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Biological evaluation of marine derived fungi *C. geniculatus* secondary metabolites

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ABSTRACT

In order to explore pharmaceutical activity of marine fungi fungal samples were collected from both marine water and marine mud of coastal lines in Andhra Pradesh, India. . Fungal samples were screened and identified as *C.geniculatus*. Screened marine fungi were evaluated for therapeutic activities which include antimicrobial, Anti-oxidant, Antidiabetic and anti-inflammatory activities. Pure cultures of these strain were fermented for 10 to 15 days, which resulted in the production of crude extract. The crude extract was separated, condensed,, weighed and stored for further analysis. Crude extract was analyzed for antibacterial and antifungal activities using agar well diffusion method at four different concentrations. Anti-oxidant by DPPH method, Antidiabetic by alpha amylase activity and anti-inflammatory activities by 5-LOX methods were done. Antimicrobial activity was done with nine bacterial strains and five fungal strains. *C.geniculatus* showed good inhibitory activity against bacterial *Streptococcus mutans, Lactobacillus casei and Enterococcus faecalis* strains and fungal strain *Fusarium oxysporium*. At maximum concentration DPPH method showed 92.58% radical scavenging activity, amylase activity showed 79.26% activity and 5 LOX method showed moderate activity at 51.72% of anti-inflammatory activity at this concentration.

Keywords: Marine fungi, *C.geniculatus*, Fermentation, crude compound, Biological activities

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INTRODUCTION

Marine fungi are taxonomically, physiologically and economically well-defined group of organisms. Some fungi are parasitic and some other are saprophytic in marine ecosystem. Literature reviewed in many articles on marine derived fungi, most compounds are active in antibacterial and antifungal activities [1]. Antibacterial and antifungal compounds are most usually used drugs in these days. But due to increase in resistance of bacterial and fungal pathogens demand for identifying new antibacterial and antifungal compounds growing widely. Natural products from marine fungi gain huge importance because of vast fungal diversity and their improvement in the fermentation and genetic breeding processes. Digging of antibacterial and antifungal compounds from marine derived fungi gain much importance from 2010 onwards [2].

In these days' hyperglycemia is major classical risk factor in development of diabetes. The patient with diabetes results ROS generation which ultimately leads to increased oxidative stress in various tissues [3]. This results increase production of free radical species that cause oxidative stress defenses which initiates the progression of complications in diabetes associated disorders [4]. Many strategies have been proposed for early treatment of glycaemia, to prevent, further development. Inhibition of alpha amylase that digests carbohydrates pay way to suppress hyperglycemia [6].

Inflammation is a protective mechanism, it show response on trauma or infection, noxious stimuli. Improper regulation to this protective mechanism leads to chronic inflammatory disorders. In inflammation mechanism immune system host complex respond to stimuli like microbial infection, endotoxins, burns, injury to tissues. Macrophages are the first defense cells against infections and play a major role in inflammation development [8]. Since four decades of research marine fungi are proved to be the magnificent source of bioactive secondary metabolites and various compounds reported *invitro* and *invivo* immune stimulatory activity [9].

MATERIALS AND METHOD

Sample Collection:

Samples ware collected from marine water of Krishna lanka beach (Bapatla) and machili patanam beach, Krishna dist, Coastal line of Andhra Pradesh, India. The samples were collected from different regions of beaches ranging from surface to depth of water After sample collection the bottle is tightly packed and safely transferred to laboratory with in limited period as possible and stored. The plating technique which we used consisted of spreading samples of sea water with a bent glass rod on the surface of a solid isolation medium. These sample inoculated Petri plates were incubated for 48rs. Isolated fungal colonies were observed on each dilution in Petri plates

after period of incubation. The colony with specific characters was identified and colony was transferred to double sterilized potato dextrose agar slants. These fungal culture slants act as working stocks for further. [10]

Identification:

Marine fungal strain was identified based on microscopic and macroscopic description . Riddel's simple method of slide culturing (Mycologia 42:265, 1950) permits us to study fungi virtually in situ with as little disturbance as possible. Microscopic test was done by preparing permanent slides and stained with lacto phenol cotton blue and observed under 100X oil immersion. Macroscopic study was done on the basis of size of the colony, color of the colony, pigmentation of the colony and mycelia growth of the colony. With these characters the taxonomy was identified [11].

Preparation of production medium

Saline water was collected from marine sources in required quantity. Initially, saline water was filtered twice through watt Mann filter paper and sterilized in autoclave for two times. The autoclaved saline water was once gain filtered through Whatman filter and again sterilized. These sterilized saline water are ready to use in production medium.

Potato dextrose broth medium with saline water:

For 1 lit of potato dextrose broth medium 2.4 gms of powder was dissolved in 500ml double distilled water. 500ml sterilized saline water was added in 1:1 proportion and sterilized for cultivation of fungal strains.

