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Antimicrobial activities of some Basidiomycetous fungi

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In the present investigation the medical benefits of the higher fungi, which are used by the rural people and the '*Ojhas*', the tribal doctors of West Bengal for the treatments of ailments, were studied. All together six higher fungi, namely, *Ganoderma applanatum* (Pers. Ex Wallr.) Pat., *Lenzites reparida* (Pers.) Fr., *Polyporus zonalis* Berk., *Trametes corrugata* (Pers.) Bres., *Tricholoma crassum* (Berk.) Sacc. and *Gymnopilus chrysomyces* (Berk.) Sacc. were collected during rainy season from the forest beds, infected logs and bamboos of West Bengal. In the laboratory tissue cultures were prepared on malt agar slants from the fresh basidiocarps of these fungi and maintained under refrigeration and by regular subculturing for future use. The antibiotic activity of all the six fungi were determined by growing the fungi in glucose-asparagine medium and following agar plate cup diffusion assay and using *Bacillus subtilis* as test organism. All the six fungi were found to produce antibiotic substances and of these *Tricholoma crassum, Trametes corrugata* and *Ganoderma applanatum* were found to have maximum inhibitory activity. The data of the fermentation revealed that the production of antibiotic started on 6th day of fermentation in all the test-fungi and the maximum production was observed at 20th, 18th and 16th day of fermentation in case of *Tricholoma crassum, Trametes corrugata* and *Ganoderma applanatum* respectively. The terpenoids and the polysaccharides from the mycelia and basidiocarp of *Tricholoma crassum, Trametes corrugata* and *Ganoderma applanatum.* were found to possess antibacterial and antifungal activities.

Key Words : Antimicrobial activities, Basidiomycetous fungi

INTRODUCTION

In India, the researches on the higher fungi are gaining tremendous importance day by day and there are several laboratories where works on different aspects of higher fungi are being done. The main lines of investigation are taxonomical, nutritional, physiological and biochemical aspect of different fungi and cultivation of several edible fungi. But, until now investigation on medicinal uses of higher fungi has not been done in India except the reports of Bose (1952, 1953) although, there are several such reports in international fields.

The State of West Bengal is situated in the eastern part of India. It is surrounded by Bangladesh on the east, Nepal and Bhutan on the north, Bihar and Orissa on the west and Bay of Bengal of the south. The rural population is about 80 per cent. The natural forests occupy about 11.2 per cent of the total land area.

Several tribal people reside in the hilly areas of the state and the total tribal population of the state is about 40 per cent. The socio-economic condition of these tribal people are below the proverty line. These tribal doctors collect the medicinal plants including several mushrooms from the nearby forests and use them for the treatment of all types of ailments. Several higher fungi including

mushrooms are found to be used for the treatment of several ailments, namely, ear infection, cough, cold, itching, burns, abscess, cut to stop bleeding, rheumatism, gout, jaundice, dropsy, poultice for inflammed eyes, gargle for inflammation of throat, heart ailment, lowering of blood pressure etc.

Erkel *et al.* (1991) demonstrated an inhibitor of RNA–directed DNA–polymerases from *Clavicorona pyxidata.* Mizuno *et al.* (1992) demonstrated antitumour activity of some polysaccharides isolated from the fruit-body and clutured mycelia of *Polyporus confluens.*

