JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Chemical Composition and Antioxidant Properties of Extracts of Fresh Fruiting Bodies of *Pleurotus sajor-caju* (Fr.) Singer

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ABSTRACT: The chemical composition and in vitro antioxidant activity of aqueous butanol and ethyl acetate extracts of *Pleurotus sajor-caju* were investigated in this study. Twenty-two compounds comprising methyl esters, hydrocarbon fatty acids, ethyl esters, and sterols were identified in ethyl acetate extracts, while cinnamic acid, nicotinamide, benzeneacetamide, and 4-hydroxybenzaldyhde were identified in butanol extracts by gas chromatography−mass spectrometry and NMR analysis. The antioxidant activity was determined by a β-carotene bleaching method, ferric reducing antioxidant power, trolox equivalent antioxidant capacity, and lipid peroxidation assays, while the total phenolic content in *P. sajor-caju* was assessed by Folin−Ciocalteau's method. The aqueous and butanol extracts exhibited the highest antioxidant activity, corresponding to the total phenolic content. The subfractions from the ethyl acetate extract (EP1, EP2, EP3, and EP4), however, showed moderate antioxidant activity. The regular consumption of *P. sajor-caju* as a part of our diet may render nutritional and nutraceuticals benefits for good health.

KEYWORDS: *Pleurotus sajor-caju*, secondary metabolites, ergosterol, antioxidant activity, total phenolic content

INTRODUCTION

Mushrooms, long valued as delicious and nutritional foods in many countries, are now increasingly studied for their chemical and nutritional characteristics.[1] A variety of compounds with important pharmacological/nutraceutical properties have been isolated from mushrooms such as *Pleurotus* spp. They include lectins with immunomodulatory, antiproliferative, and antitumor activities; phenolic compounds with antioxidant activities; and polysaccharides, polysaccharopeptides, and polysaccharide proteins with immunoenhancing and anticancer activities.[2] In many of the life-threatening diseases, antioxidants may help reduce or even prevent the occurrence or severity of the disease. Antioxidants are substances that can fight and destroy excess free radicals and prevent oxidative damage in biomolecules.[3] Chinese herbs and mushrooms are among the traditional medicines that have been used for centuries.[4,5] Furthermore, mushrooms belonging to the *Pleurotus* genus, best known as oyster mushrooms, alluding to its appearance, have been reported to have functional biomolecules responsible for antioxidant activities.[6] The fruiting bodies of *Pleurotus* species have a characteristic texture and pleasant flavor that makes them popular as culinary mushrooms worldwide.[7] Furthermore, *Pleurotus* spp. have been shown to modulate the immune system, possess hypoglycemic activity and antitrombotic effects, lower blood pressure and blood lipid concentrations, and inhibit tumor growth, inflammation, and microbial action.[8] Among the *Pleurotus* spp., *Pleurotus sajor-caju*, *Pleurotus ostreatus*, *Pleurotus citrinopileatus*, and *Pleurotus florida* have been reported as commonly available edible mushrooms.[9] Currently, *P. sajor-caju* is cultivated all around the world.[2] *P. sajor-caju* is the second most important mushroom in production in the world. It has been gaining importance throughout the years for its medicinal properties. However, studies are focused on the optimization of processes involving biomass production and their medicinal properties such as antioxidant and antimicrobial activity. The studies on their chemical composition are few. These include the chemical composition reported previously by Caglarirmak [10] and Chu et al.[11] These studies are mainly on the isolation and quantification of ergosterol.

Despite the continuing search for active principles from the extract of *P. sajor-caju*, there are limited chemical investigations on the fruiting bodies. It is therefore of interest to identify the secondary metabolites that may be responsible for the bioactivities including antioxidant activities present in *P. sajor-caju*. The aims of this study were (i) to investigate the antioxidant activity and the phenolic content of the crude and fractionated extracts and (ii) to correlate the antioxidant activity of extracts with the chemical components. The findings of this study may be valuable in the formulation of nutraceuticals and functional food for the prevention of life-threatening diseases in human.

MATERIALS AND METHODS

Mushroom Samples. *Fresh Fruiting Bodies.* Fresh fruiting bodies of *P. sajor-caju* (10 kg) were collected from Juan Kuan's mushroom farm in Semenyih (location −3°21′19.20″N 101°14′36.35″E), Selangor Darul Ehsan, Malaysia.

Extraction and Fractionation of Chemical Components from Fruiting Bodies of P. sajor-caju. The fresh fruiting bodies (5 kg) were washed, sliced, and freeze-dried (Christ, United Kingdom) for 2 days. The freeze-dried mushrooms were ground to a fine powder using a

Received: October 22, 2010
Accepted: January 28, 2011
Revised: January 27, 2011
Published: February 24, 2011

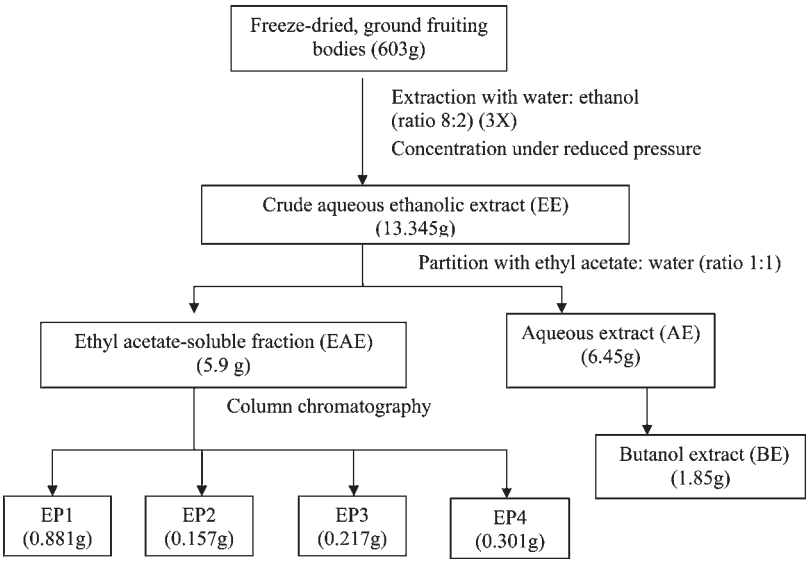

Figure 1. Fractionation and isolation of compounds from *P. sajor-caju*.

