RESEARCH ARTICLE

In vitro Antioxidant and Antibacterial Activities of Fractionized Extracts of Edible Mushroom *Pleurotus djamor* var. *roseus*

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Abstract

This study determined *in vitro* antioxidant and antibacterial activities of five different (petroleum ether, hexane, chloroform, ethyl acetate and fractionized methanol extract) fractions from the crude methanolic extract of an edible mushroom *Pleurotus djamor* var. *roseus* (GenBank database, Maryland, USA−Accession number GU350628). Five different fractions were screened for antioxidant potential by three different assays viz., ferric thiocyanate (FTC), thiobarbituric acid (TBA) method and (DPPH) free radical-scavenging assay and antibacterial activity against clinically important bacterial strains *Staphylococcus aureus* (ATCC 2079), *Bacillus cereus* (ATCC 11778), *Escherichia coli* (ATCC2567)**,** *Klebsiella pneumoniae* (ATCC 29665) and *Pseudomonas aeruginosa* (ATCC 2036) determined by well diffusion method. Results indicated that the fractionized methanol and hexane fractions of *P. djamor* var. *roseus* exhibited both antioxidant and antibacterial activity. Hexane extract exhibited strong antioxidant activity with an IC_{50} value of 2.7 mg/mL followed by methanol extract (IC_{50}-2.862 mg/mL). FTC and TBA tests showed strong inhibition of lipid-oxidation in hexane fraction evident from its low absorbance value compared to other fractions. The total phenolic content was high in the fractionized methanol extract (2.25 mg/g GAE) followed by hexane extracts (0.35 mg/g GAE). Hexane extract exhibited maximum zone of inhibition against the test pathogenic bacteria followed by fractionized methanol extract.The present study showed that five different solvent fractions of *P. djamor* exhibited varying antioxidant and antibacterial activities. Further, this edible mushroom may be a potential source of natural antioxidants and antibacterial agents.

Keywords: Antioxidant, antibacterial, *Pleurotus djamor* var. *roseus*, lipid peroxidation, phenolic content.

Introduction

In recent years, antioxidants and antimicrobial activities from natural food and plant extracts have found many applications in pharmaceuticals and alternative medicines. The antioxidants of synthetic origin such as butylated hydroxyl anisole, butylated hydroxyl toluene have been proposed for use in the treatment of various free radicals related diseases (Lee *et al.,* 2004) but the commercialized synthetic compounds have toxic effects (Grice, 1986). Antibiotics exists in large numbers in today's pharmaceutical market, despite that, their usage is becoming increasingly restricted. The reason is attributed largely to the development of antibiotic resistance among microorganisms. For safe antibacterial and antioxidants, increased attention has been directed towards natural resources such as plants and mushrooms. Mushrooms have been valued throughout the world as both food and medicine for thousands of years (Lindequist *et al.,* 2005). Mushrooms valued as highly tasty and nutritional food, rich content of proteins, amino acids, carbohydrates, vitamins and minerals. Mushrooms as functional food are easily digestible and have low calories and fat (Manzi and Pizzoferrato, 2000).

The antioxidant potential observed in the mushroom is part of their natural defence mechanism against noxious events causing oxidative damage (Jeng-Leun *et al.,* 2002). Among the edible mushrooms, oyster mushrooms (*Pleurotus* spp.) are very popular for their culinary and medicinal properties. The beneficial effects of *Pleurotus* spp. like the anticancer, antiviral, antibiotic and anti-inflammatory and cholesterol lowering activities have been reported (Bobek *et al.,* 1991; Shamtsyan *et al.,* 2004). *Pleurotus djamor* var. *roseus* belongs to the family Pleurotaceae, commonly called as roseus mushroom or pink oyster mushroom or salmon-pink mushroom. However, the antioxidant and antibacterial effects of the edible *P. djamor* var. *roseus* is yet to be explored in the production of nutraceutical. The present study was aimed to investigate the *in vitro* antioxidant and antibacterial activities of the different fractionized extracts of *P. djamor* var. *roseus* using petroleum ether, hexane, chloroform and ethyl acetate. The *in vitro* antioxidant potential of *P. djamor* var. *roseus* fractions were screened by DPPH free radical scavenging and Lipid peroxides assays. In addition, total phenolic content present in various extracts were also determined.

The fractions were also tested for the antibacterial activity against two gram positive and three gram negative clinically important bacteria by well diffusion method.

