ISOLATON AND IDENTIFICATION OF BIOACTIVE COMPOUNDS FROM FRESH FRUITING BODIES OF *PLEUROTUS SAJOR-CAJU*.

3.1 Introduction

A wide range of bioactive compounds have been isolated from various medicinal plants and mushrooms, and these compounds have shown to possess promising antitumor, immune modulating, cardiovascular, hypercholesterolemic, antiviral, antibacterial, and antiparasitic activity through their ability to modulate macrophage function (Xie et al., 2008; Wasser & Weiss, 1999b). Moreover, most plant or mushroom derived bioactive compounds are non-toxic and do not cause severe side effects, which are a major problem in treating various diseases (Shu, 1998). Thus plant and mushroom derived bioactive compounds represent ideal candidates for therapeutics treatments.

The systematic drug development programs from natural sources are based on the bioassay-guided isolation of natural products, taking into consideration the folklore uses (ethnopharmacological applications) of local plants. A number of bioassays have been developed recently to direct the isolation work. The most common method used in the isolation of bioactive compounds from natural products is column chromatography. Column chromatography is based on adsorption chromatography. It uses a mobile phase (e.g. solvents) to move a mixture of substances through stationary phase (e.g. silica gel beads). The different compounds of the sample have different affinities for the mobile and stationary phase, and appear from the stationary phase at different times. The stationary phase and mobile phases are chosen based on the nature of the sample mixture in order to achieve the best possible separations of compounds. Column chromatography is advantageous over most other chromatographic techniques because it can be used in both analytical and preparative applications. Besides that, not only can column chromatography be used to determine the number of components of a mixture, but it can also be used to separate and purify substantial quantities of those components for subsequent analysis (Harborne, 1973).

The aims of this part of study were to isolate and identify the secondary metabolites and polysaccharides in the fruiting bodies of *P. sajor-caju* by extracting and fractionating the ethanol aqueous and hot aqueous extracts using column chromatography and precipitation methods. Finally, the isolation and identification of compounds from the fractionated extracts were carried out using various spectroscopic techniques.

3.2 Materials and Methods

3.2.1 Materials

Agilent Technologies, Germany

- Gas Chromatography 6890 N equipped with a 5979 Mass Spectrum detector.
- HP-5ms capillary column (5% Phenyl-methylpolysiloxane) capillary column (30.0 m × 250 μm ID × 0.25 μm film thickness)
- Screw cap vials (1.5 ml) and blue screw caps

Beckman Coulter Centrifuges,

Beckman centrifuge, J2-MI

Bruker BioSpin, United States of America

• Nuclear Magnetic Resonans (NMR), Bruker Avance DPX-500

Butchi, Switzerland

- Chiller B-741
- Rotavapor R-114, Waterbath B-480
- Vacuum System V-700

Christ, United Kingdom

Freeze-dryer

Merck, Germany

- Acetone, (CH₃)₂CO
- Butanol, C₄H₉OH
- Ethanol, CH₃CH₂OH
- Ethyl acetate, CH₃COOCH₂CH₃
- Hexane, C_6H_{14}
- Methanol, CH₃OH
- Thin Layer Chromatography plates 60 F₂₅₄ (20.25 mm thickness)

Sigma-Aldrich, United States of America

- DEAE cellulose dialysing tube
- Silica gel beads (size: 0.063 0.200nm, mesh: 70 230)

3.2.2 Methodology

3.2.2.1 Mushrooms samples

a) Fruiting body collection

Fresh fruiting bodies of *Pleurotus sajor –caju* (10 kg) was collected from a mushroom farm in Semenyih, Selangor.

b) Extraction and fractionation of fruiting bodies of *P. sajor-caju*

The fresh fruiting bodies were washed, sliced and freeze-dried for 2 days. The freeze-dried mushrooms were ground to a fine powder using a blender. The dried, ground sample was then soaked in 20% water in ethanol (ethanol:water = 4.1) (1.5 L) for 3 days at room temperature. The solvent-containing extract was then decanted and filtered. The extraction of the ground sample was further repeated ($3\times$) with 20% water in ethanol (1.5 L each time). The filtrate from each extraction was combined and the excess solvent was evaporated under reduced pressure using a rotary evaporator. The ethanol extract was further partitioned between ethyl acetate and water (1:1) to give an ethyl acetate-soluble fraction and aqueous-soluble fraction. The aqueous layer was freeze-dried and was further extracted with *n*-butanol. 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; initial elution with hexane followed by stepwise addition of hexane enriched with increasing percentages of acetone. The extraction and fractionation of the fresh fruiting bodies of *P. sajor-caju* is shown in Figure 3.1.