blender. The dried, ground sample was then soaked in a mixture of ethanol and water at a ratio of 8:2 (1.5 L) for 3 days at room temperature. The solvent-containing extract was then decanted and filtered. The extraction process was repeated three times, the filtrates were combined, and the excess solvent was evaporated under reduced pressure using a rotary evaporator to give a light-yellowish viscous extract. The crude extract (EE) was further extracted with a mixture of ethyl acetate and water (1:1) to give an ethyl acetate-soluble fraction (EAE) and an aqueous-soluble fraction (AE). The aqueous layer was freeze-dried to yield a light brown-colored extract, which was further extracted with butanol (BE) and concentrated under reduced pressure to yield a dark brown viscous extract. Evaporation of solvent from the ethyl acetate layer gave an ethyl acetate extract, which was further subjected to column chromatography using acetone:hexane as the eluting solvent. Figure 1 shows the fractionation and isolation of compounds from *P. sajor-caju*.

Isolation of Secondary Metabolites. *Sample Preparation.* EAE (5 g) was mixed with silica gel (0.063−0.200 nm; mesh, 70−230; 30 g) (Merck). This mixture was dried at 40 °C in an oven to yield a powdery consistency.

Thin-Layer Chromatography (TLC). TLC was carried out using precoated TLC plates 60 F_{254} (20.25 mm thickness) purchased from Merck and were visualized in UV light (254 and/or 365 nm) and/or iodine vapor. Various percentages of hexane and acetone were used as the developing solvents.

Column Chromatography. The powdered mixture of EAE and silica was subjected to column chromatography initially eluting with 100% hexane followed by hexane enriched with increasing percentages of acetone. Fractions of 25 mL volume were collected in numbered vials. The eluted compounds were monitored using TLC. The fractions were pooled according to the spots on TLC plates. The excess solvent in the pooled fractions was evaporated under reduced pressure using a rotary evaporator. Components in the isolated fractions were identified using gas chromatography−mass spectrum (GC-MS) and nuclear magnetic resonance (NMR).

Instrumentation. *GC-MS Analysis.* The GC-MS analysis was performed on a Agilent Technologies 6890 N (United States) gas chromatography equipped with a 5979 Mass Selective Detector (70 eV direct inlet) and a HP-5 ms (5% phenylmethylpolysiloxane) capillary column (30 m × 0.25 mm i.d. × 0.25 mm film thickness) initially set at 150 °C, then programmed to 280 °C at ramp rate of 5 °C per min using helium as the carrier gas at a flow rate of 1 mL/min. The total ion chromatogram obtained was autointegrated by Chemstation, and the constituents were identified by comparison with the mass spectral database (NIST Library, 2005).

NMR. NMR spectra were recorded on a Bruker Avance DPX-500 (USA) spectrometer for ^{1}H, ^{13}C, correlation spectroscopy (COSY), distortionless enhancement by polarization transfer (DEPT), heteronuclear multiple bond coherence (HMBC), and heteronuclear multiple quantum coherence (HMQC) NMR. The internal standard used in ^{1}H NMR spectra was TMS (δ: 0.00) for deuterium chloroform ($CDCl_3$); in ^{13}C NMR, it was $CDCl_3$ (δ: 77.0).

Antioxidant Activity. The antioxidant potential in the extracts of *P. sajor-caju* was investigated using the following standard assays.

β-Carotene-Linoleate Bleaching Assay. The antioxidant activity of *P. sajor-caju* extracts was assayed based on the β-carotene bleaching method described by Cheung et al.[12] In the β-carotene bleaching assay, linoleic acid produces hydroperoxides (ROS), and this oxidizes the β-carotene at 50 °C. The presence of antioxidants in the extract will minimize the oxidation of β-carotene by hydroperoxides. Hydroperoxides formed in this system will be inactivated by antioxidants from extracts. Butylated hydroxyanisole (BHA) was used as the standard. β-Carotene (0.2 mg in 1 mL of chloroform), linoleic acid (0.02 mL), and Tween 80 (0.2 mL) were transferred into a round-bottomed flask. Chloroform was removed at room temperature using a rotary evaporator. Following evaporation, 50 mL of distilled water was added to the mixture and shaken vigorously to form an emulsion. Two milliliters of aliquots of the emulsion was pippeted into test tubes containing 0.2 mL of *P. sajor-caju* extracts and immediately placed in a water bath at 50 °C. The absorbance was read at 20 min intervals for 2 h at 470 nm using UV−vis spectrophotometer (Shimadzu, Japan). The antioxidant activity of extracts was expressed based on two different parameters, namely, antioxidant activity (A_A) and the oxidation rate ratio (R_{OR}).

