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Research Paper

Investigation on Antibacterial Activities of Terpenoid and Polysaccharide From *Scleroderma Citrinum* Pers. Pers. and *Tremetes Versicolor* Fr. Quel

Biological Science

KEYWORDS : Antibacterial agents; Inhibition ; Bacteria; Mushroom

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ABSTRACT

Polysaccharides and terpenoids were isolatedfrom fruit bodies of basidiomycetous fungi Scleroderma citrinumPers. Pers. andTrametesversicolorFr. Quel.,which were collected from the plain of West Bengal,India.Their antibacterial activities were assayed by agar plates cup diffusion techniques against three Gram + ve bacteria (Staphylococcus aureus,Micrococcusroseus and Bacillus brevis) and two Gram – ve bacteria (Ralstonia solanacearum and Escherichia coli).Terpenoid isolated fromTrametesversicolorFr. Quelwas most active in inhibiting the growth of all five bacteria. Terpenoid inhibited maximum (19 ±2.4mm)growth against Staphylococcus aureus and minimum against Micrococcus roseus (17±1.1mm). The MIC of terpenoid isolated from T. versicolor was 5µg/ml against S.aureus and R.solanacearum and it was similar to ampicillin (5µg/ml).The polysaccharides isolated from these two mushrooms were less active against the test five bacteria.The terpenoids isolated from Scleroderma citrinum Pers.Persalso inhibited the growth of the test bacteria.

Introduction

Although, the huge diversity of antibacterial compounds is present, bacterial resistance to first-choice antibiotics has been alarmingly increasing. Some examples are microorganisms such as Klebsiella spp. and Escherichia coli, which produce broad-spectrum β-lactamase or present resistance to third-generation cephalosporins. Other examples include MRSA, Enterococcus spp., which is resistant to vancomycin (Harbarth et al., 2001; Segal-Maurer, 1996), Acinetobacter spp. with an increasing resistance to carbapenems and colistin (Kempf and Rolain, 2012), and Pseudomonas spp., which is resistant to aminoglycosides, carbapenemics. and/or cephalosporins (Harbarth et al., 2001). Diseases that were easily healed are now-a-days becoming a serious problem due to emergent antibiotic resistance (WHO, 2000; Peres-Bota et al., 2003). The association between multiresistant microorganisms and hospital infections certainly highlights this genuine problem and the urgent need for solutions (Pittet, 2005). WHO (2012) advised all countries to implement control procedures for the propagation of drug multiresistant bacteria, highlighting the risks associated to the absence of alternative therapies against those microorganisms. Therefore, the research of new antimicrobial substances effective against pathogenic microorganisms resistant to current drugs is very essential. New groups of organisms, such as mushrooms could be an alternative source for new antimicrobials. Polysaccharides or peptideglycan, terpenoids pharmaceutically active mushroom compounds, continue to be the subject of most researches, including isolation, chemical structures and experiments in vitro or in vivo. New sesquiterpenoid hydroquinones, produced by the European Ganoderma species Ganoderma pfeifferi Bres. and named ganomycins, inhibit the growth of methicillin- resistant Staphylococcus aureus and other bacteria (Mothana et al., 2000). There are many taxonomical works of fungi in India but scientific research on medicinal uses of mushrooms in India are very limited (Bhattacharrya et al., 2006). In abroad, there are many reports on scientific researches on medicinal uses of macrofungi. In West Bengal, one state of India, there are several uses of macrofungi by village people or ojahas (Quack), for people for treatments of many diseases like jaundis, dysentery, ear infection etc. Antitumour activity of polysaccharides isolated from basidiomes of some macrofungi such as Ganoderma tsugae and Polyporus confluens were reported (Mizuno ,1992; Stark 1991). Polysaccharopeptides produced from Coriolus versicolor has great commercial interest in anticancer therapy (Cui and Chisti 2,003). Mizuno et al. (1995) recorded antitumour activity of heteroghlycan isolated from Tricholoma gigantium. A new antibiotic named strobilurin –m from Mycena sp. was first reported by Daferner et al. (1999). Antibacterial activity of organic extracts (70% methanol, 100% chloroform) from 10 lignicolous mushroom and fungal species—Meripilus giganteus, Laetiporus sulphureus, Coriolus versicolor, Flammulina velutipes, Ganoderma lucidum, G. applanatum, Pleurotus ostreatus, Piptoporus betulinus, Panus tigrinus, and Fistulina hepatica—were analyzed against 18 strains of bacteria using the agar-well diffusion assay. The level of antimicrobial effect was determined by dilution susceptibility tests in micro-plates. Antibiotic susceptibility assay of the bacterial strains to common antibiotics was done with the disk-diffusion method. The extracts of Piptoporus betulinus were the most active. The examined extracts, including M. giganteus and P. tigrinus, which were tested for the first time, inhibited mostly Gram-positive bacteria (Bacillus sp., Rhodococcus equi, and Staphylococcus aureus) resistant to common antibiotics. Minimal inhibitory concentrations ranged from 17.5 to 9000 µg/mL. The results deamonstrate that lignicolous mushrooms and fungi may be rich sources for new biologically active products. (Karaman et al., 2009). Dulger and Gonuz (2004) reported the antimicrobial properties of 4 different extracts of macrofungus (Cantharellus cibarius) against 50 important human pathogens. He observed good antimicrobial activity with ethanol and acetone extracts against most of the pathogens. Cowan (1999) reported that the most active components are generally water insoluble, hence it is expected that low polarity organic solvents would yield more active extracts. In the present experiments, antibacterial chemicals such as polysaccharides and terpenoids were isolated from fruit bodies of two basdiomycetous fungi and their antibacterial efficacies were screened against five bacteria (Gram +ve and Gram –ve bacteria).