Materials and methods

Mushroom collection: Mushrooms (basidiocarps) belonging to the family Pleurotaceae were collected from the Indian Institute of Technology (IIT) campus, Chennai, India. Based on the macro and microscopic characteristics, the strain was confirmed as edible mushroom *P. djamor* var. *roseus.* The basidiocarps were cut into small pieces and dried in the shade to constant weight. The dried pieces were then ground using an electrical blender into powder prior to assay.

Extract preparation for in vitro antioxidant and antibacterial activity: The coarse powder of *P. djamor* var. *roseus* (1000 g) was soaked in methanol (1:10) for 72 h at room temperature (maceration method). The mixtures were then filtered. The filtrate was then concentrated on a rotary evaporator at 45°C. The dried methanolic extract was subjected to sequential fractionation with the following solvents of increasing polarity namely petroleum ether, hexane, chloroform and ethyl acetate (Fig. 1). The remaining fraction was considered as a fractionized methanol extract.

Quantification of total phenolic content: Total phenolic content were determined according to Singleton and Rossi (1965), using gallic acid as standard. One mL of the fractionized extract from the respective solvents were mixed with equal volume of Folin and Ciocalteu's phenol reagent (purchased from Sigma) and incubated for 3 min at room temperature. Then, 1 mL of saturated sodium bicarbonate (3.5%) was added and made up to 10 mL with distilled water. The reaction mixtures were kept in dark for 90 min and absorbance was read at 650 nm. The calibration curve was constructed with different concentrations of gallic acid (0.01 mM to 0.1 mM) as standard. The total phenolic compounds were calculated from the calibration curve ($y = 2.9671x + 0.0199$, $R^2 = 0.9761$) of gallic acid standard. The total phenolic content was expressed as gallic acid equivalents (GAE) in mg/g extract.

Antioxidant assays
1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay: In vitro antioxidant activities of the five different fractions of *P. djamor* var. *roseus* were evaluated by DPPH radical scavenging method of Shimada *et al.* (1992). The principle of this assay is based on the measurement of the scavenging ability of the antioxidant towards the stable radical. The free radical DPPH is reduced to the corresponding hydrazine when it reacts with hydrogen donors. The stability was evaluated by the decolouration assay which determines the decrease in absorbance at 517 nm produced by the addition of the antioxidant to a DPPH solution in ethanol.

Assays were performed in 1.5 mL reaction mixtures containing 1 mL of 0.2 mM DPPH ethanol solution and 1 mL of different concentrations of fraction extracts (1-10 mg/mL) of *P. djamor* var. *roseus* were added. After 30 min of incubation at 37°C in dark, the absorbance of the reaction mixtures were measured at 517 nm. IC_{50} is the concentration of the sample required to scavenge 50% of DPPH free radical. Percentage of inhibition is calculated by subtracting the absorbance of the sample from the absorbance of the control divided by absorbance of the control.

Ferric thiocyanate assay (FTC) for initial stage of lipid peroxidation: The FTC method was adapted from Osawa and Namiki (1981). Samples (4 mg or 4 mL) in 99.5% ethanol were mixed with 2.51% linoleic acid in 99.5% ethanol (4.1 mL), 0.05 M phosphate buffer, pH 7.0 (8 mL) and distilled water (3.9 mL) and kept in screw cap containers under dark conditions at 40°C. To 0.1 mL of this solution, 9.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate was added. Precisely 3 min after addition of 0.1 mL of 2×10^{-2} M ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance of the red colour was measured at 500 nm each 24 h until one day after absorbance of the control reached maximum. The control and standard were subjected to the same procedure as the sample except for the control, where there was no addition of sample and for the standard, where 4 mg of sample were replaced with 4 mg of α-tocopherol or L-ascorbic acid.

Thiobarbituric acid test (TBA) assay for end stage of lipid peroxidation: The test was conducted according to the methods of Kikuzaki and Nakatani (1993); Ottolenghi (1959). The same samples as prepared for the FTC method were used. To 1 mL of sample solution, 20% trichloroacetic acid (2 mL) and thiobarbituric acid solution (2 mL) were added. This mixture was then placed in a boiling water bath for 10 min. After cooling, it was centrifuged at 3000 rpm for 20 min. Absorbance of supernatant was measured at 532 nm. Antioxidative activity was recorded, based on absorbance on the final day.

