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Research Article

ANALYSIS ON ESSENTIAL FATTY ACID ESTERS OF MUSHROOM *PLEUROTUS EOUS* AND ITS ANTIBACTERIAL ACTIVITY

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ABSTRACT

The enormous increase in our population has necessitated more and more food production through alternate resources such as mushroom. *Pleurotus* mushrooms are the second most important mushroom in its production in the world. The present investigation deals with Gas Chromatography [GC] – Mass Spectrometry [MS] analysis of petroleum ether extract of dried fruiting bodies of *Pleurotus eous* and its evaluation of antibacterial activity. Five compounds were identified by GC-MS and they are fatty acids esters such as Cyclopentanetridecanoic acid, methyl ester (25%); Tartronic acid, (p-ethoxyphenyl), diethyl ester (16.3%); 7, 10-Octadecadenoic acid, methyl ester (40.3%); Heptadecanoic acid, 16-methyl, methyl ester (13.5%) and 9-Octadecenoic acid [Z]-, 2-hydroxyl-1-[hydroxymethyl] ethyl ester (4.9%). This is the first report on the composition of the fatty acids of these fruiting bodies. Petroleum ether, ethyl acetate, methanol and aqueous extracts of *P.eous* were obtained by hot continuous Soxhlet extraction and were screened for its antibacterial property against *Staphylococcus aureus, Bacillus subtilis, Bacillus cereus, Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella pneumoniae.* Among all the crude extracts tested, only petroleum ether extract showed strong antibacterial activity by inhibiting the growth of both gram positive and gram negative bacterial isolates. The minimal inhibitory concentration (MIC) values of the petroleum ether extract against *S. aureus* is (4.4µg/ml), *B.subtilis* (3.1µg/ml), *B.cereus* (4.2µg/ml), *P.aeruginosa* (8.8µg/ml), *E.coli* (3.1µg/ml) and *K. pneumoniae* (4.4µg/ml). Hence, based on these observations it can be clearly concluded that petroleum ether extract is found to have antimicrobial activity. The GC-MS studies on this extract further confirms that this activity is due to the presence of fatty acid esters that are identified in the present study. The investigation therefore supports the traditional uses of the mushroom in the treatment of infectious diseases.

Keywords: Mushroom, fatty acids, antimicrobial agent, Basidiomycetes fungi, GC-MS analysis.

INTRODUCTION

Basidiomycetes mushroom have been valued as both food and medicine for thousands of years. They have high nutritive and medicinal values and contribute to a healthy diet because of their rich source of vitamins, minerals and proteins[1]. Not only do mushrooms provide food, but their waste can be recycled into fertilizers and additives that improve tree plantations and soil conditions. They are low calorie food with very little fat and are highly suitable for obese persons[2]. This entire *Pleurotus* mushroom has various bioactivities which render them valuable active ingredients of medicines. Oyster mushrooms (*Pleurotus* genus) are a good choice for beginners involved in mushroom cultivation because they are easier to grow than many of the other species, and they can be grown on a small scale with a moderate initial investment[3]. Among all the mushrooms, *Pleurotus* mushrooms contain higher composition of cancer-fighting properties and some aid the body's immune system. For centuries people have been trying to alleviate and treat disease with different mushroom extracts and formulations[4]. It is therefore essential that efforts should be made to introduce new medicinal mushrooms to develop cheaper drugs. Mushrooms still represent a large untapped source of structurally novel compounds that might serve as lead for the development of novel drugs.

In search of new drugs from mushrooms GC-MS techniques have been widely used. Fhernanda R Smiderle et al., (2006) isolated and identified polysaccharide such as xylomannans and β-glucan from the edible mushroom *Flammulina velutipes* by using GC-MS and NMR techniques[5]. Using Ion Trap detector in GC-MS, Barbara et al., (2009) identified thirty fatty acids from twelve wild edible mushroom species[6]. According to Assya Petrova et al., (2007) GC-MS studies on genus *Agaricus* mushrooms reveal the presence of fatty acids and their esters, amino acids and sugar alcohols[7]. Amino and fatty acids of 15 wild edible mushroom species belonging to the genus *Boletus* (phylum *Basidiomycota*) was characterized by using HP-GC mass selective detector[8]. Thus, it is evident that GC-MS is widely used method to identify different substances within a test sample[9].

In our earlier experimental studies on *Pleurotus eous*, it has been found to have strong analgesic, anti-inflammatory and antipyretic activity[10, 11]. Crude extracts of *P.eous* also found to exhibit significant *in vitro* free radical scavenging property and antiplatelet activity[12].