Small scale fungi cultivation

Small scale fermentation is initial step of fermentation. In which, fungal cultures were grown on slants and petri dishes before fermentation. For small scale fermentation, firstly fungal samples were inoculated and incubated in petri dishes. Later when growth reaches 2mm surface circles, mycelium of isolated fungi were inoculated into 250ml of potato dextrose broth. Cultures were fermented at 25^0c Time of incubation varies for each and every fungal strain. Rapid growth of fungal strains was seen in seven days and slow growth strains were incubated till 28 days [12]

Small scale fermentation

Production of biologically active compounds at favorable conditions:

In this work, production of biologically active compounds from the suitable medium is an important criteria. Providing all the conditions for medium, may result in bulk compound production. Suitable medium, pH, temperature, incubation period and RPM wasmaintained for production of secondary metabolites.

The selected strain was inoculated in potato dextrose broth as one of the favorable medium with pH and incubated in shaker for 15 days at 150 rpm at pH 4, and temperature at 28 ºC. .

Extract filtration:

Extract filtration is the final step for collection of secondary metabolites. Extract is filtered. Extraction of mycelia and extraction of extract are two steps taken. **Extraction of mycelia**: Before extraction of mycelia ethyl acetate solvent is added to broth in 1:1 proportion of broth quantity. Flasks were stirred for 30 min periodically for 5 to 6 hours prior to mycelia filtration. After stirring mycelia was separated and the filtrate was collected.

Extraction of filtrate:

The above collected filtrate was transferred to separating funnel. The filtrate in the funnel is stirred and set to stand for constant position for few minutes , then for few minutes till the separation of medium and solvent layer separation is clearly seen through our naked eye. Broth is discharged through out let and solvent layer is collected separately. The collected solvent is condensed by Rota vapor at particular temperature and required rpm. The crude extract is collected. The crude extract compound is weighed and stored for evaluating the biological activities.

Antibacterial Activity

The *in vitro* antibacterial activity of marine derived fungal crude extract was studied against eight bacterial strains include *Bacillus megaterium , Staphylococcus aureus, Streptococcus. mutans Lactobacillus casei Lactobacillus acidophilus, Enterococcus faecalis, Xanthomonas campestris*

Enterococcus faecalis. Antibacterial activity was done by agar cup diffusion method.. Agar well diffusion bioassay was employed for testing antibacterial activity**.** The nutrient agar medium after autoclave was poured into sterile Petri dishes under aseptic conditions in a laminar flow chamber. When the medium petri plates was solidified,1 ml of (week old) culture of test organism was inoculated and evenly spread over the agar medium surface with a sterile L-shaped rod. Crude extract compound were prepared by dissolving in DMSO and different concentrations were made. After spreading with culture, cups were scooped out with sterile cork borer To each cup, different concentrations of test solutions were added. Controls were maintained with DMSO. The plates were kept at 37^0 C for 48 h. Inhibition zones were measured and the diameter was calculated in millimetres [11].

Antifungal activity

The *in vitro* antifungal activity of the collected crude extract was studied against the fungal strains, viz., *Candida rugosa*, *Fusarium oxysporium Saccharomyces cerevisiae*, *Rhizopus oryzae and Aspergillus flavus* by agar cup diffusion method. Strains were obtained from the Institute of Microbial Technology, Chandigarh. The potato dextrose agar medium after autoclave was poured into sterile Petri dishes under aseptic conditions in a laminar flow chamber. When the medium in petri plates was solidified, 1 ml of (week old) culture of test organism was inoculated and evenly spread over the agar medium surface with a sterile L-shaped rod. Crude extract compound were prepared by dissolving in DMSO and different concentrations were made. After spreading with culture, cups were scooped out with sterile cork borer . To each cup, different concentrations of test solutions were added. Controls were maintained with DMSO. The test and the controls were kept at 27^0C for 48 h. Inhibition zones were measured and the diameter was calculated in millimeter [11]

Antioxidant Activity:

Determination of 1, 1- Diphenyl-2-Picrylhydrazyl (DPPH) Radical Scavenging Activity

The scavenging activity for DPPH free radicals was measured according to the procedure described [13]. An aliquot of 3 ml of 0.004% DPPH solution in methanol and 0.1 ml of plant extract at various concentrations were mixed. The mixture was shaken vigorously and allowed to reach a steady state at room temperature for 30 min. Decolorization of DPPH was determined by measuring the absorbance at 517 nm. A control was prepared using 0.1 ml of respective vehicle in the place of plant extract/ascorbic acid. The percentage inhibition activity was calculated as

DPPH scavenging activity (%) = $\left[\frac{(A0-As)}{A0}\right] * 100$

Where, **A_0** is the absorbance of the control and **As** is the absorbance of the plant sample,

Concentration of working extract is 1mg/ml

Antidiabetic activity[14]:

Initially 250µl of amylase solution (1mg/ml phosphate buffer) was mixed with 100µl of sample except to blank and incubated at 37^0C for 20 mins in water bath To this 250µl of substrate solution (0.5% starch in phosphate buffer) was added and incubated at 37^0 C for 15 mins. The reaction was stopped by adding 2ml of DNS (Dinitrosalicyclic acid reagent) (40 mM DNS, sodium potassium tararate, 0.4% M NaOH). The readings were recorded spectrophotometrically at 540nm and percentage of inhibition was calculated

% of inhibition= optical density Test-OD / Control OD X100

Anti-inflammatory activity:

***In vitro* 5-Lipoxygenase inhibition:**

5-LOX enzyme inhibitory activity of extracts were measured for fungal compound.The assay mixture contained 80 µM linoleic acid and 10 µl potato 5-LOX in 50 mM phosphate buffer (pH 6.3). The reaction was initiated by the addition of enzyme buffer mix to linoleic acid and the enzyme activity was monitored as the increase in absorbance at 234 nm for 120 sec and the inhibitory potential of the test substances was measured by incubating various concentrations of test substances for two minutes before addition of linoleic acid. Percentage of inhibition was calculated.

