ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Studies on effects of supercritical CO_2 extraction on yield and antioxidant activity of *L.edodes* extract.

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ABSTRACT

Lentinus edodes with a number of chemical groups is considered to have direct beneficial effects on human health besides its potential applications in the pharmaceutical and nutraceutical industries. The inherent difficulties in screening and production of the bioactive molecules have led to the development of advanced technologies. Various novel techniques viz. ultrasound and microwave assisted, supercritical fluid and accelerated solvent extraction for the extraction of nutraceutical compounds/elements have been developed in order to shorten the extraction time, decrease the solvent consumption, increase the extraction yield, and to enhance the quality of extracts in terms of number of compounds present. In this study, supercritical CO_2 extraction of *Lentinus edodes* was investigated and the chemical composition of the mushroom extract derived was analysed by GC-MS. Comparison was made with the extract obtained with the use of solvents. The yield of the extract obtained with the supercritical extraction was found to be 1.02%. Fatty acid esters, fatty acids, triterpenes, diterpene alcohols and phytols were identified as the major chemical groups in the *L.edodes* extract. The extracts obtained showed strong anti oxidant activity i.e. DPPH radical scavenging activity, polyphenol content and FRAP. The present study indicated that the extract obtained with supercritical method although gave low yield but it produced quality extract with more number of organic compounds superior over extract obtained by solvent extraction method, could be successfully used in pharmaceutical applications.

Keywords: *L.edodes*, Supercritical, yield, GC-MS, antioxidant

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INTRODUCTION

Mushrooms (fungal sporocarps) represent one of the world's greatest untapped resources of nutritious and palatable food as they pose extensive enzyme complexes which enable them to flourish successfully on a wide variety of inexpensive substrates. They are rich in proteins, fibre and have high vitamin B and folic acid content which are uncommon in vegetables [1]. Shiitake (*L.edodes*), is the third largest cultivated, most popular mushroom in the world [2] that not only has several functional properties but also has many antioxidative minerals. It is known to posses significant antitumor, cardio protective, anti diabetic and hepatoprotective and antifatigue effects [3]. These beneficial health attributes are due to the presence of secondary metabolites and bioactive compounds. Since these bioactive molecules exhibit broad diversity of functionalities therefore they act as an excellent pool of molecules for the production of nutraceuticals, functional foods, and food additives.

Development and manufacture of functional foods from medicinal mushrooms has been a marked trend in the food industry and is a great success in the last few years. These products are hence considered in the category of healthy foods as more often they are free from synthetic chemicals and are rich in bioactive molecules [4]. Inclusion of mushrooms as functional food can help in the early intrusion of sub-healthy states in humans and it might avert the consequences posed by life threatening diseases [1]. These active components are found in lesser amounts and therefore their extraction from the mushrooms remains a challenge. Besides, the structural diversity, complexity of these molecules make their chemical synthesis unprofitable [5]. Up to now, numerous methods of extraction have been developed with the objective of obtaining extracts with higher yields and lower costs. Such is the case of extraction with organic solvents, such as methanol, ethanol and acetone [6]. But the utilization of such solvents are toxic [7] for human health and environment. The inherent difficulties in screening and production of these molecules have led to the advancement of emerging alternative technologies to address these precincts in the extraction procedures.
Supercritical carbon dioxide (SC-CO_2) is considered an attractive alternative to organic solvents and has immense benefits. 1) it is a clean technology, also it uses high pressure solvents due to which the extracts obtained have high purity. 2) easy removal of solvents is achieved due to temperature elevation and pressure reduction. 3) at low temperature, thermally sensitive compounds can be separated. 4) the process involves low heat demand as compared to the distillation process. 5) rapid extraction due to the low solvent viscosity, high diffusivity and solvation power. 6) CO_2 has many advantages over other organic solvent as it is inflammable, cheap, chemically inert, non hazardous and extracts obtained are of high purity. Since carbon dioxide is apolar molecule, polar molecules are not extractable due to poor solubility [8]. Other solvents added are in small amounts in order to enhance the solubility [9].

Keeping this in mind, the study was designed to carry out supercritical extraction of shiitake mushroom and to evaluate its antioxidant property.

MATERIALS AND METHODS

Cultivation of Shiitake Mushroom:

The strain of *Lentinus edodes* OE-388 was procured from germplasm collection bank of Directorate of Mushroom Research (DMR), Solan. The culture was maintained on Potato dextrose agar (PDA) slants and spawn was prepared on the wheat grains. The cultivation technology was followed as prescribed by Puri et al. [10]. The bags filled with sawdust were inoculated and kept for 90 days for incubation. After 90 days, the fruit bodies were harvested. They were dried and powdered for further analysis.

