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Isolation of Polysaccharides and Terpenoids from Some Basidiomycota and Their Antibacterial Activities

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Author's contribution

This whole work was carried out by the author SKG.

Original Research Article

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ABSTRACT

Aims: Antibacterial chemicals were isolated from fruit bodies of three basidiomycota [*Coltricia perennis* (L) Murrill*, Onnia tomentosa* (Fr.) P. Karst.*,* and *Polyporus mori* (Pollini) Fr.] fungi and their antibacterial potential were screened against five bacteria.
Study Design: All experiments were performed thrice in completely randomized design (CRD) each, with five replications per treatment (antibacterial activity). The data was subjected to ANOVA. Means of three observations were compared with Duncan's Multiple Range Test (DMRT).
Place and Duration of Study: Molecular Mycopathology Laboratory, Department of Botany, Ramakrishna Mission Vivekananda Centenary College, Rahara, Kolkata, between January 2012 and February 2013.
Methodology: During the rainy season in the year of 2012, a survey for mushroom collection in the forest beds, infected logs in the plain of west Bengal was conducted .The fruit bodies of some basidiomycota were collected in sterile biodegradable polythene begs and brought to laboratory. The morphology, anatomy of fruit bodies and measurement of reproductive organs were recorded. The spore prints of all collected basidiocarps were taken.The collected basidiomycota were identified. The polysaccharides from the basidiocarps of the test fungi were isolated employing the methods of Mizuno *et al.* [17] and Wang *et al.*[12].Terpeniods were isolated according to the method followed by Anke and Werte [24] and Chairul *et* al. [25].

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Their antibacteral activities were assayed against five bacteria [three Gram positive bacteria (*Staphylococcus aureus, Micrococcus roseus* and *Bacillus brevis*) and two Gram negative bacteria (*Ralstonia solanacearum* and *Escherichia coli*)] following the agar plates cup diffusion techniques.

Results: Terpenoid isolated from *Coltricia perennis* was most active in inhibiting the growth of all five bacteria. This terpenoid inhibited maximum (25 ±2.4mm) growth against *Staphylococcus aureus* and minimum against *Micrococcus roseus* (17±1.1mm). The polysaccharides isolated from these three mushrooms were less active against the test five bacteria. The terpenoids isolated from *Onnia tomentosa* and *Polyporus mori* also inhibited the growth of the test bacteria.

Conclusion: These three basidiomycetous mushrooms have antibacterial activity. After further research, their activity can be employed in medical science.

Keywords: Antibacterial agents; Inhibition; Bacteria; Mushrooms.

1. INTRODUCTION

Despite the huge diversity of antibacterial compounds, bacterial resistance to first-choice antibiotics has been drastically increasing. According to the WHO [1] “the bacterial infections which contribute most to human disease are also those in which emerging and microbial resistance is most evident: diarrhoeal diseases, respiratory tract infections, meningitis, sexually transmitted infections, and hospital-acquired (nosocomial) infections.” Moreover, the association between multiresistant microorganisms and nosocomial infections highlight the problem, and the urgent need for solutions. Natural resources have been exploited in the last years and among them, mushrooms could be an alternative source of new antimicrobials [2].

It is estimated that there are about 140,000 species of mushrooms on earth, and of these only 22000 are known and only a small percentage (5%) has been investigated. Therefore, there is much to explore about mushroom properties and potential applications [2,3].

There are many taxonomical works of fungi in India but scientific research on medicinal uses of mushrooms in India are very limited [4]. In West Bengal, one state of India, there are several uses of macrofungi by village people or “Ojahas”, for tribal people for treatments of many diseases like jaundis, dysentery, ear infection etc.