The antioxidant activity (A_A) was expressed as a percent of inhibition relative to the control, using the following formula:

$$A_A = \left[\left(R_{control} - R_{sample\ or\ standard}\right)/R_{control}\right] \times 100$$

where $R_{control}$ and R_{sample} represent the bleaching rates of β-carotene without and with the addition of antioxidant, respectively. Degradation rates (R_D) were calculated according to the first-order kinetics:

$$R_D = \ln(A_t/A_x) \times 1/t$$

Table 1. Chemical Constituents in EP1

chemical constituents	RT (min)	MW	MF	percentage[a](%)	method of identification
ethyl myristate	7.897	256.24	$C_{16}H_{32}O_2$	0.21	MS
metyl palmitate	10.099	270.26	$C_{17}H_{34}O_2$	3.83	MS
ethyl palmitate	11.350	284.27	$C_{18}H_{36}O_2$	16.33	MS
methyl linoleate	13.052	294.26	$C_{19}H_{34}O_2$	14.83	MS
methyl stearate	13.496	298.29	$C_{20}H_{40}O_2$	1.02	MS
ethyl linoleate	14.421	308.27	$C_{20}H_{36}O_2$	53.00	MS
ethyl octadecanoate	14.715	312.30	$C_{20}H_{40}O_2$	4.94	MS

[a] Percentages were calculated based on results obtained from the total ion chromatogram. RT, retention time; MW, molecular weight; MF, molecular formula; and MS, mass spectrum.

where A_t is the initial absorbance at 470 nm at $t = 0$ and A_x is the absorbance at 470 nm at $t = 120$ min.

The oxidation rate ratio (ROR) was calculated as:

$$R_{OR} = R_{sample}/R_{control}$$

where R_{sample} and $R_{control}$ are described earlier.

Ferric-Reducing Antioxidant Power (FRAP). The FRAP assay proposed by Benzie and Strain [13] was modified to be performed in 96-well microplates and expressed as $FeSO_4 \cdot 7H_2O$ equiv. The working FRAP reagent was prepared by mixing 50 mL of 300 mM acetate buffer with 5 mL of 10 mM TPTZ (2,4,6-tripyridyl-*s*-triazine) in 40 mM hydrochloric acid and with 5 mL of 20 mM ferric chloride. Ten microliters of extracts of various concentrations was added to wells of 96-well microtiter plate in triplicate, and 300 μL of freshly prepared FRAP reagent was added to the extracts to yield the final concentration of extracts in the range of 4−20 mg/mL. The FRAP reagent (without sample) was used as a negative control (A_{blank}). The plate was placed in a spectrophotometer (Bio-Tek Instruments Inc., United States) at 593 nm, and the temperature was maintained at 37 °C for 4 min. The absorbance values (A_{sample}) were measured after 4 min. The change in absorbance ($A_{sample} - A_{blank}$) was calculated. BHA was used as a positive control. The results are mean values of triplicate assays and were expressed as μM $FeSO_4 \cdot 7H_2O$ equiv/g of fresh mushroom.

Trolox Equivelants Antioxidant Capacity (TEAC). The antioxidant activity was assessed as described by Re et al.[14] 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was dissolved in water to yield a 7 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12−16 h before use. The $ABTS^{\bullet+}$ solution was further diluted with ethanol, to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30 °C. The $ABTS^{\bullet+}$ solution (100 μL) was added to 10 μL of sample, and the absorbance reading was measured after 1 min. $ABTS^{\bullet+}$ diluted in ethanol was used as a negative control. Ascorbic acid was used as a positive control. The results are mean values of triplicate assays and were expressed as mM Trolox equiv/g of fresh mushroom.

Lipid Peroxidation (LPO). The LPO assay was carried out based on a method reported previously[15] but with minor modifications. Egg yolk (rich in lecithin) was mixed with the same volume of phosphate buffer saline (PBS), pH 7.45, and stirred vigorously using a magnetic stirrer to yield a smooth suspension, which was diluted 40 times with PBS prior to use. The assay mixture was comprised of 0.5 mL of yolk suspension, 1 mL of *P. sajor-caju* extracts, and 0.5 mL of 24 mM $FeSO_4$ in PBS, which was incubated at 37 °C for 15 min. After the addition of 0.5 mL of trichloroacetic acid (20% v/v) and 1 mL of 2-thiobarbituric acid (0.8% v/v), the incubation mixture was heated at 100 °C for 15 min. The reaction mixture was centrifuged at 5000 rpm for 30 min, and the absorbance of the resulting supernatant was measured at 532 nm using a spectrophotometer (Sunrise, India). BHA was used as a positive control. All determinations were carried out in triplicate. The results are expressed as mM 1,1,3,3-tetraetoxypropane equiv/g of fresh mushroom.

Total Phenolic Content (TPC). The concentrations of phenolic compounds were determined according to the method described by Cheung et al.[12] 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 40 °C 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.

Statistical Analysis. Data are shown as the mean ± standard deviation (SD). Analysis of variance was used to determine any significant differences between groups using STATGRAPHICS Plus software (version 3.0, Statistical Graphics Corp., Princeton, NJ). Statistical significance was accepted at $p < 0.05$. Duncan's multiple range tests (DMRT) were used to determine the significant differences between groups.

RESULTS AND DISCUSSION

Identification of Compounds in EAE. Column chromatography of the EAE yielded 10 fractions. However, only the nonpolar fractions were identified and were labeled as EP1, EP2, EP3, and EP4, respectively. All components present in the fractions were identified by GC-MS analysis. Tables 1−4 show the chemical constituents in fractions EP1, EP2, EP3, and EP4, respectively; meanwhile, Table 5 shows the chemical constituents in BE.

Palmitic acid exhibited a parent ion at m/z 256 in the EI-MS spectrum that is consistent with the molecular formula $C_{16}H_{32}O_2$. The base peak at m/z 60 is a characteristic peak resulting from the McLafferty rearrangement. Besides the McLafferty rearrangement peak, the spectrum also showed loss of clusters of 14 (CH_2) mass units typical of long chain carboxylic acid.

