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Original Article

Isolation of bioactive compounds from exudate of edible fungus, *Pleurotus ostreatus*

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Abstract
The present study was aimed at to investigate the bioactive compounds from exudate of a fungus, *Pleurotus ostreatus*. Exudate as liquid droplets on the mycelium was checked for antimicrobial activity against bacterial strain, *Bacillus subtilus*. Biochemical techniques, thin layer chromatography, high performance liquid chromatography and gas chromatography/mass spectrometry, were employed to study fungal exudate. A total of fifteen different metabolites were detected in the sample by GC/MS. The detected metabolites could be classified into chemical groups of 2 esters, 4 alcohols, 3 benzoic acids, ketone, aldehyde, phenyl and amide groups containing compounds. Some of these may be classified into aroma containing compounds.
Keywords: Antimicrobial compounds; edible mushroom; *Pleurotus ostreatus,* GC/MS.

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INTRODUCTION

Mushrooms are very popular in the market for their nutritional and medicinal value and have especially been widely used as a food or flavoring material for their unique and subtle flavor. Mushrooms are also recognized as an important source of biologically active compounds (Cheung *et al*., 2003; Cheung and Cheung, 2005; Cui *et al*., 2005; Jayakumar *et al*., 2006; Turkoglu *et al*., 2007; Qazi and Naeem, 2012; Vasundhara *et al*., 2016) such as phenolic compounds, terpenes etc which usually possess antioxidant activities (Chipault *et al*., 1952, 1956). Various phenols are present in mushrooms, which are very effective scavengers against peroxy radicals (Murcia *et al*., 2002). Mycelia of mushrooms are also used as food, food-flavoring material and in the formulation of nutraceutical foods (Lee *et al*., 2007). The hyperoxide radical, which is mainly involved in human ageing process, has been removed by *Ganoderma lucidum* extract (Liu *et al*., 1997). The potent scavenging of hydroxyl radicals and inhibition of lipid peroxidation activities have been found in *P. florida* extract prepared in ethyle acetate and methanol (Jose and Janardhanan, 2000). Mattila *et al*. (2002). reported that *Pleurotus ostreatus* contains higher concentrations of cystine, methionine and aspartic acid as compared to the other edible mushrooms such as *Agaricus bisporus* (brown), *A. bisporus* (white) and *Lentinesedodes*. Levostatin and its analogues are reported to be the best therapeutic agents for correcting hypercholesterolemia obtained from *Pleurotus* spp. (Endo, 1988). Jayakumar *et al*. (2006) reported that *P. ostreatus* extract can be used against oxidative stress to protect vital organs like heart, brain and liver of aged rats. Besides this, it has good reducing power on ferric ions (Lin, 1999). Many researchers have focused their research on the dietary value of edible mushrooms; but few reports are giving information regarding to exudate production by fungi and its possible role as an antioxidant activity or to inhibit oxidative stress. The present investigation was conducted to determine the possible role of exudate secreted from the mushroom, *P. ostreatus*. Exudates have frequently been observed as a natural common phenomenon on a number of fungi as liquid droplets adhering to the mycelium. Various biochemical techniques, [Thin layer

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chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC/MS)], were used for the detection of organic compounds from the mushroom's exudate.

MATERIALS AND METHODS

Experimental procedure and inoculum preparation

Potato dextrose agar (PDA) was used for the cultivation of fungus, *P. ostreatus* (Ilyas *et al*., 2012) prepared by dissolving 3.4 g of PDA in 100 mL of distilled water. Petri dishes containing sterilized PDA medium were inoculated with 7 mm pure inoculum of *P. ostreatus* and incubated at 28°C for 7 to 14 d. Tiny deep yellow droplets were appeared on the surface of mycelium. Culture grown (7–14 d) on YPD broth medium containing 2% glucose, 1% yeast extract, and 2% bacto-peptone also exhibited tiny droplets on the mycelium surface.

Detection of antimicrobial activity

Agar diffusion method was used for determining exudate bioactive compounds. The exudate was checked for antimicrobial activity against *Bacillus subtilus*. For this purpose LB medium [1% tryptone, 0.5% yeast extract, 1% NaCl and 1.5% agar (pH 6.5)] agar plates was prepared and inoculated with tested bacterial strain at 37°C for 24 h. Wells (5 mm) were made with the help of sterilized micro tips. Exudate (80 µL) was loaded on the wells, left for one hour to diffuse and incubated for 12 h at 37°C and zone of inhibition was measured.