In abroad there are many reports on scientific researches on medicinal uses of macrofungi. Numerous mushroom extracts have been reported as having antimicrobial activity against gram-positive bacteria. The methanolic extract of *Agaricus bisporus* revealed MIC = 5 µg/mL against *Bacillus subtilis*, even lower than the standard ampicillin (MIC = 12.5 µg/mL) [5], and also showed activity against *Bacillus cereus, Micrococcus luteus, Micrococcus flavus, Staphylococcus aureus,* and *Staphylococcus epidermidis* [6,7,8]. Other *Agaricus* species have also demonstrated antimicrobial activity. *Agaricus bitorquis* and *Agaricus essettei* methanolic extracts showed an inhibitory effect upon all the tested gram-positive bacteria [6]. *Agaricus silvicola* methanolic extract also showed antimicrobial properties against *Bacillus cereus* (MIC = 5 µg/mL), *Bacillus subtilis*(MIC = 50 µg/mL), and against *Staphylococcus aureus* (MIC = 5 µg/mL), lower than the standard ampicillin (MIC = 6.25 µg/mL) [31]. The mycelium of *Agaricus cf. nigrecentulus* and *Tyromyces duracinus* (ethyl acetate extracts) showed activity only against *Staphylococcus saprophyticus* [9]. *Clitocybe alexandri* methanolic extract presented significant activity against *Bacillus subtilis, Micrococcus luteus ,Enterobacter aerogenes and Escherichia coli* [10]. Beatttie et al. [11] reported anti-

Pseudomonas aeruginosa activity of the genus *Cortinarius* and its subgenus, *Dermocybe* (methanolic extracts). Ethanolic extracts of *Armillaria mellea* fruiting bodies revealed better antimicrobial activity than chloroform extracts and mycelium extract upon gram-negative bacteria [12,13].Antitumour activity of polysaccharides isolated from basidiomes of some macrofungi such as *Ganoderma tsugae* and *Polyporus confluens* were reported [14,15]. Polysaccharopeptides produced from *Coriolus versicolor* has great commercial interest in anticancer therapy [16]. Mizuno et al. [17] recorded antitumour activity of heteroglycan isolated from *Tricholoma gigantium.* A new antibiotic named strobilurin –m from *Mycena* sp. was first reported by Daferner et al. [18]. The objectives of the present experiments were to isolate antimicrobial chemicals (terpenoids and polysaccharides) from fruit bodies of three basidiomycota fungi and to screen their antibacterial potentiality against five bacteria (Gram positive and Gram negative).

2. MATERIALS AND METHODS

2.1 Collection of Fruit Body and Identification of Fungi

During the rainy season in the year of 2012, a survey for mushroom collection in the forest beds, infected logs in the plain of West Bengal was conducted. The fruit bodies of some basidiomycota were collected in sterile biodegradable polythene bags and brought to laboratory. The morphology, anatomy of fruit bodies and measurement of reproductive organs were recorded. The spore prints of all collected basidiocarps were taken. The collected basidiomycota were identified by consulting with standard keys of basidiomycota published by Bakshi [19], Pacioni [20], Philips [21] and Jordan [22].

2.2 Biological Assay

2.2.1 Isolation of test compounds: Polysaccharides

The polysaccharides from the basidiocarps of the test fungi were isolated employing the methods of Mizuno *et al.*[17] and Wang et al. [23].The surface of the basidiocarps collected were cleaned by running tap water and air dried for 24 - 48 hrs. The cleaned and dried fruit body was crashed and powdered in Wiley mill (Wiley India Pvt.Ltd, Kol.01, India) and sieved by 60 mash screen. The powdered basidiocarp (ca: I kg) was treated by 85% ethanol (Merck, Shiv Sagar Estate, A, Dr.Annie Basant Road, Mumbai-400018, India) repeatedly at room temperature (30ºC ± 2ºC) for 24 hrs. The residue was then treated with hot water (100ºC) for 4 hrs and filtered by whatman filter paper – IV. The filtrate was collected and evaporated completely and this was the fraction -1 (F-l) of polysaccharides. The residue was then treated with 1% ammonium oxalate (Merck, Shiv Sagar Estate, A, Dr. Annie Basant Road, Mumbai-400018, India) solution at 100ºC for 5 hrs. The filtrate was collected and evaporated completely under reduced pressure by suction pump (Precivace Engineering Pvt. Ltd. N.S. Road Kol.01,India). It was taken as Fraction - II (F - II) of polysaccharides .The remaining residue was treated with 5% sodium hydroxide (Merck, Shiv Sagar Estate, A, Dr.Annie Basant Road, Mumbai -400018, India) solution at 80ºC for 5 hrs. The filtrate was collected and evaporated under reduced pressure. This was taken as Fraction - III (F- III) of polysaccharides.

All fractions (F -1, F - II & F - III) were mixed and tested against test bacteria.