Ergosterol was obtained as colorless needles and identified using GC-MS analysis and NMR (^1H, ^{13}C, DEPT, COSY, HMBC, and HMQC NMR) analysis. The proton NMR spectrum was consistent with the structure of ergosterol. Two singlets at δ 0.61 and 0.92 were assigned to the 18- and 19-methyl protons, respectively, while doublets at δ 0.78 (^1H, d, $J = 2.7$ Hz) and δ 0.82 (^1H, d, $J = 2.7$ Hz) were consistent with the 26- and 27-methyl protons. The 21- and 28-methyl protons were expected to resonate at lower fields (δ 0.90, 1.02) as they were in

Table 2. Chemical Constituents in EP2

chemical constituents	RT (min)	MW	MF	percentage[a](%)	method of identification
palmitic acid	10.768	256.24	$C_{16}H_{32}O_2$	19.4	MS
oleic acid	13.721	282.26	$C_{18}H_{34}O_2$	6.75	MS
ethyl linoleate	14.090	302.87	$C_{20}H_{36}O_2$	23.78	MS
ethyl oleate	14.165	310.29	$C_{20}H_{38}O_2$	5.49	MS
α-linoleic	17.862	306.26	$C_{20}H_{34}O_2$	25.84	MS

[a] Percentages were calculated based on results obtained from the total ion chromatogram. RT, retention time; MW, molecular weight; MF, molecular formula; and MS, mass spectrum.

Table 3. Chemical Constituents in EP3

chemical constituents	RT (min)	MW	MF	percentage[a](%)	method of identification
ergosterol	9.799	396.34	$C_{28}H_{44}O$	73.59	MS
lichesterol	9.876	396.34	$C_{28}H_{44}O$	10.05	MS
5,6-dihydroergosterol	9.905	398.36	$C_{28}H_{46}O$	4.14	MS
neoergosterol	10.005	380.31	$C_{27}H_{40}O$	3.58	MS
ergosta-5.,8-dien-3-ol	10.449	398.36	$C_{28}H_{46}O$	3.91	MS
7-ergostenol	10.662	400.37	$C_{28}H_{48}O$	2.10	MS

[a] Percentages were calculated based on results obtained from the total ion chromatogram. RT, retention time; MW, molecular weight; MF, molecular formula; and MS, mass spectrum.

Table 4. Chemical Constituents in EP4

chemical constituents	RT (min)	MW	MF	percentage[a](%)	method of identification
myristic acid	7.359	228.21	$C_{14}H_{28}O_2$	0.41	MS
linoleic acid	14.234	208.24	$C_{18}H_{32}O_2$	71.76	MS
ergosta-5,8(14)-dien-3-ol	32.111	398.36	$C_{28}H_{46}O$	0.76	MS
ergosta-5-en-3-ol	33.944	400.37	$C_{28}H_{48}O$	0.41	MS

[a] Percentages were calculated based on results obtained from the total ion chromatogram. RT, retention time; MW, molecular weight; MF, molecular formula; and MS, mass spectrum.

close proximity to the double bond at C-22. Both methyl protons appeared as doublets with a coupling constant of 8.1 Hz each. The two olefinic protons at δ 5.56 (^{1}H, dd, J = 2.7, 5.4 Hz) and δ 5.36 (^{1}H, m) were assigned to H-6 and H-7, respectively, while superimposed dd peaks at δ 5.15−5.32 were assigned to the olefinic protons H-22 and H-23. Multiplets centered at δ3.62 were consistent with H-3. The C-13 spectrum showed the presence of 28 carbons consistent with 10 methine carbons, seven methylene, six methyl, one olefinic methine, two quatenary carbons, and two quatenary olefinic carbons. Table 6 shows the ^{1}H and ^{13}C assignments of ergosterol by DEPT, HMQC, and HMBC. Figure 2 shows the structure of ergosterol. Even though mushrooms are deficient in vitamin D_2, previous researchers have found them to be rich source of ergosterol. Ergosterol was the major component in *P. sajor-caju* and acted as a biological precursor to vitamin D_2. It can form viosterol by ultraviolet light, irradiation temperature, and moisture,[16] which then converts to ergocalciferol, a form of vitamin D_2 used for pharmaceutical applications and food supplements. Linoleic acid is a member of the group of essential fatty acids (EFAs) called ω-6 fatty acids, so-called because they are essential dietary requirement for all mammals. EFAs are fatty acids that cannot be synthesized by humans and therefore must be obtained from the diet.[17] Linoleic acid is the raw material for a number of compounds vital for health (e.g., arachidonic acid, which is involved inflammation response). Linoleic acid produces compounds called prostaglandins, and prostaglandins are substances that regulate a wide range of functions, including blood pressure, blood clotting, blood lipid levels, the immune response, and the inflammation response to injury infection and are found in every cell for the body's health maintenance.[18]

Nicotinamide, also known as niacinamide and nicotinic acid amide, is the amide of nicotinic acid (vitamin B_3/niacin). Nicotinic acid is converted to nicotinamide, and the two are identical in their vitamin functions. In a recent study, nicotinamide was reported to play an important role in aging and development by maintaining genome stability and regenerative fitness and protection from injury.[19] Thus, nicotinamide could provide new approaches in treating some of the most common disorders of human memory: those associated with age. Hitherto, many studies have been done to evaluate the effects of cinnamic acid on health benefits. The acute toxicity experiment on white rats, white mice, and guinea pigs showed that cinnamic acid is safe for consumption and also has been proven to be effective for antimutagenecity, anticarcinogenecity, and anticytotoxicity.[20] Table 7 shows the fragmentation pattern for each compound identified in EAE and BE of *P. sajor-caju*.

Antioxidant Activity. The potential antioxidant activities of the fractions (EP1, EP2, EP3, EP4, EE, EAE, BE, and AE) from *P. sajor-caju* were evaluated in different in vitro tests such as β-carotene bleaching method, FRAP assay, TEAC assay, and LPO assay.