Extraction of biomolecules from L.edodes

Supercritical CO_2 extraction

SFE reported in this paper was conducted by as per the method by Pradhan et al. [11] with slight modifications. The CO_2 extraction was performed with a supercritical-fluid extraction system (Thar Technology, USA). Carbon dioxide was compressed to the desired pressure by using a diaphragm compressor. The extraction vessel was heated with a heating jacket, and temperature was controlled by a thermostat (±1 C). Pressure was controlled by a backpressure regulator. *L.edodes* powder (100 g) were loaded into a 400 ml

vessel covered by glass wool and extracted with CO_2 at a flow rate of 40 g/min. The temperature was kept 50 C and the extractions were performed at a pressure of 30 MPa. The extracts were collected in another vessel attached to the depressurization valve, which were held in a circulating refrigerated bath at 0 °C. The collected fractions were stored in a refrigerator (5 °C) for further analysis.

Solvent Extraction

Soxhlet extraction was performed according to the 920.39 C method of A.O.A.C. [12] at least in duplicate. The procedure consisted of 150 mL of solvent recycling over 5 g of dried sample, in a Soxhlet apparatus for 6 h at the boiling temperature of the solvent used. The extraction of *L.edodes* was performed with the following solvents: ethyl acetate (EtAc) and methanol (MeOH). The solvents of the resulting extracts were evaporated under reduced pressure in a rotary evaporator to obtain the crude extracts. All extracts were stored in sealed amber glass bottles at −18 ◦C. The extraction global yields of all method/solvent systems were determined by the ratio between the mass of extract obtained and the mass of raw material used (wet basis).

GC-MS Analysis

GC-MS analysis was carried out with GCMS-QP2010 Plus, Shimadzu, Japan fitted with programmable head space auto sampler and auto injector. The capillary column used was DB-1/RTX-MS (30 metre) with helium as a carrier gas, at a flow rate of 3 mL/min with 1 µL injection volume. Samples were analysed with the column held initially at 100°C for 2 min Dier injection, then increased to 170°C with 10°C/min heating ramp without hold and increased to 215°C with 5°C/min heating ramp for 8 min. Then the final temperature was increased to 240°C with 10°C/min heating ramp for 15 min. The injections were performed in split mode (30:1) at 250°C. Detector and injector temperatures were 260°C and 250°C, respectively. Pressure was established as 76.2 kPa and the sample was run for 40 min. Temperature and nominal initial flow for flame ionization detector (FID) were set as 230°C and 3.1 mL/min, correspondingly. MS parameters were as follows: scan range (m/z): 40-650 atomic mass units (AMU) under the electron impact (EI) ionization (70 eV). He constituent compounds were determined by comparing their retention times and mass weights with those of authentic samples obtained by GC and as well as the mass spectra from the Wiley libraries and National Institute of Standards and Technology (NIST) database.

Identification of components

Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology [13] having more than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library.

Antioxidant activity

DPPH radical scavenging activity

The scavenging activity of the free and bound extracts of mushrooms on DPPH radical was measured according to the method of Cheung et al. [14] with some modifications. Aliquots of 0.8 ml of 0.2 mM DPPH ethanolic solution was mixed with 0.2 ml of the extracts. The mixture was vigorously shaken and left to stand for 10 min under subdued light. The absorbance was measured at 520 nm. The DPPH radical scavenging activity (%) was calculated by the following equation

$$\text{Radical scavenging activity (\%)} = (1 - A_{sample}/A_{control}) \times 100$$

where, A_{sample} is the absorbance in the presence of sample and $A_{control}$ is the absorbance in the absence of sample, respectively. All extracts were analyzed in triplicate.

Total Polyphenol content

The concentrations of phenolic compounds were determined according to the method described by Cheung et al. [14]. A 0.02 mL aliquot of extracts at different concentrations ranging from 4 to 20 mg/mL and negative control (methanol) were mixed with 1.58 mL of distilled water and 0.1 mL of Folin-Ciocalteu's

reagent. After 3 min, 0.3 mL of saturated sodium carbonate (Na_2CO_3) (≈35%) solution was added to the mixture. The contents were vortexed for 15 s and then left to stand at 40ºC for 30 min. Absorbance measurements were determined at 765 nm using a spectrophotometer (Shimadzu, Japan). A calibration curve, using gallic acid with concentrations ranging from 50 to 500 mg/L gallic acid, was prepared as a standard. Estimation of the phenolic compounds was carried out in triplicate. The results were expressed as mg GAE (gallic acid equiv)/g of fresh mushroom.

FRAP (ferric reducing antioxidant power) assay

The FRAP reagent contained 10 mM of 2,4,6-tris(2-pyridyl)-striazine (TPTZ) solution in 40 mM HCl, 20 mM FeCl3.6H2O, and acetate buffer (300 mM, pH 3.6) (1:1:10, v/v/v). The extract solutions with different concentrations (100 µl) were added to the FRAP reagent (3 ml), and the absorbance was measured at 593 nm in an Agilent 8453 spectrophotometer after incubation at room temperature for 6 min, using the FRAP reagent as a blank [15]. The reducing power was obtained directly from the absorbances and the result was expressed as EC50 value (the extract concentrations providing 0.5 of absorbance), calculated from the graph of absorbance at 593 nm against extract concentrations. Trolox was used as positive control.

RESULTS AND DISCUSSION

Yield of mushroom extract

The results pertaining to shiitake extract yield, comparing both the techniques (with solvents i.e methanol and ethyl acetate and SFE with pure CO_2 and with CO_2 along with the co-solvents at 5% concentration) is shown in fig.1.