Thin layer chromatography

TLC was carried out to know the activity of compounds in the exudate. Sample drop was placed on TLC plate by using a sterilized pasteur pipette. The plates were air dried with the help of dryer. The process was repeated by superimposing suitable quantity (2–5µg) of sample drops on the original spot of the plate. The 10% MeOH/CH_2Cl_2 solvent system used to develop the plates. Ultraviolet light was used to observe the plates at 254 and 366 nm and the parts showing absorbance were photographed. The TLC plate was sprayed with Ehrlich's reagent and Anisaldehyde /H_2SO_4 reagent for further localization of interesting compounds.

High performance liquid chromatography

The exudate analysis was done by Sykum HPLC system. This system has two pressure pumps (Syknm S1122 delivery system), an injection port with a 20 µL loop (Syknm S 5111 injector valve bracket) and a UV detector (Syknm S 3210 UV/Vis detector). Thecolumn used was RP C18 (Thermo Hypersil Keystone, 250 x 4.6 mm 5µm Hypersil). Methanol and acetonitrile (1:1) were used to prepare mobile phase and the flow rate was adjusted to 1.0 mL/min. The 50 µL exudate was dissolved in methanol (200 µL). A microsyringe was used to inject the sample (20 µL) and the sample was run for 15 min. Finally, UV absorbance was taken at 254nm.

GC/MS analysis

Gas chromatographical analysis was made by following the method of Hübschmann (2008). Exudate (50 µL) in methanol was evaporated to dryness and reconstituted in methanol (2 µL). Aliquot was injected into the column with the injector heater at 250°C. Total running time was 60 min and Helium (He) was used as a carrier gas at constant flow rate of 1 mL per minute. In conclusion, the exudate as liquid droplets obtained from *P. ostreatus* was checked for antimicrobial activity against bacterial strain, *Bacillus subtilus*. A total of 15 different metabolites were detected by GC/MS. The detected metabolites could be classified into chemical groups of 2 esters, 4 alcohols, 3 benzoic acids, ketone, aldehyde, phenyl and amide groups containing compounds. Some metabolites have antimicrobial activity and some of these may be classified into aroma containing compounds.

RESULTS AND DISCUSSION

Various biological compounds with anti-tumor, anti-cancer, antiproliferative, cytotoxic as well as antibiotic properties have been isolated from fungal sources. Fungi are potent producers of bioactive secondary metabolites (Hasan *et al*., 2015). The crude extract collected from fungal metabolites has complex chemical nature which is difficult to identify and characterize. In this study, one of the compound extracted was benzoic acid. In past, benzoic acid and its related compounds have been reported. Benzyl benzoate is one of the primary prescribed ointments to treat scabies (Goutam *et al*., 2016). In the present study, exudate from *P. ostreatus* was examined in an attempt to understand its possible role and composition. Intact droplets were seen above the mycelium surface after

14d. A zone of inhibition (10 mm) was seen against bacterial strain *B. subtilus* indicating the ability of the chemicals present in the exudate to inhibit the microbial growth (Fig. 1).

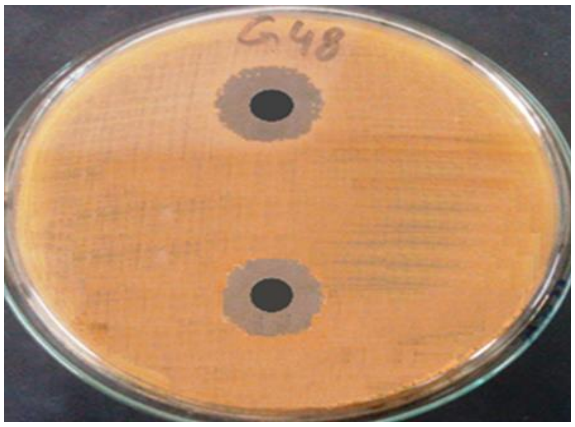
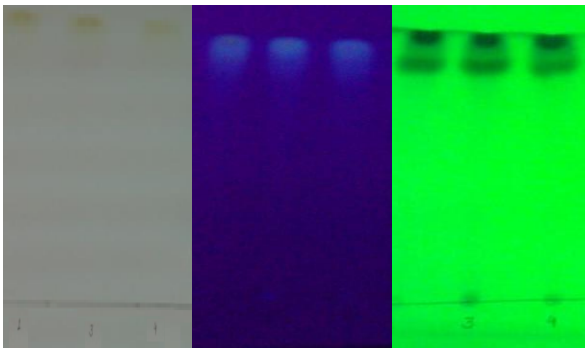


Figure 1: Zone of inhibition caused by exudate obtained from *P. ostreatus* against *B. subtilis*.