β-Carotene Bleaching Method. In this study, we evaluated the antioxidant activity of different extracts of *P. sajor-caju* by the β-carotene linoleate bleaching method because β-carotene

Table 5. Chemical Constituents in BE

chemical constituents	RT (min)	MW	MF	percentage[a] (%)	method of identification
4-hydroxybenzaldyhde	3.356	122.04	$C_7H_6O_2$	10.06	MS
benzeneacetamide	3.781	135.62	C_8H_9NO	20.06	MS
nicotinamide	3.719	122.05	$C_6H_6N_2O$	31.95	MS
cinnamic acid	3.919	148.17	$C_9H_8O_2$	49.02	MS

[a] Percentages were calculated based on results obtained from the total ion chromatogram. RT, retention time; MW, molecular weight; MF, molecular formula; and MS, mass spectrum.

Table 6. ^{1}H and ^{13}C NMR Assingments of Ergosterol by DEPT, HMQC, and HMBC[a]

position	δ_C	DEPT	δ_H	HMBC (H → C)
3	70.47	CH−OH	3.62, m	
4	40.81	CH_2	2.48, broad doublet	
5	141.33	C		C-19
6	119.58	CH	5.56, dd	
7	116.29	CH	5.36, m	
8	139.78	C		
9	40.37	CH	2.30, broad triplet	
10	42.84	C		
13	37.04	C	2.00, multiplet	
14	54.56	CH		
18	12.03	CH_3	0.61, s	C-12, C-14, C-17
19	16.28	CH_3	0.92, s	C-5
21	17.58	CH_3	0.90, s	C-22
22	132.00	CH	5.15−5.26, superimposed multiplet	
23	135.56	CH	5.15−5.26, superimposed multiplet	C-28
26	19.62[b]	CH_3	0.78, dd	
27	19.32[b]	CH_3	0.82, d	
28	21.08	CH_3	1.02, d	C-23

[a] ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 500 MHz), DEPT, and HMQC were performed on A Bruker Avance DPX-500 spectrometer. [b] Assignment may be interchangeable.

shows strong biological activity and is a physiologically important compound.[21] Thus, the degradation rate of β-carotene-linoleate depends on the antioxidant activity of the extracts. There was an inverse correlation between the degradation rate and the bleaching of β-carotene; the extract with the lowest β-carotene degradation rate showed the highest antioxidant activity. The AE, BE, EE, and EAE extract showed a similar activity with BHA. The antioxidant activity of the extracts in descending order was BE > AE > EE > EAE > EP1 > EP4 > EP2 > EP3, as shown in Table 8. There was a significant difference between the AE, BE, EE, and EAE and the fractions from the ethyl acetate extract ($p < 0.05$). The oxidation rate ratio exhibited an inverse relationship with antioxidant activity. It seems that AE, BE, and EE showed a comparable antioxidant activity with the fruiting bodies of *P. citrinopileatus* with antioxidant activities of 71.7−87.9% at 10−20 mg/mL. In addition, AE, BE, and EE showed higher antioxidant activities compared to fruiting bodies of *Agaricus bisporus*, *Pleurotus energii*, *Pleurotus ferulae*, and *P. ostreatus* that reported antioxidant activities of 74.2−84.0, 73.4−84.0, 64.5−79.2, and 68.5−84.6% at 10−20 mg/mL, respectively.[22]

FRAP Assay. The FRAP assay gives fast and reproducible results. Evaluation of the total antioxidant capacity of extracts of *P. sajor-caju* by FRAP assay was selected because it had not been determined previously by others using this method. FRAP values of the extracts of *P. sajor-caju* are shown in Table 9. The descending order of ferric reducing capacity per gram of fresh mushroom was AE > BE > EE > EAE > EP2 > EP3 > EP4 > EP1. There was a significant difference ($p < 0.05$) between FRAP values for AE and BE extracts. Among the fractions from the ethyl acetate extracts, EP2 displayed the highest total antioxidant capacity, which was 3.44 ± 0.79 μmol of $FeSO_4 \cdot 7H_2O$ equiv/g of fresh mushroom. When comparing the FRAP values of extracts of *P. sajor-caju* with fresh vegetables and fruits,[23] FRAP values of AE and BE were higher than chili pepper (23.54 μmol of $FeSO_4 \cdot 7H_2O$ equiv/g of fresh weight), red bell pepper (20.98, $p < 0.05$ of $FeSO_4 \cdot 7H_2O$ equiv/g of fresh weight), orange (20.50 μmol of $FeSO_4 \cdot 7H_2O$ eguiv/g of fresh weight), and cultivated strawberry (22.74 μmol of $FeSO_4 \cdot 7H_2O$ equiv/g of fresh weight). However, the water extract of *P. ostreatus* (43.80 μmol of $FeSO_4 \cdot 7H_2O$ equiv/L) showed better reducing activity than *P. sajor-caju*. Besides that, it also reported that oyster mushrooms exhibited better antioxidative reducing power ability than shiitake and golden mushrooms.[24]

TEAC Assay. TEAC was determined using the method described by Re et al.[14] Table 9 illustrates TEAC values (mM Trolox equiv/g of fresh mushroom) of different extracts of *P. sajor-caju*. AE, BE, EE, EAE, EP1, EP2, EP3, and EP4 were tested for TEAC values using the described TEAC assay. The TEAC