Well diffusion method for antibacterial activity: Evaluation of antibacterial activity of fractionized extracts of *P. djamor* var. *roseus* was performed by well diffusion method. The test bacteria obtained from ATCC was maintained on brain heart infusion agar slope at 4°C and subcultured 24 h before use. Isolated colonies of the bacteria were placed into individual tubes containing 5 mL of sterile brain-heart infusion broth (BHIB) (Himedia) and incubated at 37°C, before adjusting the tubes with 0.5 McFarland Units using sterile BHIB. Turbidity was also verified using spectrophotometric comparison with a 0.5 McFarland standard. The dilutions were used within 15 min of preparation and gently vortexed prior to use. Sterile Mueller-Hinton agar (pH 7.2-7.4) plates were prepared by pouring the

sterilized media in sterile petri plates under aseptic conditions. The standardized inoculum (1.5×10^8 colonies forming unit (cfu)/mL, suspension of turbidity equal to a McFarland standards 0.5) were introduced on the surface of sterile Mueller-Hinton agar (pH 7.2–7.4) plates using sterile cotton swabs (by streak plate method). Wells about 5 mm in dia were made in the agar plates using the sterile borer. Then each fractions was dissolved in 10% (v/v) of dimethyl sulfoxide (DMSO) and 50 µL (200 µg) of each fractionized extract were introduced in to the wells using sterile micropipette. Tetracycline (30 mcg) was used as a positive control and negative control wells contained 10% DMSO. The triplicates plates of each fractionized extracts were incubated at 37°C for 24 h. The zones of inhibition were measured.

Statistical analysis: All results were obtained in triplicates and data were expressed as mean ± SD. The data were subjected one-way and univariate analysis of variance (ANOVA) to determine the significance of individual differences at $p<0.05$ level. Significant means were compared by the Duncan's multiple range test. All statistical analyses were carried out using SPSS statistical package (SPSS, Version 10.0 for Windows, SPSS Inc., Chicago, USA).

Results

Pleurotus djamor var. *roseus* (NCBI-GenBank– Accession number GU350628) is an edible mushroom isolated from decomposed wood materials in the forest located in Indian Institute of Technology (IIT) campus, Chennai, India. The mushroom has not been commercialized. The nutraceutical and medicinal beneficial of the mushroom was likely to be lost if this edible *P. djamor* var. *roseus* was not documented.

Extract preparation: A yield of 65.80 g of crude methanolic extract was obtained from 1 kg dried powder of *P. djamor* var. *roseus.* The different fractions namely viz., petroleum ether, hexane, chloroform, ethyl acetate and fractionized methanol were 4.85 g, 16.20 g, 10.60 g, 6.85 g and 26.35 g, respectively (Fig. 1). All extracts were dried at room temperature and stored at 4°C for further usage.

Phenolic content in fractionized extracts: The fractionized methanolic extract exhibited highest phenolic content (2.25±0.04 mg/g dry weight, expressed as gallic acid equivalents), followed by hexane (0.35±0.013 mg/g), chloroform (0.3±0.015 mg/g), ethyl acetate (0.225±0.01 mg/g) and petroleum ether fraction (0.05±0.008) (Fig. 2).

DPPH free radical scavenging activity: Figure 3 and Table 1 showed the free radical scavenging activities of different fractions of methanol of *P. djamor* var. *roseus.* Among the five fractions, the maximum percentage of antioxidant activity and IC_{50} value of 86.89%; 2.862 mg/mL was observed in fractionized methanol

fraction followed by hexane fraction 83.83%; 2.7 mg/mL, chloroform fraction 81.89%; 3.691 mg/mL and ethyl acetate fraction 78.28%; 4.795 mg/mL, respectively. The results showed that statistically not significant different ($p<0.05$) to those of the standards α-tocopherol and ascorbic acid. The minimum inhibition percentage was recorded in petroleum ether fraction (72.2; 5.474 mg/mL). It is evident from the results that the methanol and hexane fraction showed strong antioxidant activity in a dose-dependent DPPH radical scavenging activity.