TLC results had shown that the bioactive compounds fluoresce under UV light. Ehrlich's reagent was used for staining the colored spots indicated the presence of different functional groups like amines derivatives in the exudate (Fig. 2). Staining with anisaldehyde/H_2SO_4 reagent resulted in a colored spots explaining the presence of phenols and terpenes in the exudate. Lee *et al*. (2007) found that the major naturally occuring antioxidant components in *P. citrinopileatus* were phenols.



A B C

Figure 2: TLC plate spotted with exudate showing. A: after staining with anisaldehyde/H_2SO_4 reagent. B: under UV at 366 nm. C: under UV at 254 nm.

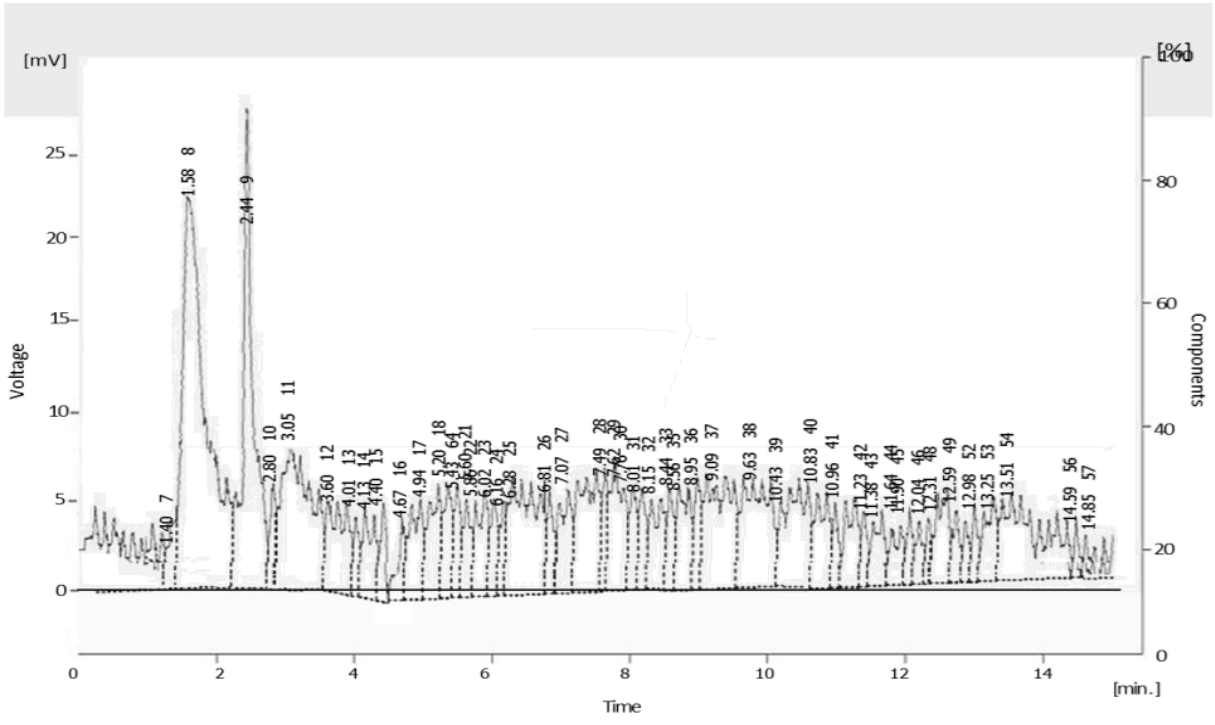


Figure 3: HPLC analysis of exudate obtained from *P. ostreatus*. Mobile phase of methanol: acetonitrile (1:1) was used at 1.0 mL/minute using C-18 column.

Madhavi *et al*. (1996) reported that BHT and gallate due to their scavenging and chelating abilities against free radicals and ferrous ions, are effective antioxidants (Lotito and Fraga, 1998), anti-mutagenic and anti-cancer properties (Ahmad and Mukhtar, 1999). HPLC-UV results also estertained various detectable peaks of different active compounds at various retention times (tR) (Fig. 3). GC/MS analysis revealed 2 esters, 4 alcohols, analdehyde and a ketone, 3 benzoic acid, phenyl and amide groups containing compounds (Table I).