Lieu *et al.* (1993) found that *Ganoderma lucidum* is a herbal medicine with tumoricidal activity capable of inhibiting the proliferation of mouse Sarcoma 180 cells both *in vitro* and *in vivo.* Wang *et al.* (1993) reported strong antitumour activity of polysaccharides isolated from *Ganoderma tsugae.* Zhuang *et al.* (1993) obsrved antitumour activity of protein containing polysaccharides from fruit-body of *Pleurotus sajor-caju.* Bobek *et al.* (1993) observed that *P. ostreatus* reduced by 33 percent the level of serum and liver cholesterol at the end of the 10th week. The level of serum triacylglycerols was also reduced by 13 percent in liver. Ren *et al.* (1993) reported that *Coriolus versicolor* as an effective biological response modulator and could be used as adjuvant measures in the treatment of cancer. Mizuno *et al.* (1995) reported production of antitumour active heteroglycans from *Tricholoma gignateum.* Yang and Liau (1998) reported the cultivating conditions of mycelium of *Ganoderma lucidum* in submerged culture. Engler *et al.* (1998) reported antibiotic production by *Collybia nivalis, Omphalotus olearius,* a *Favolaschia* and a *Pterula* species. Tanaka *et al.* (1998) reported a terpenoid antibiotic from *Fomitella fraxinea* capable in inhibiting DNA–polymerases of cat and rat. Daferner *et al.* (1999) reported a new antibiotic, strobilurin-M, from *Mycena* sp.

In the present investigation attempts have been made to screen the antimicrobiological activity of some higher fungi and to find out the optimum conditions for the production of mycelia biomass along with the yield of antimicrobiological compound(s) present in these fungi.

MATERIALS AND METHODS

During the rainy season the fungi were found to grow luxuriantly in forest beds and on infected logs, bamboos etc. These were collected from several areas spread over the plains of West Bengal and immediately brought to laboratory. Spore prints of the fungi were taken and hand sections were cut to observe the tissue arrangements. The anatomical and morphological studies of all the fungi were made along with measurements.

Several tissue cultures were prepared from the basidiocarps of the different fungi collected in this investigation and maintained on PDA medium. All these tissue cultures of the fungi were stored in refrigerator at 10°C and by regular subculturing at 30 days interval the cultures were maintained.

All the fungi were initially grown stationary in liquid synthetic medium i.e. glucose-asparagine medium (Lilly and Barnett. 1951) in conical flasks for studying the growth and production of antimicrobial compounds(s).

A small protion of the activiely growing mycelium in PDA was pounched out aseptically by a sterilized cork borer (5 mm diameter) and was transferred aseptically to Erlenmeyer flask (150 ml) of Borosil, Bombay, India containing 30 ml. of sterial glucose-asparagine liquid medium. The inoculated flask was incubated for 7 days in complete darkness in a shaking incubator (120 rpm) at 30°C. After harvesting the mycelia were repeatedly washed with sterile distilled water to remove any trace of medium and were fragmented into small pieces aseptically in a waring blender. Then the frangmented mycelia were again washed with sterilized distilled water and suspended in phosphate buffer (pH 6.0) medium for 24 h to overcome the shock encountered during blending. From this fragmented mycelial suspension 1 ml was used as inoculum.

Glucose asparagine liquid medium was prepared and distributed (30 ml/150 ml flask) among several Erlenmeyer flasks (150 ml) according to the requirement of each experiment. These flasks were sterilized at 121°C for 15 minutes.

All the experimental flasks were inoculated with 1.0 ml of mycelial cell suspension of each test fungi separately and incubated in shaking incubator (150 rpm) and other conditions of each individial experimental conditions were maintained.

The mycelia were separated from the medium after the incubation period by filtration. The filtered mycelia were washed with distilled water three times to make them free of liquid medium. The washed mycelia were dried in oven at 60°C for 24 h and then cooled in descicator and weighed to a constant weight.

This dry weight of mycelia was treated as index to growth of the test organisms.

Several flasks were inoculated in order to have at least three replications of each treatment of every experiments.

Biological assay

The antimicrobial assay of the test compounds were done against some selected bacteria and fungi. The antibacterial assay was done using nutrient agar medium (Difco) following the agar plate cup diffusion technique. The antifungal assay was performed using Malt agar medium following the agar plate disc diffusion technique. The concentration of the test compound solution was 20 µg/ml.

After 24 h incubation in case of bacteria and 72 h incubation in case of fungi were considered and the diameter of the inhibition zone (in mm) was recorded and presented as the test data.