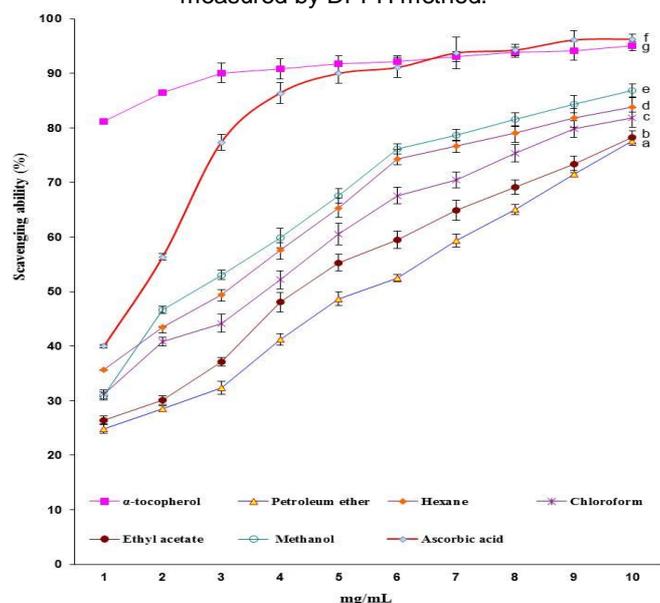Fig. 1. Extraction of various fractions of *P. djamor* var. *roseus* by sequential method.
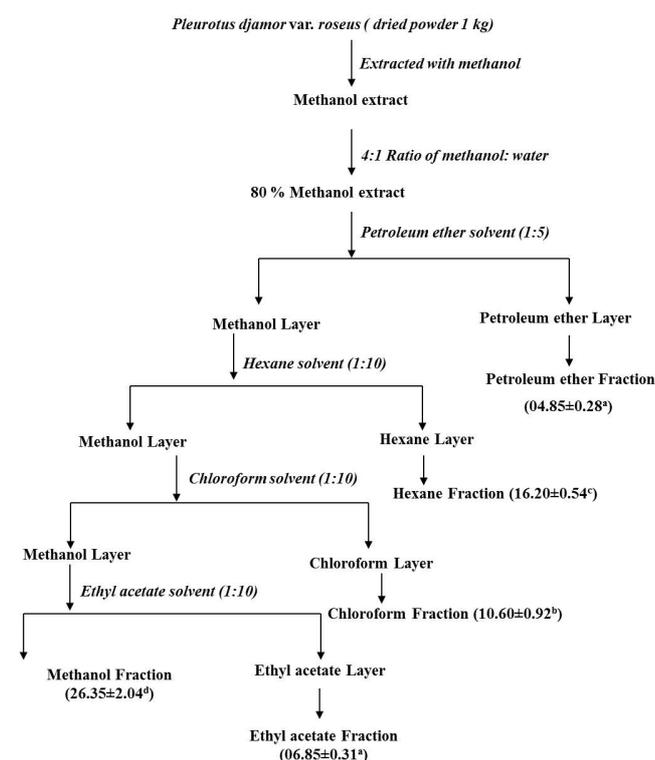
Values expressed are means ± S.D of triplicate measurements. Significantly different at P<0.05, ANOVA, Duncan[a]. Yield of the fractions in g.

Fig. 2. Concentration of total phenolic content present in different extracts of *P. djamor* var. *roseus.*
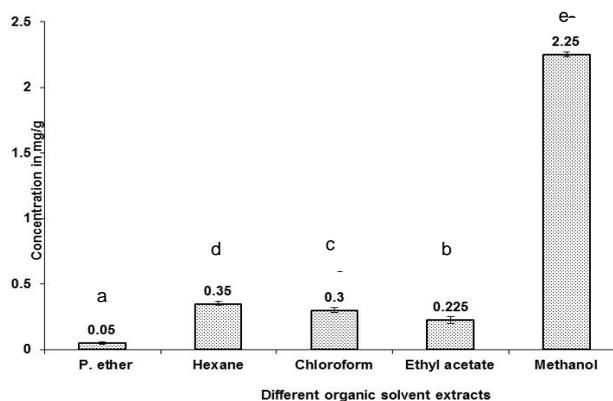
Values expressed are means ± S.D of triplicate measurements. Significantly different at P<0.05, ANOVA, Duncan[a,b].