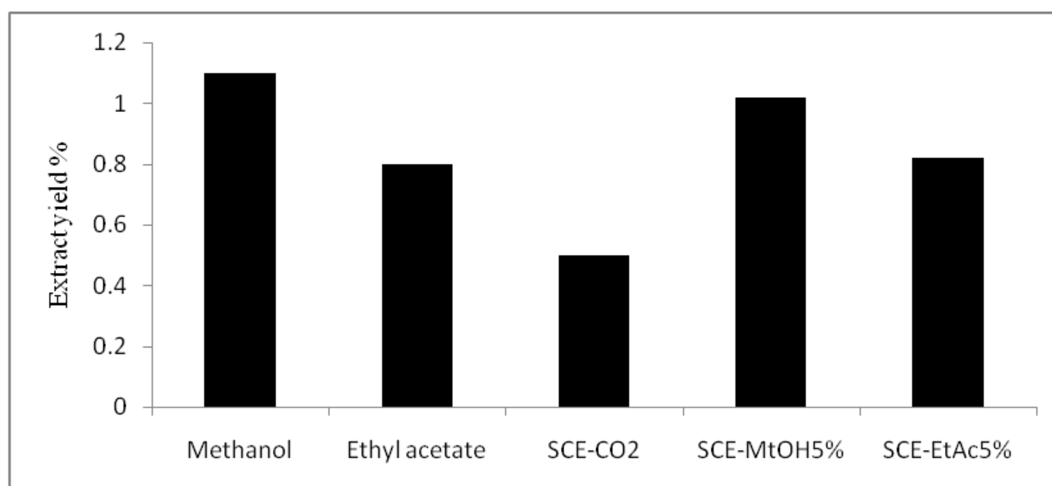


Fig 1: Yield % of shiitake extracts using different techniques

The highest yield of the mushroom (*L.edodes)* extract was 1.18% obtained with methanol solvent and the lowest 0.56 % from SFE-CO_2 extract without co-solvent. These results indicate that the amount of extract is related to the solvent power. The greater yield in conventional method using organic solvents may be due to the solvent solute interaction that contributes to the higher solubilisation of components from raw materials (max. yields). Moreover due to the boiling temperature of the solvent, the surface tension and viscosity is low which facilitates the solvent to reach the active sites inside the solid matrix far more easily thereby promoting solubilization [16].

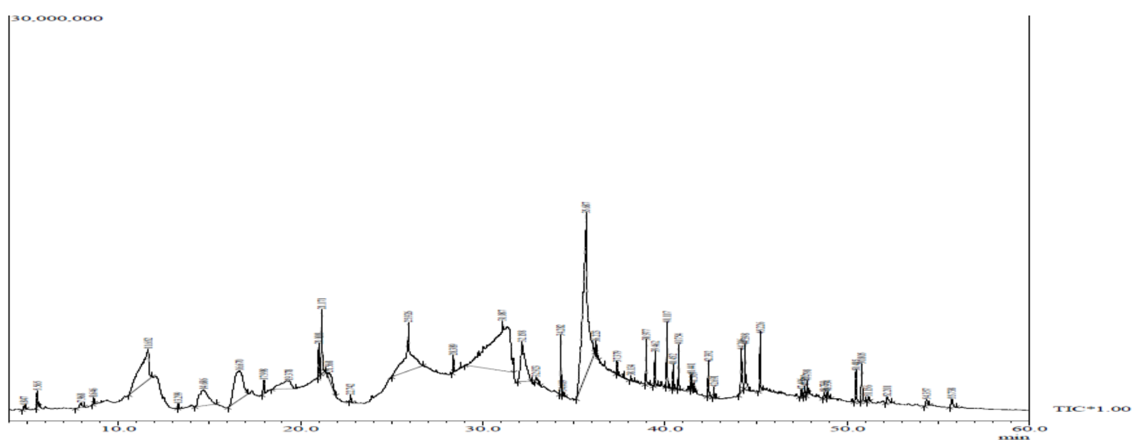
The increase in the mushroom extract yield from 0.5% (with pure CO_2) to 1.02% (CO_2 with 5% methanol) indicates the presence of polar compounds along with non polar ones in the mixture i.e. MeOH/CO_2. Similar results have also been reported by Kitzberger et al. [17] where the yield of shiitake extract increased after addition of ethanol at 5% with a yield of 1.00%. Despite not having achieved high extract yields, supercritical method can be considered important technology to get a large number of compounds/molecules. The low critical temperature of CO_2 allows its use in the supercritical state, and it is sufficient for extraction of

the active principle since most of these compounds are hydrolyzed and thermolabile. Another significant factor of this technology is that the extract is free of contaminants, since CO_2 is a nontoxic, contamination-free solvent [18]. Yield of approximately 0.6% at extraction temperature of 40 °C has been reported by Gil Ramirez et al. [19] in *Agaricus brasiliensis*