Isolation of polysaccharides

(i) From the basidiocarps

The polysaccharides from the basidiocarps of the test-fungi were isolated according to the methods of Mizuno *et al.* (1992) and Wang *et al.* (1993).

Fresh besidiocarps of the test-fungi were collected and brought into laboratory. The surface of the basidiocarps were cleaned and air dried for 24 to 48 h. The dried mass was powdered in Wiley mill attached with 60 mesh screen.

The powdered basidiocarp (ca 1 kg) was treated with 85 per cent ethanol (by 10 volumes) repeatedly (4 times) at room temperture. 30°C ± 2°C for 24 hours to remove the lower molecular weight substances. The residue was then treated with hot water (100°C) for 3 h and filtered. The filtrate was collected neatly and evaporated completely and this was the Fraction I (F1) of the polysaccharides. The residue was treated with 1 per cent ammonium oxalate solution at 100°C for 6 h. The filtrate was collected and evaporated completely under reduced pressure. This was Fraction 2 (F2) of the polysaccharides. The residue was further treated with 5 per cent sodium hydroxide solution at 80°C for 6 h. The filtrate was collected and evaporated completely under reduced pressure. This was collected as Fraction 3 (F3) of the polysaccharides.

All the F1, F2 and F3 polysaccharides were stored carefully and used in experiments as basidiocarp — polysaccharides.

(ii) From the mycelial biomass

The isolation of polysaccharides from submerged mycelia of the test-fungi was done following the methods of Mizuno *et al.* (1992) and Wang *et al.* (1993).

Mycelia of the test-fungi were harvested aseptically from 30-day-old liquid culture in fermentor (IL) by filtration through Sintered IG3 disc using vacuum pump and the harvested mycelia were washed and then the entire biomass was air dried and ground in Wiley Mill attached with 60 mesh screen.

The powdered mycelial biomass (ca 500 g) was treated with 85 per cent ethanol (by 10 volumes) repeatedly (4 times) at room temperture. 30°C ± 2°C for 24 h to remove the lower molecular weight substances. The residue was then treated with hot water (100°C) for 3 h and filtered. The filtrate was collected neatly and evaporated completely and this was the Fraction I (F1) of the polysaccharides. The residue was treated with 1 per cent ammonium oxalate solution at 100°C for 6 h. The filtrate was collected and evaporated completely under reduced

pressure. This was Fraction 2 (F2) of the polysaccharides. The residue was further treated with 5 per cent sodium hydroxide solution at 80°C for 6 h. The filtrate was collected and evaporated completely under reduced pressure. This was collected as Fraction 3 (F3) of the polysaccharides.

(L) Isolation of terpenoids

(i) From the basidiocarps

The isolation of terpenoids from the basidiocarps was done following the methods of Shiao *et al.* (1986), Anke and Werte. (1990) and Chairul *et al.* (1991).

Fresh basidiocarps (1 kg) were cut into small pieces, air dried and ground in Wiley Mill and powdered mass was passed through 60 mesh screen. The power mass was extracted with methanol. The methanolic extract was evaporated and the solid residue was dissolved in saturated sodium bicarbonate solution three times. The sodium bicarbonate solution was acidified with 10% HCL and extracted three times with ethyl acetate. The entire acidified ethyl acetate extract was chromatographed on a silica gel column with hexane-ethyle acetate (1:1) mixture and three fractions (F1, F2 and F3) were obtained. These crude fractions were used as terpenoids in all the experiments.

(ii) From the mycelial biomass

The isolation of terpenoids from submerged mycelia of the test-fungi was done following the method of Shiao *et al.* (1986), Anke and Werle (1990) and Chairul *et al.* (1991).