RESULTS AND DISCUSSION

Table represents Antibacterial activity of *C.geniculatus:*

S.no	Organism	25 µg	50 µg	75 µg	100 µg
1	*Staphylococcus aureus*	12	14	16	20
2	*Streptococcus mutans*	14	14	18	24
3	*Lactobacillus casei*	16	18	18	24
4	*Lactobacillus acidophilus*	14	16	18	20
5	*Enterococcus faecalis*	18	20	22	24
6	*Bacillus megaterium*	14	16	20	22
7	*Xanthomonas campestris*	14	14	16	18
8	*Escherichia coli*	12	14	16	18

The above table evaluated the antibacterial activity of *Cochliobolus geniculatus* crude extract. To perform antibacterial activity ,eight different bacterial strains include *Staphylococcus aureus, Streptococcus mutans, Lactobacillus casei, Lactobacillus acidophilus, Enterococcus faecalis, Bacillus megaterium, Xanthomonas campestris, Escherichia coli* were tested against 4 different concentration aliquots of crude extract. Four aliquots include 25 µg, 50 µg, 75 µg and 100 µg.

Crude extract is dissolved in DMSO. DMSO is taken as control against all above bacterial strains. It resulted no activity against any of bacteria. Crude extract aliquots showed good activity against all bacterial strains. At 100 µg concentration *Streptococcus mutans, Lactobacillus casei and Enterococcus faecalis* showed 24mm zone of inhibition. *Bacillus megaterium* showed 22mm. *Staphylococcus aureus* and *Lactobacillus acidophilus* showed 20mm. *Xanthomonas campestris* and *Escherichia coli* showed 18 mm zone of inhibition.

Table represents Antifungal activity of *C.geniculatus*:

S.No	Organism	50 µg	100 µg	150 µg	200 µg
1	*Candida rugosa*	15	16	19	22
2	*Fusarium oxysporium*	14	16	18	24
3	*Saccharomyces cerevisiae*	14	16	16	20
4	*Rhizopus. oryzae*	16	16	20	22
5	*Aspergillus flavus*	12	14	16	16

Antifungal activity *C.geniculatus* crude extract was performed by agar well diffusion. Crude extract is weighed and dissolved in DMSO. Four aliquots were prepared as 50 µg, 100 µg, 150 µg and 200 µg. aliquots were tested against five fungal strains include *Candida rugose, Fusarium oxysporium, Saccharomyces cerevisiae, Rhizopus Oryzae* and *Aspergillus flavus*. DMSO taken as control and tested, resulted no activity. At 200 µg *Fusarium oxysporium* showed 24mm zone of inhibition, *Candida rugose and Rhizopus Oryzae* showed 22mm, *Saccharomyces cerevisiae* showed 20mm and *Aspergillus flavus* showed 16mm.

Anti-oxidant, Antidiabetic and anti-inflammatory activities:

Table represents results of Anti-oxidant, Antidiabetic and anti-inflammatory activities:

S.no	Concentration µg/ml	% of inhibition		
		Anti-oxidant	Antidiabetic	anti-inflammatory
1	50	39.84	42.51	28.7
2	100	52.19	51.64	34.92
3	150	63.69	59.50	39.45
4	200	77.54	68.29	45.65
5	250	92.58	79.26	51.72

Crude extracts were prepared in five different concentrations. Based on the result, the activity of inhibitory concentration was measured. The effect of crude extracts of *C.geniculatus* strain with anti-oxidant (DPPH method), antidiabetic (amylase test) and anti-inflammatory (5-LOX) scavenging activity was investigated. Crude extracts were made from 50 to 250µg/ml concentration in different aliquots. The DPPH, amylase and 5-LOX inhibition activity was increased with compound concentration increases from 50 to 250µg/ml. At 250µg/ml concentration DPPH showed 92.58% radical scavenging activity, 79.26% amylase activity and 5

LOX method showed moderate activity at 51.72% of anti-inflammatory activity at this concentration.

CONCLUSION

Marine organisms have a great reserve of bioactive compounds. Marine fungi can be easily isolated and cultured.. Many fungal species are screened, cultured, and purified products are tested for therapeutic activity. In the present work, isolated fungi *C.geniculatus* crude extract showed good antibacterial, antifungal, anti-diabetic, antioxidant and anti-inflammatory activities.

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