ataxia type 2" with the duration of one year. Essential Qualifications are M.Sc. degree in Biology with good academic record. CSIRNET/UGCNET/GATE qualified candidates will be preferred. Last date for applications is 15th October 2010. Also Applications are invited from Indian Nationals to work as Post Doctoral Research Associate (RA) for two positions in a the same research project funded by Department of Science & Technology (DST). Essential Qualifications are Ph.D degree in Biology, chemistry, physics, engineering or allied sciences with good academic record. Last date for applications is 30 October 2010. Applicants should send the application by email addressed to bio_app@iiserpune.ac.in attaching duly filled PA_ Application_Form.doc (for Project Assistant – Grade II applicants) or RA_ Application_Form (for Post Doctoral Research Associate applicants) available on the website under this advertisement on or before the last date (Oct. 15 for PA and Oct. 30 for RA).

BIOCON, 20th KM, Hosur Road, Electronics City, Bangalore Calls for Executive/Sr.Executive-QualityAssurance (Biologicals) Opening(s). Candidate with 2-5 years experience in protein and fermentation based products and have completed UG - B.Pharma – Pharmacy, B.Sc – Any Specialization, Bio-Chemistry, Microbiology PG - Any PG Course – Any Specialization, Post Graduation Not Required, M.Pharma – Pharmacy, M.Sc – Any Specialization, Bio-Chemistry, Chemistry, Microbiology are eligible. For More Details Please go through - http:// www.biocon.com/ or Contact: Ramitha.Ravindran, Biocon Limited, 20th KM, Hosur Road, Electronic City, Bangalore, Karnataka, India - 560100. Email Address: ramitha.ravindran@biocon.com. Contact: (D) 080-28082204

## SEMINARS/CONFERENCES

National Conference on Emerging trends in Biopharmaceuticals: Relevance to Human Health & 4th Annual Convention of Association of Biotechnology & Pharmacy: A national conference on Emerging trends in Biopharmaceuticals: Relevance to Human Health & 4th Annual Convention of Association of Biotechnology & Pharmacy was going to held on November 11-13, 2010 organized by the Department of Biotechnology & environmental sciences, Thapar University, Patiala, Punjab, India. Abstracts of Scientific papers not exceeding 300 words inclusive of Title, names and Addresses of author's should be submitted by email only to pharmacy@thapar.edu on or before 15th October 2010. For further information contact: Organizing Secretary, NCETB & ABAP, TIFAC-CORE, Thapar University, Patiala - 147004, Telephone: (0175)-2393743, 2393736, 9463930451. Email: pharmacy@thapar.edu.

**International Conference on Biodiversity and Aquatic Toxicology:** An international conference on Biodiversity and Aquatic Toxicology was going to held on February 12-14, 2011 organized by the Department of Zoology & Aquaculture, Acharya Nagarjuna University, Guntur, A.P, India at Hotel Fortune Murali Park, Vijayawada, A.P, India. Abstracts of Scientific papers not exceeding 300 words inclusive of Title, names and Addresses of author's should be submitted by email only to int.sympaqtbd @gmail.com on or before 31st October 2010. For further information contact: Dr.K.Veeraiah, Secretary of the conference, Department of Zoology & Aquaculture, Acharya Nagarjuna University, Nagarjuna Nagar - 522510, Guntur, A.P, India, Telephone: 9490880831. Email: veeraiah.kotturu@gmail.com.

Prasada Rao S. Kodavant, Senior Neurotoxicologist working at United States Environmental Protection Agency (USEPA), Research Triangle Park, North Carolina has been Awarded the highest level Scientific and Technological Achievement Award (STAA), given each year for outstanding scientific and technological papers published by EPA scientist. Each year, EPA recognizes outstanding papers written by the Agency's staff and published in scientific and technical journals, and evaluated by a panel convened by EPA's Science Advisory Board (SAB). The SAB convenes an experienced group of scientists and engineers who review and evaluate the nominations. The SAB review panel then produces a set of recommendations that ORD uses to select the actual awards. The Level I STAA Award carries cash prize of $10,000, a congratulatory plaque, a letter of appreciation, and a certificate. Dr. Prasada Rao' s research contributions highlighting the Use of Genomics in Understanding Mode of Action in Developmental Neurotoxicity has been recognized for the award. Dr. Prasad Rao received his Ph.D degree in Zoology from Sri Venkateswara University and had extensive postdoctoral training in different institutions in USA before he accepted a scientist assignment in USEPA, where he is currently working. He has received several research awards, written books and serving on the editorial board of several International Research Journals.

Prasada Rao S. Kodavant, Senior Neurotoxicologist working at United States Environmental Protection Agency (USEPA), Research Triangle Park, North Carolina has been Awarded the highest level Scientific and Technological Achievement Award (STAA), given each year for outstanding scientific and technological papers published by EPA scientist. Each year, EPA recognizes outstanding papers written by the Agency's staff and published in scientific and technical journals, and evaluated by a panel convened by EPA's Science Advisory Board (SAB). The SAB convenes an experienced group of scientists and engineers who review and evaluate the nominations. The SAB review panel then produces a set of recommendations that ORD uses to select the actual awards. The Level I STAA Award carries cash prize of $10,000, a congratulatory plaque, a letter of appreciation, and a certificate. Dr. Prasada Rao' s research contributions highlighting the Use of Genomics in Understanding Mode of Action in Developmental Neurotoxicity has been recognized for the award. Dr. Prasad Rao received his Ph.D degree in Zoology from Sri Venkateswara University and had extensive postdoctoral training in different institutions in USA before he accepted a scientist assignment in USEPA, where he is currently working. He has received several research awards, written books and serving on the editorial board of several International Research Journals.

Acharya Nagarjuna University and INDICASAT-AIP of Republic of Panama signed an agreement for collaborative research and grant of Ph.D program at Republic of Panama on 6-10-2010 at Panama. Ms.Marta Linares de Martinelli, Honorable first lady of Panama, Dr.Ruben Berrocal, Honorable Minister for Science and Technology, Prof.Y.R.Haragopal Reddy, Vice-Chancellor of Acharya Nagarjuna University, Dr.K.S.J.Rao, Advisor to Ministry of Science and Technology and Prof.K.R.S.Sambasiva Rao were present

## International Symposium on

# Innovations in Free Radical Research and Experimental Therapeutics

# &

# 5th Annual Convention of Association of Biotechnology and Pharmacy

**(December 15-17, 2011)**

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

Its our pleasure to invite you all to attend the International Symposium on "Innovations in Free Radical Research and Experimental Therapeutics" to be held in Karunya University, Coimbatore from December 15-17, 2011. Researchers, Scientists and Students are encouraged to participate and present their research/experimental findings. The International Symposium is organized to focus on the recent developments in various thrust areas of Free Radical Research in general and therapeutics for various diseases affecting humans. The International Symposium includes invited lectures and presentations of original research papers. We hope that you will be able to join us and enjoy an exciting meeting.

### Broad Areas of Focus

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

### Contact for further details

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