Mycelia of the test-fungi were harvested aseptically from 30-day-old liquid culture in fermentor (IL) by filtration through Sintered IG3 disc using vacuum pump and the harvested mycelia were washed at least three times with sterile distilled water in order to remove the adhering medium. Then the entire biomass was air dried and ground in Wiley Mill and passed through 60 mesh screen. The mycelial powder (ca 500 g) was extracted with methanol. The methanolic extract was concentrated under reduced pressure and the concentrated extract was dissolved in staturated sodium bicarbonate solution three times. The sodium bicarbonate solution was acidified with 10% HCL and extracted three times with ethyl acetate. The entire acidified ethyl acetate extract was chromatographed on a silica gel coloumn with hexano–ethyl acetate (1 : 1) mixture and three fractions (F1, F2 and F3) were collected. These crude fractions were used as terpenoids in all the experiments.

RESULTS AND DISCUSSION

The following fungi were collected from different localities of West Bengal and tissue cultures and basidiocarps of these fungi were used in the present investigation, viz. *Ganoderma applanatum* (Pers. ex Wallr.) Pat., *Lenzites repanda* (Pres.) Fr., *Polyporus zonalis* Berk., *Trametes corrugata* (Pers.) Bres. *Tricholoma crassum* (Berk.) Sacc. and *Gymonopilus chrysomyces* (Berk.) Lacc.

Tissue cultures were prepared from the freshly collected basidiocarp of the test-fungi on malt agar medium and maintained in malt agar medium at 20°C by subculturing at regular intervals for future uses.

Growth of mycelia and screening for antibiotic activity

The tissue cultures of all the test-fungi were grown in glucose-asparagine liquid medium in a shaking incubator (150 rpm) for 30 days at pH 6.0 and 30°C in Corning conical flasks (150 ml). The culture fluid was made free of the mycelia and tested for antibiotic activity using *Bacillus subtilis* as test organisms. The data are presented in Table 1.

The data revealed that mycelial growth was maximum in *Ganoderma applanatum* followed by *Tricholoma crassum, Trametes corrugata, Polyporus zonalis, Lenzites repanda* and *Gymnopilus chrysomyces*. The maxmium amount of mycelial growth was 13.40 g^{-1} in *Ganoderma applanatum* and the minimum amount of mycelial growth was 3.86 g^{-1} in *Lenzites repanda.*

The maximum antibiotic activity inhibition zone in

diameter was found in case of *Gonoderma applanatum* (18 mm) followed by *Tricholoma crassum* (17 mm), *Trametes corrugata* (16 mm), *Polyporus zonalis* (12 mm), *Lenzites repanda* (12 mm) and *Gymnopilus chrysomyces* (11 mm).

Table 1 : Data[a] showing the mycelial growth and antibiotic activity of the culture filtrate of the test-fungi

Test-Fungi[b]	Growth of mycelia (g^{-1})[c]	Antibiotic activity (mm)[d]
Tricholoma crassum	6.10 ± 1.6	17 ± 1.2
Trametes corrugata	10.03 ± 1.8	16 ± 1.0
Polyporus zonalis	5.10 ± 1.6	12 ± 1.4
Lenzites repanda	3.86 ± 1.2	12 ± 1.0
Gymnopilus chrysomyces	4.28 ± 1.0	11 ± 1.0
Ganoderma applanatum	13.40 ± 2.2	18 ± 1.2

[a] Data are mean of 3 replications.
[b] Tissue cultures of all the test fungi were prepared, maintained and used in the experiment.
[c] Tissue cultures of the test fungi were grown in glucose-asparagine liquid medium (pH 6.0) in shaking incubator (120 rpm) for 30 days at 30°C and the harvested filtered mycelia were dried at 60°C for 24 hours and weighed.
[d] The antibiotic activity of the culture filtrate was determined by agar plate diffusion assay using *Bacillus subtilis* as test organism and presented as diameter of the inhibition zone (mm).

Table 2 : Data[a] showing the antibacterial activity of terpenoids and polysaccharides obtained from the mycelia of *Tricholoma crassum*.