According to our knowledge, no investigations have been reported on the constituents of crude extracts of *P.eous*. Keeping this in view, the present study has been undertaken to identify the essential bioactive compounds which are present in the petroleum ether extracts of *P.eous* by GC-MS. As per the literature, *P.eous* is used as an important food source; but there are no conclusive reports on the antibacterial activity about this mushroom. Hence, the main objectives of this study are: (i) to determine the composition and identification of fatty acids, of petroleum ether extract by GC-MS and (ii) to investigate the antibacterial activity of extracts of *P.eous* by agar well diffusion method against some of the selected bacterial strains.

MATERIALS AND METHODS

Mushroom Material

The fruiting bodies of the mushroom *P. eous* were obtained from Kerala Agricultural University, Trivandrum and authenticated by Dr.Lu Lu Das, Professor, Dept of Plant Biology, College of Agriculture, Vellayani, Kerala Agricultural University, Trivandrum. Authentication No is (Reg:No.T.5365/06:61;27/08/2009).

Preparation of extracts

Mushroom fruiting bodies were dried at 40-50ºC for 48 h and powdered. About 500 g of the powdered material were extracted with petroleum ether (40-60ºC) for the removal of fatty acids, waxes and sterols. The defatted material was extracted with ethyl acetate (to separate flavonoid glycosides, phenolic compounds and flavonoids) and then with 70% methanol (for the complete removal of tannins and flavonoid compounds) for 8-10 h using Soxhlet apparatus. For the preparation of aqueous extract defatted material was extracted with hot water (70-80ºC) for 8-10hrs. Aliquots of extracts were collected and passed through Whatmann No.1 filter paper. Then the solvents were completely evaporated to dryness at 60ºC by vacuum distillation. The residues were designated as petroleum ether (PE), ethyl acetate (EA), methanol (MeOH) and aqueous (Aqs) extracts, respectively. The crude extract thus

obtained was then subjected to GC-MS analysis and for the antibacterial studies.

GC–MS Analysis

GC-MS was performed using Varian (model 3300) Gas Chromatograph linked to a Finnigan Ion Trap model 810 R-12 Mass Spectrometer. Compounds were separated on a capillary column (30mx0.25mm, internal diameter) of DB-225, held at 50°C during injection and then programmed at 40°C min^{-1} to 220°C. The carrier gas was helium, at a flow rate of 0.9 mL min^{-1}, and the injection volume was 1 µL. In mass spectrometry electron-impact ionization was performed at electron energy of 70 eV.

Compound Identification

The components were identified by comparison with Computer Library (NIST version 2.1) attached to the GC-MS instrument.

Antibacterial activity

Test Bacteria

Staphylococcus aureus ATCC 25923, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 11778, *Pseudomonas aeruginosa* ATCC 29212, *Escherichia coli* ATCC 29995 and *Klebsiella pneumonia* CCM 2318 were used as test bacteria. Nutrient Broth (NB) was used for culturing of test bacteria. All strains were stored at -20°C in the appropriate medium containing 10% glycerol, and regenerated twice before use. Gentamycin (Sigma Aldrich, India) was used as standard drug for these studies.

Preparation of Inoculum

The gram positive (*S. aureus, B.subtilis and B.cereus)* and gram negative bacteria *(P.aeruginosa*, *E.coli* and *K. pneumonia)* were pre-cultured in nutrient broth overnight in a rotary shaker at 37°C. It is centrifuged at 10,000 rpm for 5 min and the pellets were suspended in double distilled water and the cell density was standardized spectrophotometrically(A_{610}nm). The spore density of each bacterium was adjusted to obtain a final concentration approximately 10^5 spores/ml.

Agar well diffusion method

The determination of the inhibitory effect of the extracts of *P.eous* on test bacteria was carried out by agar well diffusion method. Bacterial cultures were grown at 37°C for 24 h in Nutrient Broth. The culture suspensions were adjusted by comparing against McFarland. Petri dishes with 10ml of Nutrient Agar were prepared, previously inoculated with 100µl of the culture suspension[13, 14]. The wells (7.0mm) were made and the extract which is dissolved in DMSO was added to wells (100µl) and same volume (100µl) of DMSO was used as a control. The inoculated plates were incubated for 24h. After incubation, the diameter of the inhibition zone was measured with calipers. The measurements were done basically form the edge of the zone to the edge of the well.

Determination of Minimum Inhibitory Concentration (MIC)

MIC was determined using agar dilution method[9]. Various concentrations (0.01-10µg/ml) of each extracts and standard Gentamycin were prepared in 10cm experimental tubes containing Potato Dextrose Agar (PDA) broth. Each tube contains 9ml of PDA and was sterilized by autoclaving. Upon cooling, 1ml of each extract concentration was added into the respective tubes. The mixture of PDA and extracts were poured into plates aseptically in a laminar flow cabinet. Upon solidification of the agar medium, 2µl of adjusted spore suspension were added to each plate by micropipette and incubated at 28°C for three days. The PDA without any extract served as control. The MIC was recorded as the lowest concentration of the extract that prevented the bacterial growth on the solid medium.