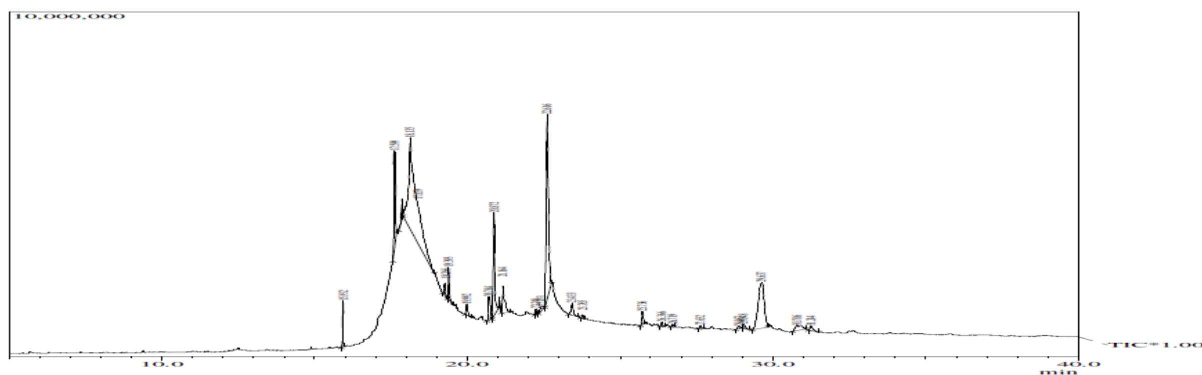
Besides the evaluation of the quantitative efficiency of extracting process, the yield values are not directly related to their qualitative efficiency. Accordingly it was important to assess the chemical profile and the antioxidant activity of the extracts.

COMPOSITION PROFILE

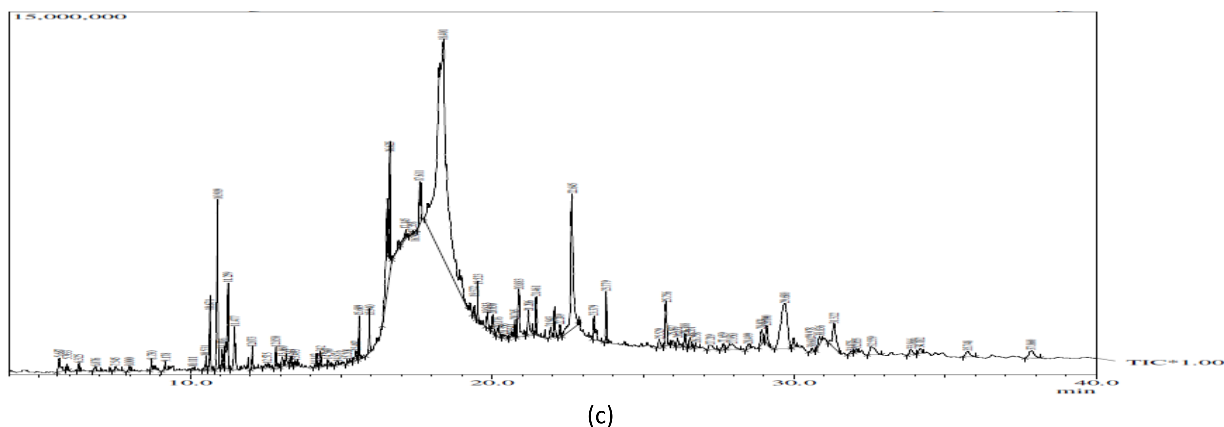
Fig.2 (a-c) shows the chromatogram of the GC-MS analysis for the conventional and the supercritical (SFE) extracts. The highest extract yield i.e SFE with 5% MeOH was subjected to GC-MS analysis for further identification of compounds and compared with the ethyl acetate and methanol solvent extracts. As seen in figure 2, more number of compounds were identified in SFE mushroom extract as compared to the conventional method. Also, the compounds that are therapeutically active are more in percentage as compared to the conventional extraction method.



(a)



(b)



(c)

Fig 2: Chromatogram of the GCMS analysis of the extract obtained by (a) Conventional method using methanol (b) using ethyl acetate and (c) SFE with 5%MeOH

The composition results are presented in Table 1, with the name of the compounds and the relative composition for the extracts obtained by supercritical fluid extraction and solvent extraction. In the solvent extraction, mostly the volatile compounds were found whereas in case of the supercritical extraction, major bioactive compounds having therapeutic attributes were identified indicating the strong nutraceutical property of the *L.edodes* mushroom. The aggregate compounds have been identified along with its medicinal property. The major identified components in terms of % area peak were Linoleic acid (41.94%), Palmitic acid (6.61%) and Ergosterol (5.82). The extracts obtained by SFE showed more fatty acids than those obtained by Soxhlet. Hexadecanoic, Tetradecanoic acid, pentadecanoic acid, pentanoic and 9-oxononanoic acid were some of the identified components in the SFE extracts. Mazzuti et al., 2012 carried out the supercritical extraction of *Agaricus brasiliensis* and reported 44.24% of linoleic acid in the extract obtained from SFE+5% ethanol. Similarly, extracts of *A. blazei* mushroom obtained by SFE with CO_2 at 40 MPa and 243.15 K have been reported to be rich in palmitic acid and oleic acid [20]. The results sustain the fact that the supercritical extraction process is very attractive since the efficiency of the process can be controlled by minute changes in pressure as well in temperature. With the density much greater than those of typical gases and slightly less than those of organic liquids and viscosity near to the typical gases and less than those of liquids [21], the supercritical carbon dioxide can easily penetrate the interior structure of *L.edodes* matrices, so more bioactive compounds were extracted from the fungi matrice. Molecular structures of the most abundant compounds found in *L.edodes* extract is shown in Figure 3.