2.2.2 Isolation of test compounds: Terpenoids

It was done according to the method followed by Anke and Werte [24] and Chairul et al. [25]. Fruit bodies were cleaned and dried at room temperature. The powder of dried fruit bodies (1 kg in weight) was extracted with methanol (85%). The extract was evaporated completely and the solid residue was subjected to dissolve in saturated sodium bicarbonate (Merck, Shiv Sagar Estate, A, Dr.Annie Basant Road ,Mumbai,400018, India) solution five times. The sodium bicarbonate was added with 10 % HCl and extracted three times with ethyl acetate. The whole acidified ethyl acetate extract was chromatographed on a silica gel (Merck,Shiv Sagar Estate,A, Dr. Annie Basant Road ,Mumbai,400018, India) column with hexane ethyl acetate (1/1) mixture (Merck, Shiv Sagar Estate, A, Dr. Annie Basant Road,Mumbai,400018, India) and three fractions (F-1. F-2&F-3) were collected. These three fractions were mixed and used as terpenoids and employed for antibacterial bioassay.

2.3 Test Bacteria

Staphylococcus aureus, *Micrococcus roseus, Bacillus brevis, Escherichia coli* and *Ralstonia solanacearum* were taken in this experiment.

2.4 Antibacterial Assay Method

The concentrated each compound (polysaccharides and terpenoids) were re-dissolved in dimethyl sulfoxide (DMSO) to make 20 ug /ml solutions. These solutions were passed through 0.03µm Milipore filter paper aseptically in Laminar Air Flow Chamber and treated against selected bacteria *(Staphylococcus aureus*, *Micrococcus roseus, Bacillus brevis, Escherichia coli* and *Ralstonia solanacearum).* It was done using nutrient agar medium (Peptone,0.5%; Beef extract,0.3%; NaCl,0.5%;Agar,2%; Distilled water,100ml; pH,7.0 : all ingredients were from Merck, Shiv Sagar Estate, A, Dr. Annie Basant Road ,Mumbai,400018, India: Systronic digital pH meter, Ahamadabad, India) following the agar plates cup diffusion techniques [26]. The prepared agar plates were inoculated with 200 µl bacteria culture by spreading evenly over the surface of agar plate using an ethanol flamed glass Drigalsky spatula (spreader). An uninoculated untreated agar plate was incubated at 35 ^{0}C for 24 hours before use to ensure sterility. Wells of 5 mm in diameter and 4 mm in depth were made on the agar using a sterile cork borer. For each test microorganism, 25 µl of each extract and of control were pipetted into different wells. The wells were then labeled to correspond with the test crude compounds and controls. The treated plates were stored in a refrigerator (DAEWOO®, Daewoo Electronics, Europe GmbH, Germany) at 4 ^{0}C for at least six hours to allow diffusion of the extracts into the agar while arresting the growth of the test microbes. The solution of ampicillin was taken in wells as positive control while solution of DMSO (20 µg /ml) was taken in wells as negative control. The plates were then incubated for 24 hours at 35 ^{0}C for bacteria. After 24 hrs, incubation of bacteria at 35 ^{0}C temperature in a BOD incubator (REMI 6, Ganash Avenue, Kol.-17,India), the diameter of the inhibition zone (in mm) was recorded.

2.5 Phytochemical Analysis

Qualitative phytochemical analysis of the isolated compounds of each of the three species of mushrooms was determined [27].

2.6 Statistical Analysis

All experiments were performed thrice in completely randomized design (CRD) each, with five replications per treatment (antibacterial activity). The data was subjected to analysis of variance (ANOVA) using Genstat statistical software. Means of three observations were compared with Duncan's Multiple Range Test (DMRT) at P=.05 for determining the statistical significance.