METERIALS AND METHODS

Collection of fruit body and identification of fungi

During the rainy season in the year of 2013, a survey for mushroom collection in the forest beds, infected logs at remote village Iswaripur, 24 Parganas (N), in the plain of West Bengal was conducted and consulted with the old medicine men for the medicinal use s of the collected fruit bodies of mushrooms. The fruit bodies of some basidiomycota were collected in sterile biodegradable polythene begs and brought to laboratory. The morphology, 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 (1971), Pacioni (1981), Philips(1981) and Jordan (1999).

Biological assay

Isolation of Test compounds: Polysaccharides

The polysaccharides from the basidiocarps of the test fungi were isolated employing the methods of Mizuno et al. (1992) and Wang et al.(1993).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.Estate, A, Dr. Annie Basant Road, Mumbai,400018, India) repeatedly at room temperature (30°C ± 2oC) for 24 hrs. The residue was then treated with hot water (100 0C) for 4 hrs and filtered by whatman filter paper–IV. The filtrate was collected and evaporated completely and this was the fraction -1 of polysaccharide. The residue was then treated with 1% ammonium oxalate (Merck, Shiv Sagar Estate A, Dr. Annie Basant Road, Mumbai, 400018, India) solution at 100 0C 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 - 2 (F-2) 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 800C for 5 hrs. The filtrate was collected and evaporated under reduced pressure. This was taken as Fraction – 3(F- 3) of polysaccharides. All fractions (F -1, F - 2, & F - 3) were mixed and the solvents were evaporated to make it dry powder in a steady air-current for about 24 hours in a previously weighed evaporation dishes (porcelain dishes). After evaporation, the dishes were re-weighed and the differences in weights before and after evaporation were calculated (Trease and Evans, 1994). The extracts powders were stored (4°C) in a clean sterile container for further use.