Test organisms[b]	Diameter of inhibition zone (mm)[c]	
	Terpenoids[d]	Polysaccharides[d]
Acinetobacter aerogenes	26 ± 2.0	20 ± 1.8
Acrobacter aerogenes	25 ± 2.0	20 ± 2.2
Arthrobacter citreus	21 ± 1.8	20 ± 2.0
Bacillus brevis	37 ± 2.0	22 ± 2.2
Bacillus subtilis	30 ± 1.4	24 ± 2.0
Corynebacterium insidiosum	17 ± 1.5	20 ± 2.4
Escherichia coli	22 ± 1.4	20 ± 1.8
Proteus vulgaris	26 ± 1.4	18 ± 1.4
Clostridium pasteurianum	20 ± 1.5	20 ± 1.6
Micrococcus roseus	18 ± 1.4	18 ± 1.4
Mycobacterium phlei	—	—
Sarcina lutea	—	—
Staphylococcus aureus	20 ± 1.5	17 ± 1.5

'—' indicate no activity.
[a] Data are mean of three replications.
[b] Test-organisms were grown on nutrient agar medium.
[c] The antibiotic activity was determined following the agar plate diffusion cup method.
[d] Terpenoids and polysaccharids were isolated from the mycelium grown in fermentor following the methods of Anke *et al.* (1990), Chairul *et al.* (1991) and Zhuang *et al.* (1993) respectively.

Table 3 : Data[a] showing the antibacterial activity of terpenoids and polysaccharides obtained from the mycelia of *Trametes corrugata*.

Test organisms[b]	Diameter of inhibition zone (mm)[c]	
	Terpenoids[d]	Polysaccharides[d]
Acinetobacter aerogenes	23 ± 1.2	18 ± 1.2
Acrobacter aerogenes	25 ± 1.4	20 ± 1.4
Arthrobacter citreus	21 ± 1.6	21 ± 1.5
Bacillus brevis	20 ± 1.0	18 ± 1.4
Bacillus subtilis	17 ± 1.5	20 ± 1.2
Corynebacterium insidiosum	22 ± 1.6	20 ± 1.2
Escherichia coli	29 ± 1.5	28 ± 1.4
Proteus vulgaris	20 ± 1.4	22 ± 1.4
Clostridium pasteurianum	21 ± 1.3	20 ± 1.4
Micrococcus roseus	22 ± 1.4	22 ± 1.4
Mycobacterium phlei	—	—
Sarcina lutea	11 ± 1.2	13 ± 1.4
Staphylococcus aureus	19 ± 1.5	20 ± 1.5

'—' indicate no activity.
[a] Data are mean of three replications.
[b] Test-organisms were grown on nutrient agar medium.
[c] The antibiotic activity was determined following the agar plate diffusion cup method.
[d] Terpenoids and polysaccharids were isolated from the mycelium grown in fermentor following the methods of Anke *et al.* (1990), Chairul *et al.* (1991) and Zhuang *et al.* (1993) respectively.

Table 4 : Data[a] showing the antibacterial activity of terpenoids and polysaccharides obtained from the mycelia of *Ganoderma applanatum*.

Test organisms[b]	Diameter of inhibition zone (mm)[c]	
	Terpenoids[d]	Polysaccharides[d]
Acinetobacter aerogenes	21 ± 1.2	18 ± 1.4
Acrobacter aerogenes	23 ± 1.3	19 ± 1.4
Arthrobacter citreus	20 ± 1.4	20 ± 1.5
Bacillus brevis	20 ± 1.2	18 ± 1.4
Bacillus subtilis	16 ± 1.4	20 ± 1.2
Corynebacterium insidiosum	22 ± 1.4	19 ± 1.2
Escherichia coli	28 ± 1.4	26 ± 1.4
Proteus vulgaris	20 ± 1.4	20 ± 1.2
Clostridium pasteurianum	20 ± 1.2	19 ± 1.4
Micrococcus roseus	21 ± 1.3	21 ± 1.3
Mycobacterium phlei	—	—
Sarcina lutea	10 ± 1.2	12 ± 1.2
Staphylococcus aureus	18 ± 1.4	19 ± 1.4

'—' indicate no activity.
[a] Data are mean of three replications.
[b] Test-organisms were grown on nutrient agar medium.
[c] The antibiotic activity was determined following the agar plate diffusion cup method.
[d] Terpenoids and polysaccharids were isolated from the mycelium grown in fermentor following the methods of Anke *et al.* (1990), Chairul *et al.* (1991) and Zhuang *et al.* (1993) respectively.