3. RESULTS AND DISCUSSION

The components isolated from three basidiomycetous mushrooms (*Coltricia perennis, Onnia tomentosa,* and *Polyporus mori*) were qualitatively tested. FI,FII and FIII components gave positive response to polysaccharideswhile F1, F2 and F3 components showed positive response to terpenoids..The data represented in the Table 1 indicated that the terpenoid isolated from *Coltricia perennis* was most active in inhibiting the growth of all test bacteria . Moreover, the maximum inhibition zone (25±2.4mm) was recorded in case of *Staphylococcus aureus* by the terpenoids isolated from *Coltricia perennis.* The minimum inhibition zone (17±1.1mm) was noted against *Ralstonia solanacearum. Staphylococcus aureus* is the dangerous causal pathogen of boils, scadded skin syndrome and impetigo contigosa of human [28].The inhibition of growth of tested bacteria (*Staphylococcus aureus* and *Micrococcus roseus)* in this experiment by terpenoid from this mushroom were statistically different to the ampicillin. On the other hand the inhibition zone against other bacteria (*Bacillus brevis, Escherichia coli* and *Ralstonia solanacearum*) by these terpenoids were statistically similar. The terpenoids isolated from *Coltricia perennis* exhibited inhibition zone (25±2.4mm) against *Staphylococcus aureus* which was superior than ampicillin(20±1.2). The highest inhibition zone size (25±2.4mm) against *S. aureus* suggested that the terpenoids could be used in the treatment of infections commonly associated with the organism. The highest antibacterial activity was seen in the extract of *Hygrophorus agathosmus* against *Staphylococcus epidermidis* and *Bacillus subtilis*, with MIC values 7.81 µg/mL lower than the reference antibiotic streptomycin (MIC = 15.62 µg/mL). MIC values (15.62 µg/mL) against *Staphylococcus aureus* were equal to the ones of streptomycin. *Suillus collitinus* showed MIC values much higher than the standard [29]. It may be mentioned that isolation of antibacterial component from *C. perennis* was not reported earlier. But isolation of antibacterial component from other mushrooms against these five bacteria has been done by many scientists [2].

The data in the Table 2 showed that maximum inhibition zone (22±2.5mm) was recorded by terpenoid from *Onnia tomentosa* against *S. aureus* followed by *Escherichia coli* (20±2.0mm) *Ralstonia solanacearum* (17±2.0 mm), *Bacillus brevis*(16±1.8mm) and *Micrococcus roseus* (14±1.0 mm) respectively. In respect of polysaccharides extracted from *Onnia tomentosa*, it was observed that maximum inhibition zone (17±1.7 mm) against *S. aureus* was recorded, the minimum inhibition zone (8±1.4mm) was recorded against *Micrococccus roseus*.

In this experiment, the effect of terpenoid from *Onnia tomentosa* against *S.aureus* (22±2.5mm) and *Ralstonia solanacearum* (17±2.0mm) were superior than the ampicillin. The antibacterial of *Onnia tomentosa* was not noted by other workers.

Table 1. **Antibacterial activities of polysaccharides and terpenoids isolated from basidiocarp of *Coltricia perennis***

Test bacteria	Diameter (mm) of inhibition zone ±SE			
	Polysaccharides	Terpenoids	Ampicillin (positive control)	DMSO (Negative control)
1.Staphylococcus aureus	16 ± 1.8 [a]	25± 2.4 [c]	20±1.2 [b]	00.0
2.Ralstonia solanacearum	12 ±1.3 [a]	17±1.1 [b]	15±1.1 [b]	00.0
3. Micrococcus roseus	9 ±1.2 [a]	20±1.9 [c]	17±1.5 [b]	00.0
4.Escherichia coli	10±2.0 [a]	22±2.3 [b]	21±1.9 [b]	00.0
5.Bacillus brevis	14 ±1.3 [a]	18±1.8 [b]	17.9 ±1,1 [b]	00.0

**The data presented average of 5 replications ; The same letter in the same row showed no different statistically but different letter in the same row indicated they are statistically different as per Duncan Multiple Test (P=.05).*

Table 2. Antibacterial activities of polysaccharides and terpenoids isolated from basidiocarp of *Onnia tomentosa*

.

Test bacteria	Diameter (mm)* of inhibition zone ±SE			
	Polysaccharides	Terpenoids	Ampicillin (positive control)	DMSO (Negative control)
1.Staphylococcus aureus	17±1.7 [a]	22±2.5 [c]	20±1.2 [b]	00.0
2.Ralstonia solanacearum	11 ±1.2 [a]	17±2.0 [c]	15±1.1 [b]	00.0
3. Micrococcus roseus	8 ±1.4 [a]	14±1.9 [b]	17±1.5 [c]	00.0
4.Escherichia coli	9±1.1 [a]	20±2.0 [b]	21±1. [b] 9	00.0
5.Bacillus brevis	12±1.3 [a]	16+1.8 [b]	17.9 ±1,1 [b]	00.0