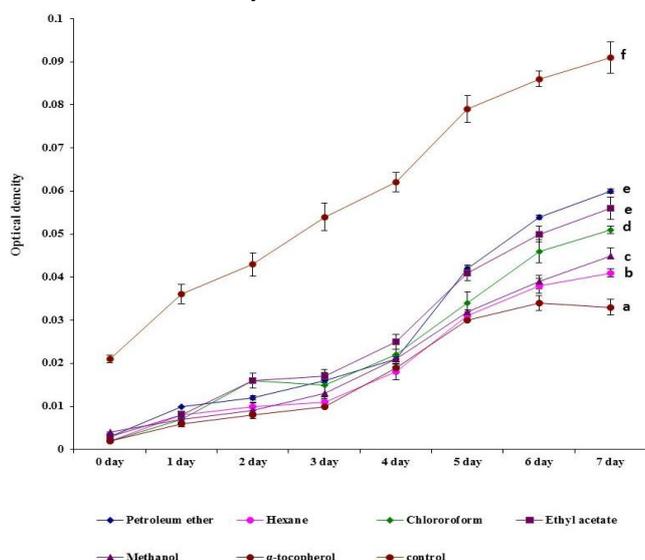Fig. 3. Antioxidant activity of *P. djamor* var. *roseus* extracts as measured by DPPH method.

Values expressed are means ± S.D of triplicate measurements. Significantly different at P<0.05, ANOVA, Duncan[a.b].

Table 1. Scavenging activity (IC$_{50}$ values) of various extracts of *P. djamor* var. *roseus* on DPPH radical.

Fractionized extracts	IC$_{50}$ values (mg/mL)	Maximum inhibition effect (%)
Petroleum ether	5.47±0.42[f]	72.21±1.81[a]
Hexane	2.7±0.26[c]	83.83±2.44[bc]
Chloroform	3.67±0.27[d]	81.89±3.04[bc]
Ethyl acetate	4.8±0.47[e]	78.28±2.39[b]
Methanol	2.87±0.29[c]	86.89±4.41[c]
α-tocopherol	0.81±0.09[a]	95.01±3.69[d]
Ascorbic acid	1.76±0.19[b]	96.28±2.58[d]

Values expressed are means ± S.D. of triplicate measurements. Significantly different (P<0.05, ANOVA, Duncan[a]).
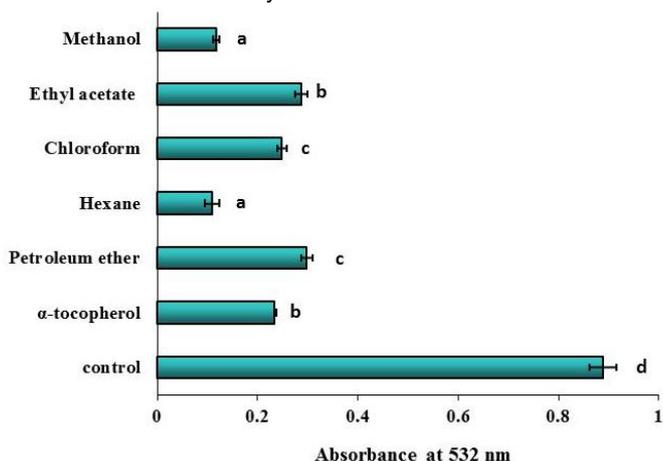
Fig. 4. The initial stage of lipid-peroxidation measured by TBA method.

Values expressed are means ± S.D of triplicate measurements. Significantly different at P<0.05, ANOVA, Duncan[a.b].

FTC the initial stage of lipid peroxides assay: Figure 4 depicts the FTC lipid peroxides activity in descending order α-tocopherol > hexane fraction > fractionized methanol > chloroform fraction > ethyl acetate fraction > petroleum ether fraction. Hexane and fractionized methanol fraction of *P. djamor* var. *roseus* displayed strong antioxidant activity that was not significantly different (p<0.05) to those of the standard α-tocopherol.
TBA the end stage of lipid peroxides assay: The results showed that *P. djamor* var. *roseus* inhibited the end stage if lipid peroxidation. The activity of fractions in descending order was hexane > methanol > α-tocopherol > chloroform > ethyl acetate > petroleum ether. No significant (p<0.05) difference was observed between the mushroom fractions and α-tocopherol. Moderate antioxidant activity was found in ethyl acetate and chloroform fractions. Among the fractions, hexane fraction showed a lower absorbance than the fractionized methanolic fraction, which signifies its antioxidant activity (Fig. 5).

Fig. 5. The end stage of lipid-peroxidation measured by TBA method.