Isolation of test compound : Terpenoids

It was done according to the method followed by Anke and Werte (1990) and Chairul et al. (1991). Fruit bodies were cleaned and dried at room temperature. The dried bodies were ground by Wiley mill to powders. The powders were sieved through 60 mash screen. The powder of dried fruit bodies (1kg 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). These three fractions were mixed and the solvents were evaporated to make it dry powder in a steady air-current for about 24 hours in a previously weighed evaporation dishes (porcelain dishes). After evaporation, the dishes were re-weighed and the differences in weights before and after evaporation were calculated (Trease and Evans, 1994). The extracted powders were stored (4°C) in a clean sterile container for further use as terpenoids and employed for antibacterial bioassay.

Test Bacteria

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

Phytochemical Analysis

Qualitative phytochemical analysis of the isolated compounds of each of the two species of mushrooms was determined (Harborne ,1973).

Biological 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 ,1%; 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 (NCCLS 2000). 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 350C 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 0C 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 0C for bacteria. After 24 hrs, incubation of bacteria at 350C temperature in a BOD incubator (REMI 6, Ganash Avenue, Kol.-17, India), the diameter of the inhibition zone (in mm) was recorded.

Determination of minimum inhibitory concentration (MIC) of the extracts

The minimum inhibitory concentration (MIC) was determined by macro-broth dilution techniques as specified by National Committee for Clinical Laboratory Standards (NCCLS 2000). A twofold serial dilution of the reconstituted extract was prepared in Mueller Hinton Broth. Each dilution was seeded with 100 µl of the standardized suspension of the test organism (1 × 106 cfu/ml) for Gram positive bacteria and (5 ×105 cfu/ml) for Gram negative bacteria and incubated for 24 h at 37°C. MIC was determined as the highest dilution (i.e. lowest concentration) of the extract that showed no visible growth.

RESULTS AND DISCUSSION

The collected fruit bodies of mushrooms were phenotypically identified as Trametes (Coriolus) versicolor Fr. Quel. and Scleroderma citrinum Pers. Pers. Qualitative phytochemical analysis of the isolated fraction F1,F2 and F3 showed that they were polysaccharide while isolated fraction FI,FII and FIII were terpenoids.

The data represented in the Table 1 indicated the terpenoid isolated from Trametes (Coriolus) versicolor Fr. Quel. was most active in inhibiting the growth of all test bacteria . Moreover, the maximum inhibition zone (19 ±2.4mm) was recorded in case of Staphylococcus aureus by the terpenoids isolated from Trametes (Coriolus) versicolor. The positive control (ampicillin) showed more or less similar inhibition zone (20±1.2) against Staphylococcus aureus. The terpenoid isolated from T. versicolor showed 15±1.8 mm inhibition zone which was equal to positive control (ampicillin) against R .solanacearum. The inhibition zone by terpenoids from this mushroom against other three bacteria were less than positive control (ampicillin). The minimum inhibition zone (12 ±1.3mm) was noted against Bacillus brevis. Staphylococcus aureus is the dangerous causal pathogen of boils, scadded skin syndrome and impedigo contigosa of human (Black ,2008). It may be mentioned here that ojahas of West Bengal, India, use fruit body of Scleroderma citrinum and Trametes versicolor for curing skin injury and boils of human skins. Polysaccharide isolated from T versicolor gave maximum inhibition (15 ± 1.7) against S aureus which was lower than ampicillin .Other bacteria were also inhibited by polysaccharide isolated. Many basidiomycetes mushrooms contain biologically active polysaccharides (Yap and Ng 2001), some of which exhibiting haematological, antiviral, antitumour, antibiotic, antibacterial, and immunomodulating activities. Adebayo et al. (2012) reported that polysaccharides isolated from Pleurotus pulmo-

narius exhibited highest zone of inhibition (30 mm) against S. aureus. This inhibition zone size was higher than all other tested synthetic antibiotics except Gentamicin andTetracycline. The polysaccharides isolated from this mushroom are less effective against the test bacteria in compare with terpenoids. Quershi et al. (2010) have studied the antimicrobial activity of various solvent extracts (40µg/ml) of Ganoderma lucidum and tested against six pathogenic species of bacteria. Acetone extract exhibited maximum antibacterial activity (31.60±0.10), while the most susceptible bacterium observed was Klebsiella pneumoniae. The antimicrobial effect of ethanol extracts of Pleurotus sajorcaju, P. florida and P. aureovillosus were tested against four species of Gram-positive bacteria, five species of Gram-negative bacteria and one species of yeast.