**The data represented average of 5 replications replications; The same letter in the same row showed no different in statistically as per Duncan Multiple Test (P=.05)*

It was observed from the data presented in the Table-3 that terpenoids isolated from *Polyporus mori* inhibited maximum growth against *Ralstonia solanacearum* followed by *S. aureus*. Polysaccharides isolated from *Polyporus mori* also showed maximum inhibition against *R. solanacearum*. *R. solanacearum* is causal pathogen of vascular wilt disease of brinjal in agricultural field [30]. During the past three decades, many polysaccharides and polysaccharides- proteins complexes have been isolated from fungi, algae, lichens and plants, homogeneous and heterogeneous polysaccharides, glycans, and glycan-protein complexes from fungi have been shown to promote good health [31]. Many Basidiomycetes mushrooms contain biologically active polysaccharides [32], some of which exhibiting haematological, antiviral, antitumour, antibiotic, antibacterial, and immunomodulating activities. Adebayo et al. [33] reported that polysaccharides isolated from *Pleurotus pulmonarius* exhibited highest zone of inhibition(30 mm) against *S. aureus*. This inhibition zone size was higher than all other tested synthetic antibiotics except Gentamicin and Tetracycline.

The maximum inhibition of Polysaccharides isolated from *Polyporus mori* against *R. solanacearum* in this experiment may lead plant pathologists for field trials using powder of basidiocarps of *Polyporus mori* against bacteria wilt of brinjal.

Table 3. Antibacterial activities of polysaccharides and terpenoids isolated from basidiocarp of *Polyporus mori*

Test bacteria	Diameter (mm)* of inhibition zone ±SE			
	Polysaccharides	Terpenoids	Ampicillin (positive Control)	DMSO (Negative control)
1.*Staphylococcus aureus*	10±1.2 [a]	15± 2.1 [b]	20±1,2 [c]	00.0
2.*Ralstonia solanacearum*	15±1.5 [a]	19±1.9 [c]	15±1.1 [a]	00.0
3. *Micrococcus roseus*	9±1.2 [a]	12±1.4 [b]	17±1.5 [c]	00.0
4.*Escherichia coli*	8±1.0 [a]	10±1.2 [b]	21±1.9 [c]	00.0
5.*Bacillus brevis*	10±1.5 [a]	12±1.6 [a]	17.9±1,1 [b]	00.0

**The data represented average of 5 replications ; The same letter in the same row showed no different in statistically but different letter in the same row indicated they are statistically different as per Duncan Multiple Test (P=05) .*

The comparison among the antibacterial activities of the three basidiomycota (Table -1, 2 and 3) indicated that the inhibition of the test bacteria varied among the terpenoids and the polysaccharides. But terpenoids isolated from these basidiomycota were always better than the polysaccharides isolated. Terpenoids constitute one of the largest group of naturally occurring compounds in plant, animal and protista kingdoms, being characterized by their great diversity of chemical structure. Various plant origin terpenoids have been developed as important medicinal drugs. In contrast fewer fungal terpenoids have been developed in the medical field [34]. In the list of bioactive terpenoids published in 1969 [35] an antibacterial agent, fusidic acid was the only fungal origin. Thereafter, siccanin, a triprenylphenol was successfully developed in Japan as an antidermatophytic agent [34,36]. However, a .Sterostreins A-E, fivenovel terpenoids, were isolated from cultures of the mushroom-Stereum ostrea. Sterostreins –A exhibited antimalarial activity [37].

The antibacterial activities of these three basidiomycota (*Coltricia perennis, Onnia tomentosa,* and *Polyporus mori*) were not reported earlier in India and abroad. But the antimicrobial activities of other basidiomycota were reported from abroad by many workers [38,39]. *Trametes versicolor* (L.)Lioyd has been considered among the 25 major medicinal macrofungi worldwide [40] mainly due to its traditional usage. Interesting polysaccharopeptides have been purified from this species, showing experimental immunomodulatory and anti-cancer effects [41,42]. Similar observation of antimicrobial activities of some basidiomycota (*Polyporus zonalis, Lenzities repanda, Ganoderma applanatum* etc) were recorded by few workers in India [4,43,44] and the results of this experiments were at per with these workers. The antimicrobial effect of ethanol extracts of *Russula delica* was tested against six species of Gram-positive bacteria, seven species of Gram-negative bacteria and one species of yeast. The test extract showed more potent activity against Gram-positive than Gram negative bacteria. The antimicrobial activities were comparable with those of commonly used antibiotics [45].Sheena et al. [46,40] have observed that three macrofungi *Ganoderma lucidum, Navesporus floccose* and *Phellinus rimosus* are showing antibacterial activity that occurring in South India. *G. lucidum*, which not only contains 120 different triterpenes but also polysaccharides, proteins and other bioactive compounds, the spectrum of detected the pharmacological activities of mushrooms is very