RESULTS AND DISCUSSION

Gas chromatography–mass spectrometry analysis (GC–MS) is a powerful tool for qualitative and quantitative analysis of various compounds present in natural products, and the technique has been widely applied in medical, biological and food research[15]. The chemical profiles of the fatty acids, the amount (%) of the individual components obtained and gas chromatographic-mass spectral data carried out for *P.eous* are summarized in Table 1.

Table 1: Gas Chromatographic and Mass Spectral data for the Fatty acid esters of *Pleurotus eous*

No	Compound name	Rt (min)	% area	Mol. Mass	Mol. Formula	Fragment ions
1	Cyclopentanetridecanoic acid, methyl ester	9.08	25	296	$C_{19}H_{36}O_2$	296,270,227,199,185,143,129,111,97,87,74
2	Tartronic acid, (p-ethoxyphenyl), diethyl ester	9.33	16.3	296	$C_{19}H_{36}O_2$	296,278,239,205,178,161,149,121,105,98
3	7, 10-Octadecadenoic acid, methyl ester	9.97	40.3	294	$C_{19}H_{34}O_2$	294,263,220,150,109,80,67
4	Heptadecanoic acid, 16-methyl, methyl ester	10.12	13.5	298	$C_{19}H_{38}O_2$	298,255,199,143,74
5	9-Octadecenoic acid [Z]-, 2-hydroxyl-1-[hydroxymethyl] ethyl ester	11.64	4.9	356	$C_{21}H_{40}O_4$	336,313,296,263,220,187,177,149,123,98

The results revealed that 7, 10-Octadecadenoic acid, methyl ester (40.3%) was found as major component followed by Cyclopentanetridecanoic acid, methyl ester (25%); Tartronic acid, (p-ethoxyphenyl), diethyl ester (16.3%) were found as the major components in the petroleum ether extract of *P.eous*. The present study also indicates the presence of Heptadecanoic acid, 16-methyl, methyl ester (13.5%) and 9-Octadecenoic acid [Z]-, 2-hydroxyl-1-[hydroxymethyl] ethyl ester (4.9%) in the fruiting bodies of mushroom *P.eous* (Figure 1 and 2). Among the five identified fatty acids, Octadecenoic acid was found to be higher in percentage composition, which has the property of anti-inflammatory and antiarthritis. The second most abundant fatty acids, Heptadecanoic acid and Tartronic acid are also possess the property of antioxidant and antimicrobial as reported by Suresh Lalitharani et al.,(2010) [16]. Hence the use of *P.eous* species as an edibile mushroom (food) and medicine is well appreciated and could be recommended.

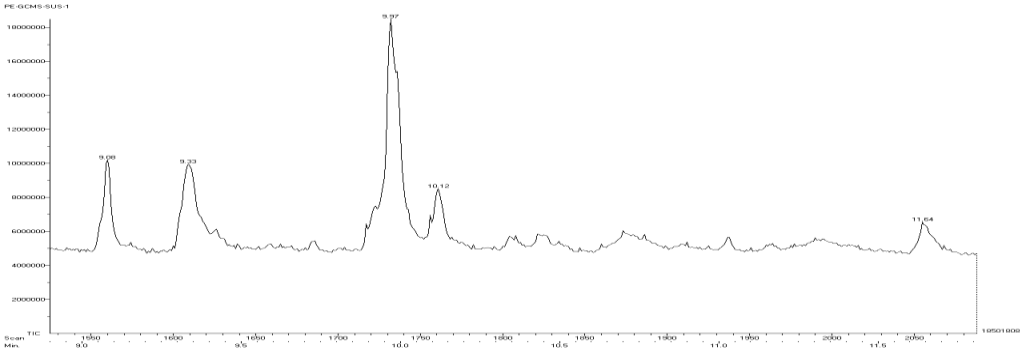

Figure.1: Gas-Chromatogram of Petroleum ether extract of fruiting bodies of *P.eous*