Table 1: Relative composition profile, in % peak area, of *Lentinus edodes* extracts obtained by conventional method (methanol & ethyl acetate and supercritical fluid extraction (SFE) with co solvents)

Name of Compound	Formula	RT (time)	SFE +5% MeOH	Solvent (MeOH)	Solvent (EtAc)	Pharmacological Activity
2(3H)-Furanone, 3-Pentenoic acid	C_5H_6O2	5.687	0.59	0.45	0.14	Antibacterial
Bicyclo-Borneol	$C_{10}H_{18}O$	5.933	0.14	--	--	
α Terpineol, L-α-Terpineol	$C_{10}H_{18}O$	6.325	0.20	--	--	
Cyclooctanol	$C_{10}H_{18}O_2$	6.876	0.20	--	--	
Decanoic acid, Capric Acid	$C_{10}H_{20}O_2$	7.545	0.19	--	--	
2-Undecanone	$C_{11}H_{22}O$	8.000	0.15	--	--	
2,Nonanol	$C_9H_{20}O$	8.730	0.25	--	--	
Valeric Acid, Pentanoic acid	$C_5H_{10}O_2$	9.178	0.23	--	--	
Cyclohexane	$C_{14}H_{28}$	10.111	0.09	--	--	
γ bisabolene	$C_{15}H_{24}$	10.531	0.31	--	--	Antiulcer
Acoradiene	$C_{15}H_{24}$	10.671	1.37	--		
Curcumene	$C_{15}H_{22}$	10.919	3.57	--	--	Antihypertensive
Zingiberene	$C_{15}H_{24}$	11.076	0.34	--		

ISSN: 0975-8585

β Bisabolene	$C_{15}H_{24}$	11.259	2.11	--	--	Antimicrobial
β sesquiphellandrene	$C_{15}H_{24}$	11.477	1.37	--	--	Anticancer
Fumaric Acid	$C_{10}H_{14}O_4$	12.073	0.43	--	--	Antibacterial
Benzene,1-(1,5 dimethyl 1-4 hexenyl) -4-methyl	$C_{15}H_{22}$	12.575	0.20	--	--	
Guaiol, Champacol	$C_{15}H_{26}$ O	12.850	0.55	--	--	
2-butanone	$C_{13}H_{22}$ O	13.060	0.27	--	--	
Sesquisabinene Hydrate	$C_{15}H_{26}O$	13.311	0.23	--	--	
Octadecane	C18H38	13.493	0.12	--	--	
3-buten-2 one	$C_{13}H_{20}O_2$	14.142	0.25	--	--	
Bicyclo [4.2.2] Dec-9-en-7one	$C_{13}H_{20}O_2$	14.342	0.27	--	--	
Tetra decanoic acid, Myristic acid	$C_{14}H_{28}O_2$	14.560	0.20	0.14	--	
Spiro [4.5]decan-7-one	$C_{15}H_{24}O_2$	14.876	0.13	--	--	
Neoisolongifolane	$C_{15}H_{26}O$	15.208	0.19	--	--	
1-Hexadecanol	$C_{16}H_{34}O$	15.497	0.34	--	--	
Pentadecanoic acid ethyl ester	$C_{17}H_{34}O_2$	15.609	0.67	--	0.43	
Hexadecanoic acid, Palmitic acid	$C_{17}H_{34}O_2$	15.940	0.72	--	0.28	
Ethyl Palmitate, Palmitic acid	$C_{18}H_{36}O_2$	16.625	6.61	0.38	3.68	Anti-inflammatory
Isopropyl palmitate	$C_{19}H_{38}O_2$	16.908	0.28	--		
Cyclohexane	$C_{20}H_{36}$	17.145	0.39	--		
Tricyclo[4.4.0.0(2,8)]decan-4-ol	$C_{10}H_{16}O$	17.358	0.06	--		
Methyl 10-trans,12-cis-octadecadienoate	$C_{19}H_{34}O_2$	17.611	1.34	--		
9,12 Octadecadienoic acid (Z,Z), Linoleic acid	$C_{18}H_{32}O_2$	18.401	41.94	20.78	15.02	Analgesic, anti-inflammatory and ulcerogenic
2-Methyltetracosane	$C_{25}H_{52}$	19.372	0.31	--		
4-Decen-3-one	$C_{17}H_{24}O_3$	19.523	0.78	--		
Isopulegol 2	$C_{10}H_{18}O$	19.813	0.47	--		
9-octadecenamide	$C_{18}H_{35}NO$	20.030	0.54	0.13	0.18	Hypolipidemic
Heptadecanoic Acid	$C_{19}H_{38}O_2$	20.176	0.43	--	--	
Acetic acid	$C_{13}H_{24}O_3$	20.356	0.24	--	--	
1,2,2-[2H(3)-4-Methoxy phenylethene	$C_9H_7D_{30}$	20.641	0.06	--	--	
1 (2H)-Naphthalenone	$C_{13}H_{16}O$	20.765	0.23	--		
14 Methyl 8 hexadecyn-1-ol	$C_{17}H_{34}O$	20.883	1.65	--		Anticancer
Heptadecane	$C_{17}H_{36}$	21.206	1.01	--	--	Anti-inflammatory
2-(7-Hydroxymethyl-3)	$C_{21}H_{28}O_3$	21.461	0.92	--		
Cedran-diol	$C_{15}H_{26}O_2$	21.943	0.27	--		
2,6,6-Trimethyl-Cyclohex-1-Enylmethane sulfonyl	$C_{16}H_{22}O_2S$	22.249	0.30	--		
E,Z-1,3,12-Nonadecatriene 5,14-diol	$C_{19}H_{34}O_2$	22.645	6.95	1.19	--	Antimicrobial
Docosanoic acid ethy ester, ethyl behenoate	$C_{24}H_{48}O_2$	23.379	0.84	--		
Squalene	$C_{30}H_{50}$	23.779	1.06	0.23	0.13	Adjunctive therapy in cancer
(N-97-Methyl-4-Aza-9-Fluorenylidene) Cyclohexylamine	$C_{19}H_{20}N_2$	25.529	0.30	--	--	
Ergosta-5,7,9 (11),22-Tetraen-3-ol	$C_{28}H_{42}O$	25.756	1.54	0.14	1.98	Precursor of Vit D
Dehydroergosterol 3,5-dinitrobenzoate	$C_{35}H_{44}N_2O_6$	25.977	0.20	--	--	
9 (11)-Dehydroergosteryl benzoate	$C_{35}H_{46}O_2$	26.133	0.33	0.25	2.12	