Values expressed are means ± S.D of triplicate measurements. Significantly different at P<0.05, ANOVA, Duncan[a].

Anti-bacterial activity: All fractions tested had varying degrees of antibacterial activity and the hexane fraction showed maximum zone of inhibition among the fractions of *P. djamor* var. *roseus* studied. Gram positive bacterial strains (*B. cereus* and *S. aureus*) were more susceptible than gram negative bacteria (*E. coli, K. pneumoniae* and *P. aeruginosa*). The petroleum ether fraction showed low antibacterial activity (Table 2). The standard antibiotic tetracycline (30 mcg) was used as a positive control.

Discussion
Natural antioxidants are being extensively studied for their capacity to protect organisms and cells from damage brought on by oxidative stress, the latter being considered as a cause of ageing and degenerative diseases (Cazzi *et al.,* 1997). Fruit bodies of mushrooms are appreciated, not only for texture and flavour but also for their chemical and nutritional properties.

Table 2. Antibacterial activity of various extracts of *Pleurotus djamor* var. *roseus.*

Test microorganism	Zone of inhibition (mm)* of fractionized extracts					
	Tetracycline (30 mcg)	Petroleum ether	Hexane	Chloroform	Ethyl acetate	Methanol
Gram positive						
Bacillus subtilis	32	13±1	25±2	19±2	20±2	24±3
Staphylococcus aureus	26	9±2	21±3	11±1	13±2	12±3
Gram negative						
Escherichia coli	16	11±2	26±3	15±2	18±2	21±3
Klebsiella pneumoniae	8±1	23±2	13±2	14±2	18±1	8±1
Pseudomonas aeruginosa	–	-	22±2	14±1	18±2	21±2

*Values are mean of three replicates; '–': no zone of inhibition.

Wild edible mushrooms are traditionally used in many Asian countries in both food and medicine (Sanmee *et al.,* 2003; Isıldak *et al.,* 2004; Turkekul *et al.,* 2004). Mushrooms accumulate a variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroids. Phenolic compounds have been found to have antioxidant activity in the inhibition of low density lipoprotein (LDL) oxidation (Teissedre and Landrault, 2000; Cheung *et al.,* 2003). Some common edible mushrooms possess antioxidant activity, which correlated with their total phenolic content. In the present study, the DPPH free radical assay, inhibition of lipid peroxides assessed by FTC and TBA assays were successfully used for the evolution of antioxidant activity of the different fractions from methanol extract derived from *P. djamor* var. *roseus.* Plants and mushrooms are in general, harmless sources for obtaining natural antioxidants. Free radical scavenging is one of the known mechanisms by which antioxidants inhibit cellular damage. DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares *et al.,* 1997). The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm, which is induced by antioxidants. The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability (Baumann *et al.,* 1979). The DPPH radical scavenging ability of fractions of *P. djamor* var. *roseus* was significantly lower than those of α-tocopherol and ascorbic acid. The fractions did show proton-donating ability and could serve as free radical inhibitors or scavengers, there by acting possibly as primary antioxidants. Proton donating ability was high in fractionised methanol followed by hexane and chloroform fractions of *P. djamor* var. *roseus.* Almost all organisms possess antioxidant defence and repair systems that have evolved to protect them against oxidative damage by enzymes, such as superoxide dismutase and catalase or compounds such as ascorbic acid, tocopherols and glutathione (Mau *et al.,* 2002). These systems are insufficient to prevent the oxidative damage entirely (Simic, 1988). However, antioxidant supplements or food containing antioxidants may be used to help the human body to reduce the oxidative damage (Yang *et al.,* 2002). Mushrooms contain wide variety of free radical scavenging molecules, such as polysaccharides and polyphenols (Mau *et al.,* 2002; Wasser, 2002).