3.2.2.2 Isolation and identification of compounds from ethyl acetate extract of *P*. sajor-caju

a) Sample preparation

Ethyl acetate extract (5.0 g) was mixed with silica gel beads. The extract mixture was further dried in oven until the mixture became powder.

b) Thin layer chromatography

Thin layer chromatography (TLC) was carried out using pre-coated TLC plates 60 F_{254} (20.25 mm thickness) and were visualized in UV light (254 and/or 365 nm) and /or iodine vapor

c) Column chromatography

Silica gel (60.0 g) was mixed with hexane and left overnight at room temperature. The resultant silica slurry was loaded onto a column (height 100 cm; diameter 10 cm). The solvent was allowed to run until it just covered the surface of the silica slurry. The sample mixture was added slowly onto the surface of the silica. Figure 3.2 shows the setup of column chromatography. Elution began with 100% hexane and polarity of eluting solvent was gradually increased using acetone. Fractions of 25 ml volume were collected in numbered vials. The separations were monitored using thin layer chromatography. The fractions were pooled according to similarities of spots on thin layered chromatography. The excess solvent in the pooled fractions was evaporated under reduced pressure using a rotary evaporator. Components in the isolated fractions were then identified using GC-MS and NMR.



Figure 3.2: A column chromatography setup

3.2.2.3 Instrumentation

a) GC – MS analysis

The GC - MS analysis was performed on a Agilent Technologies 6890 N gas chromatography equipped with a 5979 Mass Selective Detector (70 eV direct inlet); a HP-5 ms (5% Phenyl-methylpolysiloxane) capillary column (30.0 m \times 250 µm ID \times 0.25 µm film thickness) initially set at 150°C, then programmed to 280°C at 5°C per min using helium as carrier gas at a flow rate of 1 ml/min was used. The total ion chromatogram obtained was auto-integrated by Chemstation and the constituents were identified by comparison with published mass spectra data and those from the accompanying library NIST Lib, 2005.

b) Nuclear magnetic resonance (NMR)

NMR spectra were recorded on a Bruker Avance DPX-500 spectrometer for ¹H, ¹³C, COSY, DEPT, HMBC and HMQC NMR. Internal standard used in ¹H NMR spectra was TMS (δ : 0.00) for CDCl₃; in ¹³C NMR was CDCl₃ (δ : 77.0).

3.2.2.4 Isolation and purification of crude polysaccharide from hot-aqueous extract of *P. sajor-caju*

The isolation and purification of polysaccharide was done based on the method described by Roy et al., (2008). Fruiting bodies of *P. sajor-caju* was collected and washed with water. It was crushed and boiled in 500 ml of distilled water for 8 hr. The whole mixture was kept overnight at 4°C and then filtered through linen cloth. The filtrate was centrifuged at 19570 × g for 45 min at 4°C. The supernatant was collected and precipitated in ethanol (1:5. v/v). It was kept overnight at 4°C and again centrifuged at 19570 × g for 45 min. The precipitated material (polysaccharide) was washed with ethanol four times and then freeze-dried. The freeze-dried material was dissolved in 30 ml of distilled water and dialyzed through dialysis tubing cellulose membrane against distilled water for 4 hr as shown in Figure 3.3. This is done to remove low molecular weight materials. The aqueous solution was then collected from the dialysis bag and freeze-dried to yield crude polysaccharide.