Dehydroergosterol 3,5-dinitrobenzoate	$C_{35}H_{44}N_2O_6$	26.388	0.43	--		
3- (4-Isopropylphenyl)-1-Propene	$C_{20}H_{26}O_2$	26.551	0.39	--		
5,6,8,9,10,11-Hexahydrobenz(a) anthracene	$C_{18}H_{18}$	26.738	0.19	--		
1-Heptacosanol	$C_{27}H_{56}O$	27.219	0.38	--		
(1R,2S,8R,8Ar)-8-acetoxy-1-(2-hydroxyethyl)-1,2,5,5-tetramethyl-trans-decalin	$C_{18}H_{32}O_3$	27.659	0.23	--		
Ergosta-5,7,22-Trien-3-ol	$C_{24}H_{44}O$	27.950	0.61	--		
Dehydroergosterol 3,5-dinitro benzoate	$C_{35}H_{44}N_2O_6$	28.499	0.27	--		
Ergosta-5,7,9 (11),22 tetraen-3-ol	$C_{28}H_{42}O$	28.924	0.60	--	--	
Ergosta 5,8,22-trien-3-ol	$C_{28}H_{44}O$	29.090	0.77	--	0.60	
Ergosta 5,7,22-trien-3-ol	$C_{28}H_{44}O$	29.680	5.82	--	10.98	Precursor of Vit D
Ergosta-7,22-dien-3-ol	$C_{28}H_{46}O$	29.978	0.40	--	0.61	
7,2,2 ergostadienone	$C_{28}H_{44}O$	30.602	0.17	--		
Ergosta-4,7,22 trien-3-one	$C_{28}H_{42}O$	30.836	0.42	--	0.13	Precursor of Vit D
Ergost-7-en-3-ol	$C_{28}H_{48}O$	31.322	1.69	0.69	2.60	Precursor of Vit D
Ergosta-7,22-Dien-3-ol	$C_{28}H_{46}O$	31.875	0.13	--	--	
Cholest-7-en-3-one	$C_{27}H_{44}O$	32.055	0.15	--	--	
Neoergosterol	$C_{27}H_{40}O$	32.559	0.83	--		
10,13-dimethyl-17-(1,4,5-Trimethyl-hex-2-enyl	$C_{28}H_{40}O$	33.866	0.31	--		
Lanost-8 en-3-ol	$C_{31}H_{52}O$	34.182	0.31	--		
Ergosta-4,7,22-trien-3-one	$C_{28}H_{42}O$	35.748	0.42	--		
Stigmasta-4,7,22-trien-3	$C_{29}H_{46}O$	37.860	0.74	--		Antitubercular