Cyclotetrasiloxaneoctamethy, an industrial chemical silicone polymer was also identified. It is used as surfactant in certain pesticide products and as de-foamer in lubricants, cleaning products, sealants, adhesives, waxes, polishes and coatings. Antiperspirants, skin care products and deodorants use such polymers. Pharmaceuticals products also use these polymers. Tert-butyl (5-isopropyl-2-methyl phenyl) dimethylsilane is an alkaloid with no therapeutic activity reported.

Table I: Chemical compounds of exudate obtained from *P. ostreatus* using GC-MS analysis.

Sr. no.	Compounds	Formula	Molecular weight (m/z)	Retention time (min)
1	Perfouropropanimidamide, N-[3-(dimethylamino)propyl]-N'-perflouropropanol-N'-(IE)-N-[(Dimethylamino)propyl]-2,2,3,3,4,	$C_{13}H_{14}F_{14}N_4O$	508	38.033
2	Methylolacetone ($CH_3C(O)CH_2CH2OH$)	$C_4H_8O_2$	88	2.183
3	1-Di(tert-butyl)silyloxy-2-phenylethane Di (tert-butyl)(2-phenylethoxy) silane	$C_{16}H_{28}OSi$	264	3.317
4	2-(2-butoxyethoxy) ethanol	$C_8H_{18}O_3$	162	9.317
5	2-butoxy ethanol	$C_6H_{14}O_2$	118	11.092
6	Cyclotetrasiloxane, octamethy-	$C_8H_{24}O_4Si_4$	296	13.367
7	Ethanol, 2-(hexyloxy)-n-Hexyl Cellosolve Ethylene glycol monohexyl ether Ethylene glycol n-hexyl ether glycol monohexyl	$C_8H_{18}O_2$	146	18.525
8	Ethanol, 2-(hexyloxy)-n-Hexyl Cellosolve Ethylene glycol monohexyl ether Ethylene glycol n-hexyl ether glycol monohexyl	$C_8H_{18}O_2$	146	18.750
9	Benzoic acid, 2,5-bis(trimethylsiloxy)-,trimethysilyl ester	$C_{16}H_{30}O_4Si_3$	370	19.717
10	Cyclohexasiloxane, dodecamethyl-	$C_{12}H_{36}O_6Si_6$	444	25.958
11	Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl) 2-(3- acetoxy-4,10,13,14-pentamethyl-2,3,4,5,6,7,10,11,12,13,14	$C_{27}H_{42}O_4$	430	31.200
12	Butanal butaraldehyde	C_4H_8O	72	31.433
13	Undecanal,2-methyl-	$C_{12}H_{24}O$	184	31.758
14	Pentanoic acid, 5-hydroxy,2,4-di-t-butylphenyl esters 2,4 ditert-butylphenyl 5-hydroxypentanoate	$C_{19}H_{30}O_3$	306	33.025
15	Tridecanal	$C_{13}H_{26}O$	198	33.175

In this study various bioactive compounds *e.g.*, methylolacetone, 5-hydroxy,2,4-di-t-butylphenyl esters, 5-hydroxypentanoate, propanoic acid, butanal butaraldehyde, cyclotetrasiloxane and benzoic acid were found in the *P. ostreatus* exudate. Likewise, various metabolites have been isolated and characterized from the crude extract of fungus, *Fusarium proliferatum* (Dame *et al*., 2016). Similarly, Specian *et al*. (2012) reported the presence and characterization of 2(-4 hydroxyphenyl)-ethanol (Tyrosol) from a fungus, *Diaporthe helianthi*.

In present investigation, the fungal exudate inhibited the growth of *B. subtilis* while Xu *et al*. (2008) reported that extract obtained from endophytic fungi tested for its antibacterial activity. From 9 isolated endophytes, 7 showed antibacterial activity at least against 3 of the 4 tested bacteria including *Xanthomonas vesicatoria*, *E. coli*, *B. subtilis*, and *Staphylococcus haemolyticus*. These results clearly show that endophytes are an important source of biologically active secondary metabolites/compounds. Among marine organisms, fungi are prolific resources of

biologically active secondary metabolites which can easily impede other microorganisms (Swathi *et al*., 2013). The best producers of secondary metabolites like polyketide derived alkaloids, terpenes and peptides are fungi. Kossuga *et al.* (2012) reported the isolation of (*E*)-4-methoxy-5-(3-methoxybut- 1-enyl)-6- methyl-2*H*-pyran-2-one from marine fungal isolates. The purpose of the present research work was to isolate and identify the biological active compounds from *P. ostreatus***.** Various secondary compounds are present in the exudate from the fungus and further work is needed to exploit these compounds in cytotoxicity and antimicrobial assays.

Conflict of interest

The authors have declared that no competing interests exist.

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