Figure 3.3: (a) Dialysis of polysaccharide using a DEAE cellulose dialysing tube against distilled water for 4 hr (b) DEAE cellulose dialysing tube

3.3 Results and Discussion

3.3.1 Extraction and fractionation from fruiting bodies of *P. sajor-caju*

The fresh fruiting bodies of P. sajor-caju (10 kg) (Figure 3.4) were obtained from Mr. Kuan Juan's mushroom farm in Semenyih (Location – 3°21'19.20"N 101°91 14'36.35"E), Selangor Darul Ehsan, Malaysia. The fruiting bodies were washed, sliced and freeze-dried for 2 days. The freeze-dried mushrooms were ground to a fine powder using a 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 three days at room temperature. The solventcontaining extract was then decanted and filtered. The extraction process was repeated three times and 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 aqueous ethanol extract (EE) was further partitioned between a mixture of ethyl acetate and water (1:1) to give an ethyl acetate-soluble fraction 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 and concentrated under reduced pressure to yield a dark brown viscous extract (BE). Evaporation of solvent from the ethyl acetate layer gave an ethyl acetate extract (EAE) which was further subjected to column chromatography using acetone:hexane as the eluting solvent. Figure 3.5 shows the yield of all P. sajor-caju extracts obtained after extraction and fractionation of fresh fruiting bodies.



Figure 3.4: Fresh fruiting bodies of *Pleurotus sajor-caju* grown on artificial sawdust logs



Figure 3.5: Yield of each *P. sajor-caju* extracts obtained after extracting and fractionating the fresh fruiting bodies.

3.3.2 Isolation of secondary metabolites of *P. sajor-caju* from EAE.

The powdered mixture of EAE and silica was subjected to column chromatography initially eluting with 100 % of hexane followed by stepwise addition of hexane enriched with increasing percentages of acetone. Fractions of 25 ml volume were collected in numbered vials. The eluted compounds were monitored using TLC. A total of 137 fractions were collected from column chromatography. 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 GC-MS and NMR techniques.

However only the non polar fractions were identified and were labeled as **EP2**, **EP3**, **EP4** and **EP5** respectively. Table 3.1 shows the characteristics of the isolated factions.

No.	Weight	Vials	Appearance
	(g)		
EP2	0.281	5-7	Yellowish oil
EP3	0.657	8-16	Yellowish oil
EP4	0.817	17-25	Colorless needle- like structure
EP5	0.801	26-35	Colorless needle- like structure

 Table 3.1: Combination of vials and their physical properties

3.3.3 Identification of compounds in EP2, EP3, EP4 and EP5 using GC-MS analysis.

The compounds in EP2, EP3, EP4 and EP5 are identified by GCMS as shown in Table 3.2, 3.3, 3.4 and 3.5. From the total ion chromatogram, twenty-three compounds were identified from the fractions EP2, EP3, EP4 and EP5. They were three methyl esters namely, methyl hexadecanoate (methyl palmitate), methyl octadecanoate (methyl stearate) and methyl 9,12-octadecadienoate (methyl linoleate). They also include five fatty acids namely, palmitic acid, oleic acid, myristic acid, pentadecanoic acid and linoleic acid. Besides that, a phenolic compound identified as 2,4-ditertbutylphenol was also present in *P. sajor-caju* as well as six ethyl esters, namely tetradecanoic, ethyl ester (ethyl myristate), ethyl hexadecanoate (ethyl palmitate), ethyl linoleate, ethyl octadecanoate (ethyl stearate), ethyl 9,12,15-octadecatrienoic acid (ethyl linoleate) and ethyl oleate. In addition to that, eight sterols namely, ergosta-5,8,22-trien-3-ol (lichesterol), 5-dihydroergosterol, neoergosterol, ergosta-5,8-dien-3-ol, 7-ergostenol, ergosta-5,18(14)-dien-2-ol, ergosta-5-en-3-ol were also identified in *P. sajor-caju*. Figure 3.6 -3.28 shows the fragmentation patterns of each identified compounds in EAE.