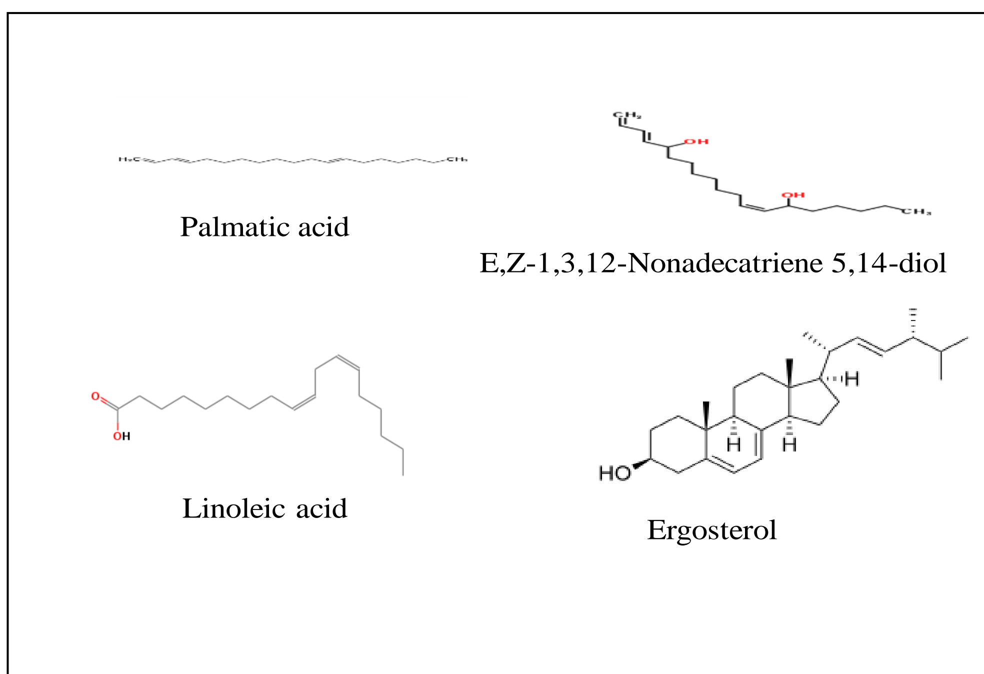


Fig 3: Molecular structures of the most abundant compounds found in *L.edodes* extracts (Source: NIST,2008)

Linoleic acid is an essential fatty acid which is not produced in human body but is obtained from diet. It takes part in a wide range of physiological functions and also reduces risks of cardiovascular diseases,

triglyceride levels, blood pressure, and arthritis [22]. Further *L.edodes* contains high amounts of ergosterol which can be converted to vitamin D_2 by UV irradiation. When mushroom fruit bodies are exposed to UV light, ergosterol undergoes photolysis to yield a variety of photo irradiation products, principally previtamin D_2, tachysterol and lumisterol [23]. It has been reported by Shu et al. [24] that the ergosterol isolated from the fungi exhibits pharmacological activities, antioxidative capacity and anti-tumour activity. As evident from the results, the conventional extraction process has low ability to extract functional compounds. Nonetheless, these methods are not selective as the process often requires further stages of fractionation to acquire desired compound. As a result, loss of compounds occurs during fractionation steps. Also, there are chances of degradation of the thermally sensitive compounds as high temperature is used during solvent/conventional extraction techniques.

ANTIOXIDANT ACTIVITY

DPPH, a free radical stable at room temperature, produces a violet solution in ethanol. In presence of antioxidant compounds the DPPH is reduced producing a non-color ethanolic solution. Fig. 4 shows the results of antioxidant activity (AA %) of *Lentinus edodes* extracts obtained using DPPH estimation method.

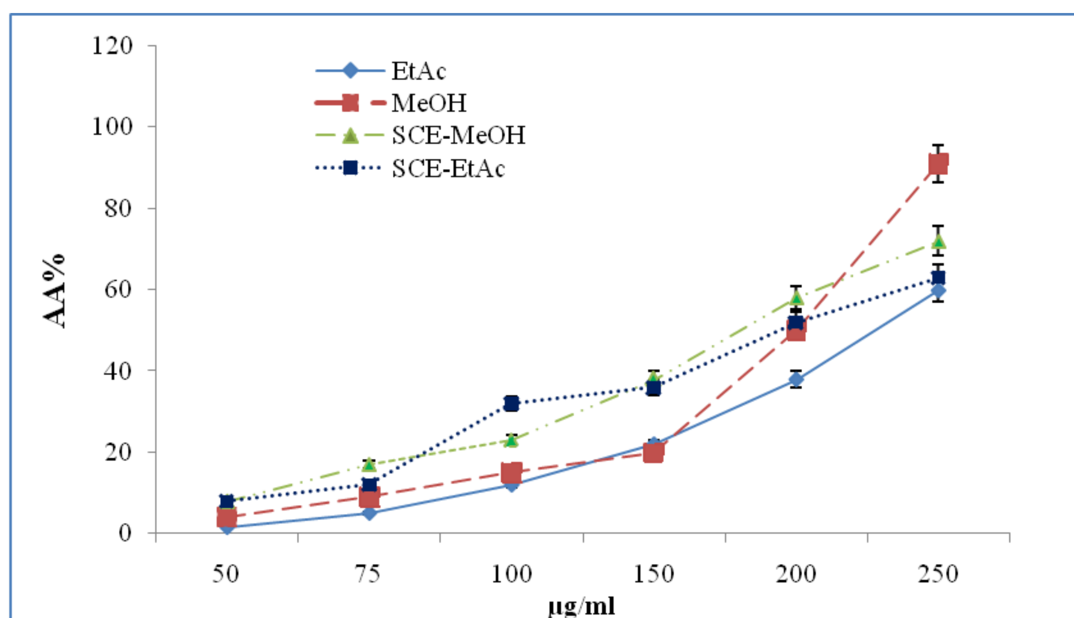


Fig 4: Antioxidant activity (AA) of *L.edodes* extracts obtained with SFE + co-solvent and organic solvents.