Table 1: Antibacterial activities of polysaccharides and terpenoids isolated from basidiocarp of Trametes(Coriolus) versicolor Fr. Quel

Test bacteria	Diameter (mm)* of inhibition zone±SE			
	Polysaccharides	Terpenoids	Ampicillin (Positive control)	DMSO (Negative Control)
1.Staphylococcus aureus	15 ± 1.7[a]	19 ± 2.0[b]	20±1.2 [b]	0.00
2.Ralstonia solanacearum	12 ±1.5[a]	15±1.8[b]	15±1.1 [b]	0.00
3. Micrococcus roseus	10±1.2[a]	14±1.5[ab]	17±1.5 [b]	0.00
4.Escherichia coli	10±1.3[a]	13±1.2[a]	21±1.9 [b]	0.00
5.Bacillus brevis	12±1.5[a]	12±1.3[a]	17.9 ±1.1[b]	0.00

*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).

The data in the Table 2 revealed that maximum inhibition zone (9±1.7mm) was recorded by terpenoid isolated from Scleroderma citrinum against S. aureus, followed by Ralstonia solanacearum (8±2.0mm), Bacillus brevis (8±1.4mm), Escherichia coli (7±1.30mm) and Micrococcus roseus (6±1.6mm) respectively. In respect of polysaccharides isolated from Scleroderma citrinum, it was observed that maximum inhibition zone (7±1.6mm) against Ralstonia solanacearum was recorded, the minimum inhibition zone (4±1.2mm) was recorded against Micrococccus roseus. The isolated terpenoids and polysaccharides from Scleroderma citrinum showed lesser effective /inhibition zone in comparison to positive control (ampicillin) against all five test bacteria. R. solanacearum is causal pathogen of vascular wilt disease of brinjal in agricultural field (Rangswami,1984). It may lead plant pathologists for field trials using basidiocarps of Tremetes versicolor against bacteria wilt of brinjal.

Table 2: Antibacterial activities of polysaccharides and terpenoids isolated from basidiocarp of Scleroderma citrinumPers. Pers

Test bacteria	Diameter (mm)* of inhibition zone ±SE			
	Polysaccharides	Terpenoids	Ampicillin (positive control)	DMSO (Negative control)
1.Staphylococcus aureus	6±1.3[a]	9±1.7[b]	20±1.2 [c]	0.00
2.Ralstonia solanacearum	7±1.6[a]	8±1.5[a]	15±1.1 b	0.00
3. Micrococcus roseus	4±1.2[a]	6±1.6[a]	17±1.5 b	0.00
4. Escherichia coli	5±1.4[a]	7±1.3[a]	21±1.9 b	0.00
5.Bacillus brevis	6±1.2[a]	8+1.4[a]	17.9 ±1.1[b]	0.00

*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).

The comparison among the antibacterial activities of the two basidiomycota (Table -1&2) indicated that the inhibition of the test bacteria varied among the terpenoids and the polysaccharides. But terpenoids isolated from two basidiomycota were always better than the polysaccharides isolated. 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 (Inouye and Yamaguchi). In the list of bioactive terpenoids published in 1969 (Martin–Smith 1969) an antibacterial agent, fusidic acid was the only fungal origin. Thereafter, siccanin, a triprenylphenol was successfully developed in Japan as an antidermatophytic agent (Inouye and Yamaguchi; Barros et al., 2007). However, sterostreins A-E, five novel terpenoids, were isolated from cultures of the mushroom-Stereum ostrea. Sterostreins –A exhibited antimalarial activity (Isaka et al., 2011).