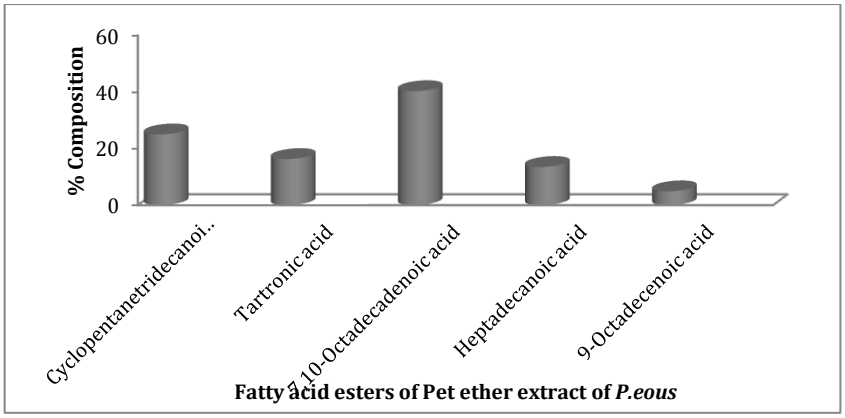

Figure.2: Percentage composition of Fatty acids esters of Petroleum ether extract of *P.eous*

The four extracts (PE, EA, MeOH and Aqs) of *P.eous* were tested against three gram positive bacteria (*Staphylococcus aureus, Bacillus subtilis and Bacillus cereus)* and three gram-negative bacteria (*Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella pneumoniae).* The results obtained for the antimicrobial studies are given in Table 2 by comparing with standard Gentamycin. It is observed that, among the four extracts PE was found to be the most effective against all organisms that were tested. EA and MeOH were found to be ineffective against all the tested organisms except *Escherichia coli. E. coli* was found to be resistant to all the mushroom extracts.

Table 2: Antibacterial Property of PE, EA, MeOH and Aqs extracts of *Pleurotus eous* against test bacteria

S.no	Test organism	Name of the extracts				Genta Mycin
		PE	EA	MEOH	AQS	
1	*Staphylococcus aureus* ATCC 25923	+	-	-	+	+
2	*Bacillus subtilis* ATCC 6633	+	-	-	+	+
3	*Bacillus cereus* ATCC 11778	+	-	-	-	+
4	*Pseudomonas aeruginosa* ATCC 29212	+	-	-	+	+
5	*Escherichia coli* ATCC 29995	+	+	+	+	+
6	*Klebsiella pneumonia* CCM 2318	+	-	-	-	+

The PE extract which showed strong antibacterial property against all the tested microorganisms is subjected to determination of minimal inhibitory concentration. The MIC values that were determined against *S. aureus* (4.4µg/ml), *B.subtilis* (3.1µg/ml), *B.cereus* (4.2µg/ml), *P.aeruginosa* (8.8µg/ml), *E.coli* (3.1µg/ml) and *K. pneumoniae* (4.4µg/ml) is tabulated in Table 3

Table 3: Minimum Inhibitory Concentrations (mg/ml) and Zone of Inhibition diameter (mm) of Petroleum ether extract of *Pleurotus eous* against test bacteria

S.NO	TEST ORGANISM	MIC (µg/ml)		INHIBITION ZONE DIAMETER(mm)	
		PE	GENTAMYCIN	PE	GENTAMYCIN
1	*Staphylococcus aureus* ATCC 25923	4.4	0.48	7±0	21±1
2	*Bacillus subtilis* ATCC 6633	3.1	0.97	9±0	20±0
3	*Bacillus cereus* ATCC 11778	4.2	0.60	5±1	18±0
4	*Pseudomonas aeruginosa* ATCC 29212	8.8	0.97	5±0	21±0
5	*Escherichia coli* ATCC 29995	3.1	0.90	11±0	20±3
6	*Klebsiella pneumonia* CCM 2318	4.4	0.48	8±1	18±2

Table 3, reveals that the inhibition zones of *P.eous* for all the test bacteria were found to be in the range of 5-11mm. The highest inhibitory activity was shown by the PE against *Escherichia coli* (11±0 mm). On the other hand, the weakest inhibitory activity was found to be against *Bacillus cereus* and *Pseudomonas aeruginosa* (5±1 and 5±0 mm). Since many fatty acids have been found to be responsible for several biological properties, including antimicrobial properties[17], it is expected that the antimicrobial activity of this mushroom species would be related to its fatty acid compounds that are identified in the present study. This could provide a rationale for the use of this mushroom in pain, fever and inflammatory disorders in folk medicine.

CONCLUSIONS

This study revealed a high level of chemical composition which is characteristic of fatty acid esters extracted from mushroom *P.eous.* The fatty acid contents that are identified in the present study contributes two significance to human; the first is their natural origin which is more safe to people and environment, the second is that they have been considered at low risk for resistance development by pathogenic microorganisms. The petroleum ether extract of the *P.eous* possessed strong antibacterial activity against both gram-positive and gram-negative bacteria. Also, the growth of food borne pathogens can be inhibited when *P.eous* is added as an extra nutrient in food products. According to the results of this investigation, *P.eous* could be suggested as a new potential source of natural antibacterial agent.

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