For screening of antibacterial activity, the mycelia of *T. crassum*, *T. corrugata* and *G. applanatum* were

extracted and polysaccharides and terpenoids were isolated and separated from the mycelial extract. These polyscchacides and terpenoids substances were tested against several bacteria by agar plate cup diffusion assay method. The data are given in Tables 2-4.

The data in Table 2 revealed that polysaccharides and terpenoids substances of *T. Crassum* were not at all active against *Mycobacterium phlei* and *Sarcina lutea.* Terpenoids were more inhibitory than polysaccharides.

The maximum inhibition was found in *Bacillus brevis* with terpenoids and minimum in *Cornybacterium insidiosum.* With polysaccharides the maximum inhibition was found in *Bacillus subtilis* and minimum in *staphylococcus aureus.*

The data in Table 3 revealed that terpenoids and polysaccharides of *Trametes corrugata* were not at all active against *Mycobacterium phlei.* In majority of the cases, the terpenoids, were more inhibitory than polysaccharides. In case of terpenoids, the maximum inhibitory activity was found in *E. coli and minimum in Sarcina lutea.* In case of polysaccharides the maximum inhibitory activity was found in *E. coli* and minimum in *Sarcina lutea.*

The data in Table 4 revealed that terpenoids and polysaccharides of *Ganoderma applanatum* were not active against *Mycobacterium phlei.* The terpenoids exhibited maximum inhibitory activity in *E. coli* and minimum inhibitory action against *Sarcina lutea.* The polysaccharides exhibited maximum inhibitory action against *E. coli* and minium against *Sarcina lutea.*

The present investigation on some aspects of medical benefits of six higher fungi of West Bengal State give an opportunity to discuss in a general way some of the salient features of the overall observations.

The six higher fungi, namely *Ganoderma applanatum, Lenzites repanda, Polyporus zonalis, Trametes corrugata, Tricholoma crassum and Gymnopilis chrysomyces* were found to be used by local people and tribal doctors, the *Ojhas,* for the treatment of several ailments of the tribal people.

In screening test, all the fungi were found to be good producer of antibacterial substance in culture filtrate. The maximum antibacterial activity was exhibited by *Ganoderma applanatum, Trametes corrugata* and *Tricholoma crassum.*

The data on the role of terpenoids and polysaccharides obtained from mycelia of the three fungi on the growth of bacteria revealed that these compounds were good antibacterial compounds. The inhibition of the growth of the bacteria varied among the terpenoids and the polysaccharides. Similar observations were reported by Stark and Anke (1988), Stark *et al.* (1991). Weber *et al.* (1990), Anke and Werle (1990) and Lauer *et al.* (1989, 1991).

The host-mediated antitumour high molecular weight compounds from basidiocarps, mycelia and culture media of several mushrooms were reported from Japan. The compounds were found to be heteroglucan, chitin, peptidoglycan, proteoglycan, lectin, RNA and undigestable dietary fiber polysaccharides in addition to-D-glucan (Mizuno *et al.,* 1992).

Coletto (1987-88) reported antibiotic activity of mycelia and culture filtrates of thirty six strains of Basidiomycetes and found that the fungi differed in their activities against the tested bacteria.

Anke (1989) reviewed excellently of production of secondary metabolites by the Basidiomycetes and pointed out the vast potentiality of utilising these compounds in human welfare.

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