broad on Multidrug resistant *Staphylococcus aureus* [47]. Of special interest are compounds with activities against multiresistant bacterial strains. Lindequist et al.[48] showed that new sesquiterpenoid hydroquinones produced by the European *Ganoderma* species *Ganoderma pfeifferi* Bres. and named ganomycins inhibited the growth of methicillin-resistant *Staphylococcus aureus* and other bacteria. Besides, they found that whole extracts of this mushroom inhibited the growth of microorganisms responsible for skin problems (*Pityrosporum ovale, Staphylococcus epidermidis,*and *Propionibacterium acnes*). When 317 strains, representing 204 species and 17 orders of basidiomycete mushrooms were screened for antimicrobial activity on human pathogens, over 45 % of the tested strains were positive for antibacterial activity [49]. The highest activity occurred among members of the *Ganodermatales*, *Poriales*, *Agaricales*, and *Stereales*. *Ganoderma applanatum i*nhibited Gram-positive bacteria such as *Bacillus cereus* and *Staphylococcus aureus* were less inhibitory against Gram-negative bacteria, including *Escherichia coli* and *Pseudomonas aeruginosa* [50]. Lentinan from shiitake mushroom *Lentinus edodes* inhibited *Mycobacterium tuberculosis* and *Listeria monocytogenes* [51].

Three antibacterial substances extracted with chloroform, ethyl acetate, or water from fruiting bodies of *L. edodes* are active against *Streptococcus* sp., *Actinomyces* sp., *Lactobacillus* sp., *Prevotella* sp., and *Porphyromonas* sp. It was suggested that these active compounds might be similar to lenthionine, disulphide derivative and lentinan types, respectively [52].

The literature regarding the exact mechanism of antimicrobial effects of mushrooms is very limited. Many antimicrobial compounds such as terpenes, lectins, polysaccharides etc. act on the bacterial cytoplasmic membrane [53,54]. Strobilurins is another class of fungicidal compounds extracted from mycelia of the mushroom *Strobilurus tenacellus*. Strobilurins A and B were highly active by inhibiting respiration of yeast and other filamentous fungi. The biological activity of strobilurins involve ubihydroquinone cytochrome C reductase, which plays a crucial role in respiration [55]. Its activity, however, depends of the presence of (E)-b-methoxyacrylate moiety [56]. The antifungal (E)-b-methoxyacrylates of strobilurin C and outdemansin B from cultures of *Xerula longipes and* Strobilurin E extracted from mycelial cultures of *Crepidotus fulvotomentosus* inhibited many phytopathogenic fungi and they also inhibited fungal respiration [57,40]. The fungicide strobilurin, F 500 enhances resistance of tobacco to the wild fire pathogen *Pseudomonas syringae* pv. *tabaci*. The mechanism of action of strobilurin F 500 is by inducing cellular responses to the pathogen attack. It induces production of endogenous salicylic acid and pathogenesis-related proteins that usually are used as molecular markers for disease resistance [58].

4. CONCLUSION

The spectrum of detected pharmacological activities of mushrooms is very broad. Dependent on increasing knowledge about chemistry, biotechnology and molecular biology of mushrooms as well as an improvement of screening methods (high throughout screening, genomics and proteomics), a rapid increase in the application of mushrooms for medicinal purposes can be expected.These three basidiomycetous mushrooms (*Coltricia perennis, Onnia tomentosa,* and *Polyporus mori*) have terpenoids and polysaccharides. These components have antibacterial activity against bacteria but terpenoids are better than polysaccharides. After further research, their activity can be employed in medical science and specially polysaccharides of *Polyporus mori* may be applied in brinjal wilt protection.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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