The shiitake fractions obtained with MeOH and EtAc show antioxidant activity (AA) of 91.08% and 60% respectively for 250 µg/ml extract concentration. It might be due to the presence of polar substances responsible for DPPH activity in the extracts. Supercritical extracts with pure CO_2 was also tested for this activity. However, very low activity, i.e. 13% AA was observed at the same concentration. The reason for the low DPPH activity might be the non-polar nature of the CO_2 that resulted in the extraction of mainly non-polar components having limited antioxidant activity. Our results are in agreement with the findings reported by Andrade et al. [25] where the extract of spent coffee ground obtained by soxhlet using ethyl acetate exhibited higher antioxidant activity and consequently lower EC_{50} value (202.23 µg/ml) as compared to the supercritical extract. Addition of methanol and ethyl acetate as co-solvents (each at 5% level) increased its AA activity upto 72% and 63% respectively at 250 µg/ml. Kitzberger et al. [17] in his study reported a limited antioxidant activity for supercritical extract obtained with pure CO_2 i.e 11% AA. Similarly, in FRAP no significant difference was found for the organic solvents used.

Phenolic compounds in plants are recognized as potent in vitro antioxidants due to their ability to donate hydrogen or electrons and to form stable radical intermediates [26]. The TPC in the extracts was expressed in equivalent of gallic acid (GAE) (g/100 g of extract) and the results for the shiitake extracts are presented in Table 2. Highest phenolic content was found in the methanolic extracts (2.14mgGAE/g) followed

by SFE + 5% MeOH extract (1.07mgGAE/g). The polyphenol content of extract obtained only by the SFE method was found to be 0.78 mg GAE/g extract. It was interesting to observe that the antioxidant capacity increased when polar organic solvent was added. The reason might be due to the fact that the important substances that show antioxidant activities are polar in nature and since CO_2 is a non polar solvent, it does not facilitate the solubilization of such components. Additionally, the enrichment of CO_2 with organic solvents improves the extraction of compounds with antioxidant activity is also due to the proportional changes in the solvent mixture characteristics/nature. Similar studies by Luengthanaphol et al. [27], Goli et al. [28], and Sanchez-Vioque et al. [29] revealed that the solvent extraction method was more effective to extract phenolic compounds compared to supercritical CO_2 extraction methods.

Table 2: Antioxidant activities of the *L.edodes* extracts

Antioxidant properties	Assay	Methanolic extract	Ethyl acetate extract	SFE	SFE-Methanol 5%	SFE-Ethyl acetate 5%
Total Polyphenols	Folin-Ciocalteu (mg GAE/g extract)	2.14±0.03^{e}	1.05±0.03^{d}	0.78±0.03^{a}	1.07±0.03^{c}	0.96±0.02^{b}
	FRAP assay (EC_{50}, mg/ml)	21.57±1.04^{a}	21.79±1.19^{a}	23.01±1.03^{c}	22.19±1.65^{b}	22.58±1.82^{b}
Radical Scavenging activity	DPPH assay (EC_{50}, mg/ml)	0.143±0.01^{a}	0.189±0.01^{b}	0.212±0.03^{e}	0.152±0.02^{c}	0.193±.03^{d}

GAE – gallic acid equivalents. For Folin–Ciocalteu, the higher values mean higher reducing power. For the other methods, the results are presented in EC50 values, meaning that higher values correspond to lower reducing power, radical scavenging activity or lipid peroxidation inhibition. EC50 is the concentration of the extract that corresponds to 50% of antioxidant activity for the DPPH or 0.5 of absorbance for the FRAP. In each row, the different letters represent significant differences between samples ($p < 0.05$).

CONCLUSION

Supercritical CO_2 of *L.edodes* mushroom was carried out. The SFE yield was of 0.56 % using pure CO_2 as solvent where as when 5% methanol was added as co-solvent, the extraction yield was higher, reaching 1.02 % due to the recovery of polar compounds. Solvent extraction was performed for comparative purposes and highest yields were obtained. Preliminary evaluation of the antioxidant potential of the mushroom extracts has demonstrated moderate antioxidant activity. Since GC-MS was not able to identify the compounds responsible for antioxidant activity, henceforth no relation was established between the chemical profile and the antioxidant activities. The major identified compounds were linoleic and palmitic acids. The SFE technique is a novel technique to obtain functional compounds from a natural source thereby increasing the aggregate value of the food products. Investigations pertaining to the anticancer activities should be performed and techniques to detect and quantify phenolic compounds in *L.edodes* mushroom should be applied.

ACKNOWLEDGEMENTS

The first author is highly grateful to the University Grants Commission (UGC), India for the financial support offered for the research work.

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ISSN: 0975-8585

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