Table 7. Mass Fragmentation of Identified Compounds in EAE and BE

compounds	mass fragmentation
nicotinamide	122 (M^+, 100), 106 ($[M - O]^+$, 80), 78 ($[M - O - C_2H_4]^+$, 90), 51
myristate acid	228 (M^+, 15), 199 ($[M - CHO]^+$, 7), 185 ($[M - CHO - CH_2]^+$, 25), 171($[M - CHO - (CH_2)_2]^+$, 20), 157 ($[M - CHO - (CH_2)_3]^+$, 20), 143 ($[M - CHO - (CH_2)_4]^+$, 20), 129 ($[M - CHO - (CH_2)_5]^+$, 50), 73(100), 60
ethyl myristate	256 (M^+,20), 227 ($[M - C_2H_5]^+$, 5), 213,199 ($[M - C_2H_5 - C_2H_4]^+$, 10), 157 ($[M - C_2H_5 - C_2H_4 - O]^+$, 20) 101 ($[M - C_2H_5 - C_2H_4 - O - C_4H_8]^+$, 50), 88 (100)
palmitic acid	256 ($[M]^+$,12), 227 ($[M - CHO]^+$, 2), 213 ($[M - CHO - CH_2]^+$, 10), 199 ($[M - CHO - (CH_2)_2]^+$, 4), 185 ($[M - CHO - (CH_2)_3]^+$, 5), 171 ($[M - CHO - (CH_2)_4]^+$, 5), 157 ($[M - CHO - (CH_2)_5]^+$, 7), 143 ($[M - CHO - (CH_2)_6]^+$, 3), 129 ($[M - CHO - (CH_2)_7]^+$, 50), 73 ($[(CH_2)_2COOH]^+$, 100), 60 ($C_2H_4O_2^+$, 100)
methyl palmitate	270 (M^+, 3), 239 ($M^+ - OCH_3$, 2, $C_{16}H_{31}O_1^+$), 87 ($[M - C_4H_7O_2]^+$, 70), 74 ($[M - C_3H_6O_2]^+$, 100), 55
ethyl palmitate	284 (M^+, 3), 241($[M - C_3H_7]^+$, 7), 157 ($[M - C_3H_7 - C_3H_7COCH_2 + H]^+$, 25), 101 ($[M - C_3H_7 - C_3H_7COCH_2 + H - C_4H_8]^+$, 50), 88 (100)
linoleic acid	280 ($[M]^+$, 6), 195, 182, 123, 109, 95, 81, 67 (100), 55
oleic acid	282 (M^+, 3), 264 ($[M - H_2O]^+$, 8), 235 ($[M - H_2O - C_2H_5]^+$, 3), 193 ($[M - H_2O - C_2H_5 - C_3H_6]^+$, 3), 55 (100)
methyl linoleate	294 (M^+, 3), 263 ($[M - CH_3]^+$, 5), 67 (100), 55
methyl stearate	298 (M^+, 5), 267 ($[M - OCH_3]^+$, 15), 227 ($[M - C_3H_7]^+$, 3), 199 ($[M - C_3H_7 - C_2H_4]^+$, 10), 143 ($[M - C_3H_7 - C_2H_4 - C_4H_8]^+$, 30), 87 ($[M - C_3H_7 - C_2H_4 - C_4H_8 - C_4H_8]^+$, 80), 74 (100)
ethyl linoleate	308 (M^+, 5), 284 ($[M - CH_2CH_2OH]^+$, 8), 220 ($[M - CH_2CH_2OH - C_3H_7]^+$, 5), 178 ($[M - CH_2CH_2OH - C_3H_7 - C_3H_6]^+$, 8), 150 ($[M - CH_2CH_2OH - C_3H_7 - C_3H_6 - (CH_2)_2]^+$, 10), 67 (100), 55
ethyl octadecanoate	312 (M^+, 5), 283 ($[M - C_2H_5]^+$, 3), 269 ($[M - C_2H_5 - CH_2]^+$, 10), 157 ($[M - C_2H_5 - (CH_2)_8]^+$, 30), 101 ($[M - C_2H_5 - (CH_2)_8 - C_4H_8])^+$, 50), 88 (100)
ethyl oleate	310 (M^+, 10), 55 (100)
α-linoleic	306 (M^+, 15), 279, 261 ($[M - CH_2CH_2OH]^+$, 5), 99 (100), 79, 55
ergosterol	396 (M^+, 35), 378 ($[M - H_2O]^+$, 10), 363 ($[M - H_2O - CH_3]^+$, 44)
neoergosterol	380 (M^+, 20), 362 ($[M - H_2O]^+$, 10), 319 ($[M - H_2O - C_3H_7]^+$, 8), 282, 267, 253, 237 (100)
5,6-dihydroergosterol	398 (M^+, 15), 383 ($[M - CH_3]^+$, 10), 355 ($[M - CH_3 - C_2H_4]^+$, 10), 313 ($[M - CH_3 - C_2H_4 - C_3H_6]^+$, 10), 55 (100)
ergosta-5,8-dien-3-ol	398 (M^+, 40), 380 ($[M - H_2O]^+$, 10), 365 ($[M - H_2O - CH_3]^+$, 100)
ergosta-5,8(14)-dien-3-ol	398 (M^+, 15), 383 ($[M - CH_3]^+$, 10), 55 (100)
ergost-7-en-3-ol	400 (M^+, 80)
ergost-5-en-3-ol	400 (M^+, 80), 385, 367, 339, 255

values of extracts varied with different concentrations. AE exhibited higher TEAC values followed by BE as compared to the other extracts. The TEAC levels of the extracts in descending order was: AE > BE > EE > EAE > EP4 > EP2 > EP3 > EP1. The TEAC value of AE and BE were 29.45 ± 0.87 mM Trolox equiv/g of fresh mushroom and 25.67 ± 2.70 mM Trolox equiv/g of fresh mushroom. There was a strong significant difference ($p < 0.05$) between these extracts. However, there were no significant difference observed ($p > 0.05$) between the fractions from ethyl acetate extract. The TEAC value for ascorbic acid was 74.48 ± 0.14 mM Trolox equiv/g of fresh mushroom. The antioxidant capacity of *P. ostreatus* was reported as 3.6−4.4 mM.[25] This seems *P. sajor-caju* showed better antioxidant capacity than *P. ostreatus*. In addition, AE and BE also exhibited better antioxidant capacity than black wines (ranging from 15.12 to 11.92 mM Trolox equiv/g), white wine (ranging from 2.48 to 1.71 mM Trolox equiv/g), vodka (0.02 mM Trolox equiv/g), and brandy (0.16 mM Trolox equiv/g).[26]