Although research was focused on the therapeutic effects of these commercial mushrooms, limited information are available about their antioxidant properties. The antioxidant activities of roseus mushroom *Pleurotus djamor* var. *roseus* was investigated in related to their total phenolic contents. Phenolic and flavonoids compounds present in several mushrooms can inhibit lipid peroxidation (Duh *et al.,* 1999; Cakir *et al.,* 2003). Lipid peroxides produced from unsaturated fatty acids via radicals cause histotoxicity and promote the formation of additional free radicals in a chain reaction type manner. In the present study, the FTC method was used to measure the peroxides formed during initial stages of lipid oxidation. During the oxidation process, peroxide was gradually decomposed into lower molecular weight compounds. The degradation products were then measured using the TBA method. Thus, in both the ferric thiocyanate (FTC), thiobarbituric acid (TBA) methods, samples with low absorbance values would indicate high antioxidative activity. This is true for both assays (FTC, TBA). Wang *et al.* (1996) has reported that the antioxidant properties of some vegetables and fruits are partly due to low molecular weight, phenolic compound, particularly the flavonoids, which are known to be potent antioxidants. In the present study, the results of both ferric thiocyanate (FTC), thiobarbituric acid (TBA) tests showed strong inhibition of lipid-oxidation by the hexane fraction. *Pleurotus djamor* var. *roseus* extracts showed antioxidant activity, which may be due to the presence of phenolic and other active compounds. The electron-transfer from the phenoxide anions of the phenolic compounds to the DPPH radicals is a possible mechanism for their reducing capacity (Huang *et al.,* 2005). Based on the previous reports, a high positive correlation between total phenols and the antioxidant and or antibacterial activities are found in many mushroom species (Alvarez-parrilla *et al.,* 2007). The antioxidant potential of the hexane fraction may be due to the better solubility of non-polar active components in hexane (Mothana *et al.,* 2000). Though fractionized methanolic fraction has high phenolic content, hexane extract showed much better antioxidant and antibacterial activity. It is thus suggested that the antioxidant and antibacterial activities of the hexane fraction were not solely contributed by phenolic compounds but by other compounds present in it.

Infectious diseases are still a major threat to human health. Such diseases are caused by various pathogens capable of invading the body and causing immune responses. A number of synthetic antimicrobial agents have been developed to kill microorganisms effectively and selectively without affecting human metabolism. However, drug resistance and organ toxicity often occur when these agents are used for an extended period. Therefore, novel antimicrobial agents with acceptable efficacy and safety are continuously sought. Solvent extracts of mushroom shiitake were first examined for the inhibition of microbial growth by an agar dilution method (Hirasawa *et al.,* 1999). The low polar organic extracts from mushrooms showed strong antibacterial activity (Mothana *et al.,* 2000). Mushrooms are traditionally been used to treat chronic infectious diseases, such as chronic hepatitis and bronchitis in Asia, when it is administered alone or more often in combination with chemotherapeutic agents. Preclinical (*in vitro* and *in vivo*) animal studies indicated that mushrooms exhibits a broad spectrum of antibacterial and antiviral activities (Reichling *et al.,* 2001). Gram positive bacteria consist of peptidoglycan, while the cell walls of gram negative bacteria consist of an inner cell membrane, a peptidoglycan layer and a thick outer layer of a lipid-polysaccharide complex (Rabe and Staden, 1997). This outer lipid membrane present in gram negative bacteria may function as a barrier and adds greater resistance for the entry of the tested extracts. The exact mechanism behind the antibacterial activity are complex to understand and could be attributed to either inhibiting the cell division or to damaging the cell walls of bacteria; which however requires to be investigated in detail.

The human pathogenic microorganisms have been developed due to indiscriminate use of commercial antimicrobial drugs commonly used for the treatment of infectious diseases. Suay and Arenal (2000) examination of over 200 species of Basidiomycetes demonstrated that about 50% of them showed significant antibiotic activity against a range of test organisms. In the present study, the fractionized extracts showed significant antibacterial activity against to clinical important gram positive and gram negative pathogens. Role of *P. djamor* var. *roseus* as antioxidants and antibacterial potential in the human body when orally ingested is unknown, but there are several possibilities. The relevance of the *in vitro* experiments in simplified systems to *in vivo* protection from oxidative damage and disease caused by the tested bacterial strains should be carefully considered. The fractionized methanol, which contains polyphenolic compounds show strong antioxidant activity, while the hexane, chloroform and ethyl acetate extracts, which contained triterpenoids and steroids including lanosterol, inotodiol, trametenolic acid and ergosterol peroxide, also had a relatively strong antioxidant effect (Kahlos *et al.,* 1989).

Conclusion
The present study reveals that *P. djamor* var. *roseus* could be a source of natural antioxidants and antibacterial agent that may have beneficial health effects on humans. However, further research would be required before such uses could be proposed with confidence. The findings of the current report warrant further research aiming to isolate and identify the specific compounds responsible for antioxidant and antibacterial activities.

Acknowledgements

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