Ergosterol (709 mg) crytallised from the ethyl acetate-soluble fraction as colourless needles and was identified by GC-MS and NMR spectral data (Figure 3.29). (Appendix A; pg 274-279)

Compound Name	Retention Time (minutes)	Percentage (%)	Molecular Weight	Molecular Formula
2,4-Ditertbutylphenol	4.169	0.14	206.17	C ₁₄ H ₂₂ O
Ethyl tetradecanoate (ethyl myristate)	7.897	0.21	256.24	$C_{16}H_{32}O_2$
Methyl hexadecanoate (methyl palmitate)	10.099	3.83	270.26	$C_{17}H_{34}O_2$
Ethyl hexadecanoate (ethyl palmitate)	11.350	16.33	284.27	$C_{18}H_{36}O_2$
9,12-Octadecadienoic acid, methyl ester (methyl linoleate)	13.052	14.83	294.26	$C_{19}H_{34}O_2$
Methyl octadecanoate (methyl stearate)	13.496	1.02	298.29	$C_{19}H_{38}O_2$
Linoleic acid ethyl ester (Ethyl linoleate)	14.421	53.00	308.27	$C_{20}H_{36}O_2$
Ethyl octadecanoate (ethyl stearate)	14.715	4.94	312.30	$C_{20}H_{40}O_2$
Total amount : 94.3%				

Table 3.2: Compounds in EP2 identified using GC-MS

2,4-Ditertbutylphenol was assigned the molecular formula $C_{14}H_{22}O$ consistent with the mass spectral data m/z (%): 206 (M⁺, 16), 191 ([M- CH₃]⁺,100).



Figure 3.6: Mass spectrum of 2,4-ditertbutylphenol

In the mass spectrum of 2,4-ditertbutylphenol, the (M^+ -CH₃) fragment ion corresponds to the base peak (m/z 191). The spectrum showed that a methyl group is lost much more readily than an α hydrogen.

Ethyl myristate was assigned the molecular formula $C_{16}H_{32}O_2$ consistent with the mass spectral data m/z (%): 256 (M⁺,20), 227 ([M-C₂H₅]⁺, 5), 213, 199 ([M-C₂H₅- C₂H₄]⁺, 10), 185, 171, 157 ([M-C₂H₅- C₂H₄ -O]⁺,20), 143, 129, 115, 101 ([M-C₂H₅- C₂H₄ –O-C₄H₈]⁺,50), 88 (100).



Figure 3.7: Mass spectrum for ethyl myristate

Methyl palmitate was assigned the molecular formula $C_{17}H_{34}O_2$ consistent with the mass spectral data m/z (%):270 (M⁺, 3), 239 (M⁺- OCH₃, 2, $C_{16}H_{31}O_1^+$), 227, 213, 199, 185, 171, 157, 143, 129, 87 ([M- $C_4H_7O_2$]⁺, 70), 74 ([M- $C_3H_6O_2$]⁺, 100), 55.



Figure 3.8: Mass spectrum of methyl palmitate

Ethyl palmitate was assigned the molecular formula $C_{18}H_{36}O_2$ consistent with the mass spectral data m/z (%): 284 (M⁺, 3), 241([M- C₃H₇]⁺, 7), 227, 213, 199, 185, 171, 157 ([M- C₃H₇ - C₃H₇COCH₂+H]⁺, 25), 143, 129, 115, 101 ([M- C₃H₇ - C₃H₇COCH₂+H -C₄H₈]⁺, 50), 88(100).



Figure 3.9: Mass spectrum of ethyl palmitate

Methyl linoleate was assigned the molecular formula $C_{19}H_{34}O_2$ consistent with the mass spectral data m/z (%): 294 (M⁺, 3), 263 ([M- CH₃]⁺, 5), 220, 192, 178, 164, 150, 135, 123, 109, 95, 81, 67 (100), 55.



Figure 3.10: Mass spectrum of methyl linoleate

Methyl stearate was assigned the molecular formula $C_{20}H_{40}O_2$ consistent with the mass spectral data m/z (%): 298 (M⁺, 5), 267 ([M-OCH₃]⁺, 15), 255, 241, 227 ([M-C₃H₇]⁺, 3),

213, 199 ([M- C₃H₇- C₂H₄]⁺, 10), 185, 171, 157, 143 ([M- C₃H₇- C₂H₄ –C₄H₈]⁺, 30), 129, 111, 97, 87 ([M- C₃H₇- C₂H₄ –C₄H₈ - C₄H₈]⁺, 80), 74(100).