Agaricus bitorquis and Agaricus essettei methanolic extracts showed an inhibitory effect upon all the tested gram-positive bacteria (Ozturk et al., 2011). 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) (Jong, 2002). In this experiment (table 3), MIC of terpenoid from T versicolor against S.aureus and R. solanacearum is 5µg/ml which is similar as showed by ampicillin against these two bacteria. The MIC of this terpenoid against other bacteria (Staphylococcus aureus, Micrococcus roseus, Bacillus brevis, Escherichia coli and Ralstonia solanacearum) was 10µg /ml which was higher than ampicillin . MIC of polysaccharide isolated from T. versicolor against all tested bacteria was 10g/ml. The MIC of polysaccharide isolated from S. citrinum was 15µg/ml against all tested bacteria which was higher than polysaccharide from T versicolor and ampicillin.The terpenoid isolated from S. citrinum showed MIC from 10-15µg/ml against all tested bacteria.

Table3: Minimum inhibitory concentrations (MIC) of the polysaccharides and terpenoids isolated from two mushrooms against all test bacteria.

Test Bacteria	Trametes(Coriolus) versicolor (MIC)*		Scleroderma citrinum (MIC)		Positive Control -ampicillin (µg/ml) (MIC)
	Polysaccharide (µg/ml)	Terpenoid (µg/ml)	Polysaccharide (µg/ml)	Terpenoid (µg/ml)	
1 Staphylococcus aureus	10.00	5.00	15.00	10.00	5.00
2. Ralstonia solanacearum	10.00	5.00	15.00	10.00	5.00
3. Micrococcus roseus	10.00	10.00	15.00	15.00	5.00
4. Escherichia coli	10.00	10.00	15.00	15.00	5.00
5.Bacillus brevis	10.00	10.00	15.00	15.00	5.00

*Results are mean of three separate experiments, each in triplicate.

The antibacterial activities of these two basidiomycota (Scleroderma citrinum Pers. Pers. and Trametes(Coriolus) versicolor Fr. Quel.) were not reported earlier in India and abroad . But the

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antimicrobial activities of other basidiomycota were reported from abroad by many workers (Chang and Miles, 2004; Stark, 1991; Wang, 1993; Wasser and Weis, 1999; Weber 1990). Similar observation of antimicrobial activities of some basidiomycota (Polyporus zonalis, Lenzities repanda , Ganoderma applanatum etc) were recorded by few workers in India (Bhattacharrya ,2006; Bose, 1953). Many antimicrobial compounds such as terpenes, lectins, polysaccharides etc. act on the bacterial cytoplasmic membrane (Lin and Chou, 1984; Yang et al., 2002). Determination of antimicrobial activity profile of Lycoperdon perlatum, Cantharellus cibarius, Clavaria vermiculris, Ramaria formosa, Maramius oreades and P. pulmunarius tested against a panel standard pathogenic bacteria and fungi indicated that the concentration of bioactive components directly influence the antimicrobial capability of the isolates (Ramesh and Pattar , 2010). Ghosh (2014) isolated terpenoids and polysaccharides from three basudiomycota (Coltricia perennis, Onnia tomentosa and Polyporus mori) and tested against three Gram +ve bacteria and two Gram –ve bacteria. 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. 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 (Lin and Chou, 1984; Yang et al., 2002). 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 (Gatti and Tzagoloff, 1990). Its activity, however, depends of the presence of (E)-b methoxyacrylate moiety (Fredenhagen, 1990).

CONCLUSION

In conclusion, this research findings suggested that the terpenoids isolated from T. versicolor may be used against human pathogen S. aureus, E coli and other bacteria.This attempt of research for isolation of polysaccharides and terpenoids from fruit body of mushrooms and screening of their antibacterial potentiality surely encourages other biologists or microbiologists to screen antimicrobial agents from other mushrooms for discovery of new generation antibiotics against multidrug resistant microbes of human. Dependent on increasing knowledge about chemistry, biotechnology and molecular biology of mushrooms as well as an improvement of screening methods (high through out screening, genomics and proteomics), a rapid increase in the application of mushrooms for medicinal purposes can be expected.

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