LPO Assay. The study of LPO processes has become a rapidly growing field in medicine and biology, based on increasing evidence that LPO is involved in the development of many chronic diseases. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) upon decomposition. The measurement of MDA has been used as an indicator of LPO. Table 9 illustrates LPO values (mM TEPs equiv/g of fresh mushroom) of different extracts of *P. sajor-caju*. AE exhibited lower values than the other extracts. The inhibition of LPO levels of the extracts in descending order was: AE > BE > EE > EAE > EP3 > EP2 > EP1 > EP4. The LPO values for the subfractions of EAE were significantly higher because the composition of these extracts is mainly unsaturated fatty acids; thus, these fatty acids generate MDA upon decomposition. Besides that, these fractions were not assayed immediately upon isolation; thus, these unsaturated fatty

Table 8. Antioxidant Activities of Different Extracts of *P. sajor-caju* by β-Carotene-Linoleate Bleaching Method[a]

extracts	A_A[b]	R_{OR}[c]
AE	91.02 c	0.09 ± 0.00 a
BE	95.39 c	0.07 ± 0.00 a
EE	89.53 c	0.10 ± 0.00 a
EAE	87.30 c	0.13 ± 0.00 a
EP1	46.86 b	0.53 ± 0.00 b
EP2	25.74 a	0.78 ± 0.00 c
EP3	24.13 a	0.94 ± 0.00 d
EP4	41.19 b	0.59 ± 0.00 b

[a] The concentration of the sample was 16 mg/mL. Values are expressed as means ± standard deviations ($n = 3$). Means with different letters (a−d) were significantly different at the level $p < 0.05$. AE is aqueous extract, BE is buthanol extract, EE is ethanol aqueous extract, EAE is ethyl acetate extract, and EP1−EP4 are the isolated fractions of the ethyl acetate extract. [b] Antioxidant activity index. [c] Oxidation rate ratio.

Table 9. FRAP, TEAC, LPO Activity and Total Phenol Content of *P. sajor-caju* Extracts[a]

extracts	FRAP (μmol of $FeSO_4 \cdot 7H_2O$ equiv/g)	TEAC (μM Trolox equiv/g)	LPO (μM TEPs equiv/g)	TPC (mg GAEs equiv/g)
AE	35.06 ± 0.86 d	29.45 ± 3.07 d	0.16 ± 0.01 a	10.48 ± 1.37 de
BE	30.23 ± 3.36 cd	25.67 ± 2.70 cd	0.35 ± 0.01 a	15.23 ± 1.31 e
EE	26.29 ± 2.82 c	16.55 ± 3.09 bc	1.35 ± 0.01 ab	9.48 ± 0.85 cd
EAE	10.26 ± 2.33 b	11.19 ± 1.22 ab	1.7 ± 0.11 ab	2.20 ± 0.43 a
EP1	1.01 ± 0.31 a	4.59 ± 1.54 a	5.63 ± 0.04 de	5.63 ± 1.39 bc
EP2	3.44 ± 0.79 a	13.61 ± 1.70 ab	5.11 ± 0.11 cd	6.14 ± 0.66 bc
EP3	2.87 ± 0.81 a	8.49 ± 3.02 ab	2.89 ± 0.06 bc	2.52 ± 0.33 ab
EP4	1.07 ± 0.58 a	13.96 ± 0.32 ab	7.78 ± 0.16 e	4.29 ± 0.54 ab

[a] The concentration of the sample was 16 mg/mL. Values expressed are means ± SDs of triplicate measurements for each sample. Different letters (a−e) within rows indicate significant differences between samples ($p < 0.05$). AE is aqueous extract, BE is buthanol extract, EE is ethanol aqueous extract, EAE is ethyl acetate extract, EP1−EP4 are subfractions of the ethyl acetate extract, and TEPs is 1,1,3,3-tetraetoxypropane.

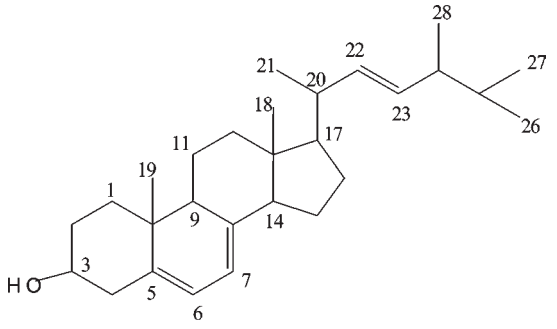

Figure 2. Structure of ergosterol.

acids will decompose naturally. The LPO value was high in EP4 as compared to other subfractions; this could this be due to the high content of linoleic acid, which is a polyunsaturated fatty acid. The LPO value for BHA was 1.04 ± 0.04 mM TEP equiv/g of fresh mushroom. *P. ostreatus* also exhibited in vitro inhibition of LPO in a dose-dependent manner against rat liver homogenate, the peroxidation of which was triggered by Fe(II)−ascorbate, and TBARS was measured. The percentage of inhibition of *P. ostreatus* was 56.20% at 10 mg/mL.[27]

TPC. A sufficiently wide range of interest has been given to phenolics or polyphenols because of their physiological function, including antioxidant, antimutagenic, and antitumor activities.[23] An investigation was thus conducted to determine the TPC of *P. sajor-caju*. The total phenolic (TPC) concentration in BE and AE was significantly stronger than the EE, EAE, EP1, EP2, EP3, and EP4 ($p < 0.05$). The phenolic contents for BE and AE were 15.23 ± 1.31 mg GAEs/g of fresh mushroom and 10.48 ± 1.37 mg GAEs/g of fresh mushroom, respectively (Table 9). To elucidate the relationship between the phenolic content and the antioxidant activity, a correlation analysis between TPC and antioxidant activity determined by different assays was done. The TPC was shown to provide the highest association with FRAP levels in the present study (R^2 = 0.7354). This result was also in agreement with Kubola et al.,[21] who found a strong positive correlation between TPC and FRAP levels. Similar results were also found for TEAC assay (R^2 = 0.7205). However, there was no significant correlation between TPC and β-carotene-linoleate bleaching and LPO levels. Figure 3a,b shows the correlation between TPC and FRAP levels and between TPC and TEAC assays, respectively. The phenolic contents of *P. sajor-caju* extracts are comparable to commonly consumed vegetables such as lettuce, celery, and cucumber.[28] Furthermore, it was reported that the antioxidant