Figure 3.11: Mass spectrum of methyl stearate

Ethyl linoleate was assigned the molecular formula $C_{20}H_{36}O_2$ consistent with the mass spectral data m/z (%): 308 (M⁺, 5), 284, 263 ([M- CH₂CH₂OH]⁺, 8), 220 ([M-CH₂CH₂OH- C₃H₇]⁺, 5), 178 ([M- CH₂CH₂OH- C₃H₇- C₃H₆]⁺, 8), 164, 150 ([M-CH₂CH₂OH- C₃H₇- C₃H₆- (CH₂)₂]⁺, 10), 123, 109, 95, 81, 67(100), 55.



Figure 3.12: Mass spectrum of ethyl linoleate

Ethyl stearate was assigned the molecular formula $C_{20}H_{40}O_2$ consistent with the mass spectral data m/z (%): 312 (M⁺, 5), 283 ([M- C_2H_5]⁺, 3), 269 ([M- C_2H_5 - CH_2]⁺, 10),

255,241, 227, 213, 199, 185, 171, 157 ([M- C_2H_5 - (CH₂)₈]⁺, 30), 143, 129, 115, 101 ([M- C_2H_5 - (CH₂)₈- C_4H_8])⁺, 50), 88(100).



Figure 3.13: Mass spectrum of ethyl stearate

The molecular ion peak of a methyl ester of saturated fatty acid is usually distinct and prominent. All the methyl esters identified in the present study displayed the molecular ion peak. Mass spectrum of all the methyl esters identified in the present study, showed peak M⁺-OCH₃ corresponding to the fragment ion R—C===O⁺. The ion R—C===O⁺ gives an easily recognizable peak for esters and occurs at M⁺- OCH₃ in methyl esters (Silverstein *et al.*, 1998). For methyl palmitate, linoleate, and methyl stearate, the molecular ion peak is weak in the range m/z 130 to ~200, but becomes somewhat more intense beyond this range. For saturated fatty ester, the most characteristic peak results from McLafferty rearrangement and cleavage one bond removed from the C=O group. Thus, methyl palmitate, methyl linoleate and methyl stearate unbranched at the α carbon gives a strong base peak at m/z 74 which is formally represented by the ion C₃H₆O₂⁺, results from McLafferty rearrangement (Silverstein et al., 1998). McLafferty rearrangement of methyl palmitate is showed in Figure 3.14



m/z = 74, (C₃H₆O₂⁺) base peak of methyl palmitate and all other saturated methyl fatty esters

Figure 3.14: McLafferty rearrangement of methyl palmitate.

Mass spectrum of methyl palmitate, methyl linoleate and methyl stearate showed peak (m/z 74) which is formally represented by the ion $[CH_3CH_2CO_2CH_3]^+$ is always more intense than its homologs. The molecular ion peak of a straight- chain, saturated hydrocarbon is always present, though of low intensity for long-chain compounds.

Compounds Name	Retention Time (minutes)	Percentage (%)	Molecular Weight	Molecular Formula
2,4-Ditertbutylphenol	4.163	2.29	206.17	$C_{14}H_{22}O$
<i>n</i> -Hexadecanoic acid (palmitic acid)	10.768	19.40	256.24	$C_{16}H_{32}O_2$
Ethyl hexadecanoate (ethyl palmitate)	11.212	4.66	284.27	$C_{18}H_{36}O_2$
9- Octadecenoic acid (oleic acid)	13.721	6.75	282.26	$C_{18}H_{34}O_2$
9,12-Octadecadienoic acid, methyl ester (methyl linoleate)	12.926	3.32	294.26	$C_{19}H_{34}O_2$
Linoleic acid, ethyl ester (ethyl linoleate)	14.090	23.78	308.27	$C_{20}H_{36}O_2$
Ethyl oleate	14.165	5.49	310.29	$C_{20}H_{38}O_2$
9,12,15-Octadecatrienoic acid, ethyl ester (ethyl linolenate)	17.862	25.84	306.26	$C_{20}H_{34}O_2$
Total amount : 99.54%				

Table 3.3: Compounds in EP3 identified using G	C-№	ЛS
--	-----	----

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 mass spectral data is as follows m/z (%): 256 ([M]⁺,12), 227([M-CHO]⁺,2), 213 ([M-CHO-CH₂]⁺,10),199 ([M-CHO-(CH₂)₂]⁺,4), 185 ([M-CHO-(CH₂)₃]⁺,5), 171 ([M-CHO-(CH₂)₄]⁺,5), 157 ([M-CHO-(CH₂)₅]⁺,7), 143 ([M-CHO-(CH₂)₆]⁺,3), 129 ([M-CHO-(CH₂)₇]⁺,50), 73 ([(CH₂)₂COOH]⁺,100), 60 (C₂H₄O₂⁺, 100). 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 (CH₂) mass units typical of long–chain carboxylic.