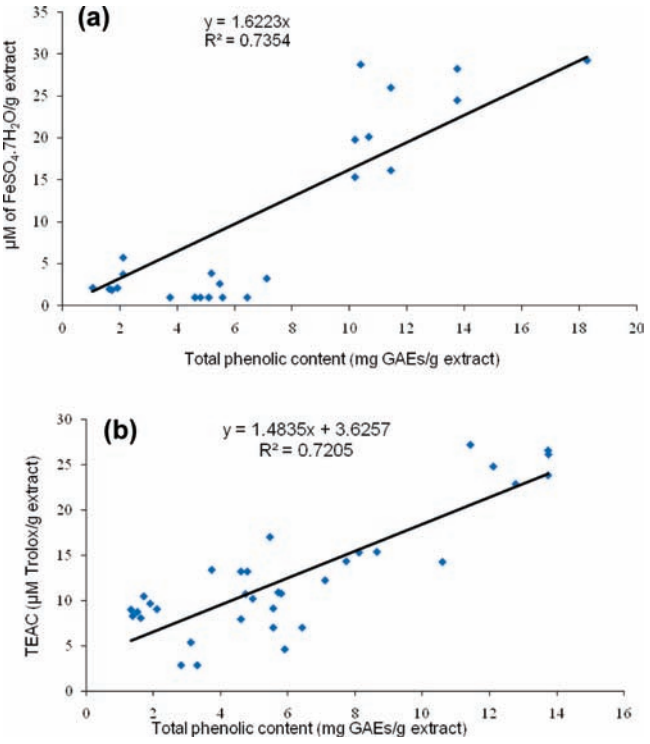

Figure 3. (a) Correlation between FRAP assay and TPC. (b) Correlation between TEAC assay and TPC.

activity by phenolics in *Ganoderma lucidum* was mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and single oxygen quenchers.[29] Flavonoids, which are also phenolic compounds with antioxidant activity,[5] were not detected in *P. sajor-caju*. Our observation agrees with findings of ref 30, and according to U.S. Department of Agriculture, mushrooms are regarded as nonsources of flavonoids. The absence of flavonoids in mushrooms may be of biological advantage in their various ecological niches since these bioactive compounds inhibit enzyme activities involved in their pigmentation, growth, and development. The antioxidant activity is not limited to phenolic compounds; hence, the possibility that some other components could contribute to the antioxidant properties should not be ruled out. Extensive studies have been done on the isolation and identification of compounds in the aqueous extract of *P. sajor-caju*. Studies showed that aqueous extract consists of polysaccharides such as soluble glucan with

(1 → 2,6), (1 → 6), (1 → 3) and nonreducing end D-glucosyl moieties and hetero polysaccharides composed of D-mannose, D-glucose, and D-galactose. The molecular weights for the polysaccharides are 2.4×10^5 and 3.5×10^4, respectively.[8,9]

The compounds present in extracts of *P. sajor-caju* are mainly low molecular weight such as triterpenoids and fatty acids. Ergosterol was found to be the major component followed by linoleic acid, thus making *P. sajor-caju* a good source of these two components. Ergosterol is the precursor of ergocalciferol, which is extensively used as dietary calcium supplement in people suffering from hypocalcinaemia and osteoporosis. Linoleic acid is important for bodily growth; maintainance of health through the production of prostaglandins; and regulation of blood pressure, blood lipid, immune response, inflammation, and apoptosis. In conclusion, the compounds linoleic acid, cinnamic acid, and nicotinamide identified in *P. sajor-caju* were shown to have antioxidant activities. All extracts obtained with different extraction solvents had varying degrees of antioxidant activity. This shows that antioxidant activity in mushrooms may not only be due to their phenolic contents. Thus, in formulation and preparation of functional food or nutraceuticals, this has to be borne in mind. The aqueous extract of *P. sajor-caju* showed a potent antioxidant activity (β-carotene bleaching assay, 91.02%; reducing capacity, 35.06 μmol $FeSO_4 \cdot 7H_2O$/g of fresh mushroom; and antioxidant capacity, 29.45 mM Trolox/g of fresh mushroom) and inhibition of LPO (0.16 mM TEP/g of fresh mushroom). Thus, the regular consumption of fresh fruiting bodies *P. sajor-caju* in human diets may be beneficial for health purposes and may have chemopreventive properties of selected disease of humankind.

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Funding Sources

This work was supported by PS139/2008B grant and Mushroom Research Grant (66-02-03-0074) funded by University of Malaya.

ACKNOWLEDGMENT

This work was supported by PS 139/200B grant, University Malaya Research Grant (RG083-09AFR) and Mushroom Research Grant (66-02-03-0074) funded by University of Malaya.

ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid; BHA, butylated hydroxyanisole; ^{13}C, carbon-13; COSY, correlation spectroscopy; $CDCl_3$, deuterium chloroform; DEPT, distortionless enhancement by polarization transfer; d, doublet; dd, doublet doublet; GC-MS, gas chromatography−mass spectrum; ^1H, proton-1; HMBC, heteronuclear multiple bond coherence; HMQC, heteronuclear multiple quantum coherence; m, multiplet; NMR, nuclear magnetic resonance; Trolox, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid

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