Figure 3.15: Mass spectrum of palmitic acid

Oleic acid was assigned the molecular formula $C_{18}H_{34}O_2$ consistent with the mass spectral data m/z (%): 282 (M⁺, 3), 264 ([M- H₂0]⁺, 8), 256, 235 ([M- H₂0- C₂H₅]⁺, 3), 222, 207, 193 ([M- H₂0- C₂H₅- C₃H₆]⁺, 3), 180, 165, 151, 137, 123, 111, 97, 83, 69,55(100).



Figure 3.16: Mass spectrum of oleic acid

Ethyl oleate was assigned the molecular formula $C_{20}H_{38}O_2$ consistent with the mass spectral data m/z (%): 310 (M⁺, 10), 284, 264, 255, 251, 241, 222, 213,199,189, 180,166,155, 137, 123, 111, 97, 83, 60, 55 (100).



Figure 3.17: Mass spectrum of ethyl oleate

Ethyl linolenate was assigned the molecular formula $C_{20}H_{34}O_2$ consistent with the mass spectral data m/z(%): 306 (M⁺, 15), 279, 261 ([M- CH₂CH₂OH]⁺, 5), 250, 236, 224, 207,198, 189, 180, 164, 151. 136, 121, 109, 99 (100), 79, 55.



Figure 3.18: Mass spectrum of ethyl linolenate

Compounds Name	Retention Time (minutes)	Percentage (%)	Molecular Weight	Molecular Formula
Ergosta-5,8,22-trien -3-ol (lichesterol)	9.799	10.05	396.34	C ₂₈ H ₄₄ O
Ergosterol	9.876	73.59	396.34	C ₂₈ H ₄₄ O
5,6-Dihydroergosterol	9.905	4.14	398.36	$C_{28}H_{46}O$
Neoergosterol	10.005	3.58	380.31	$C_{27}H_{40}O$
Ergosta-5,8-dien-3-ol	10.449	3.91	398.36	$C_{28}H_{46}O$
Ergosta-7-en-3-ol (7-ergostenol)	10.662	2.10	400.37	C ₂₈ H ₄₈ O
Total amount : 97.37%				

Table 3.4: Compounds in EP4 identified using GC-MS

Ergosta-5,8,22-trien-3-ol was assigned the molecular formula $C_{28}H_{44}O_2$ consistent with the mass spectral data m/z(%): 396 (M⁺, 60), 378 ([M - H₂O]^{+,} 20), 363 ([M - H₂O - CH₃]⁺, 100).



Figure 3.19: Mass spectrum of ergosta-5,8,22-trien-3-ol

5,6-Dihydroergosterol was assigned the molecular formula $C_{28}H_{46}O$ consistent with the mass spectral data m/z(%): 398 (M^{+,} 15), 383 ([M- CH₃]⁺,10), 355 ([M- CH₃- $C_{2}H_{4}]^{+}$,10), 313 ([M- CH₃- $C_{2}H_{4}$ - $C_{3}H_{6}]^{+}$,10), 300, 285, 271, 255, 241, 229, 107, 95, 81, 69, 55(100).



Figure 3.20: Mass spectrum of 5,6-dihydroergosterol

Neoergosterol was assigned the molecular formula $C_{27}H_{40}O$ consistent with the mass spectral data m/z(%): 380 (M⁺, 20), 362 ([M- H₂0]⁺, 10), 319 ([M- H₂0- C₃H₇]⁺, 8), 282, 267, 253, 237(100), 225, 213,195, 183, 165, 153, 141, 128,109,95, 81,69,55.



Figure 3.21: Mass spectrum of neoergosterol

Ergosta- 5,8- dien 3-ol was assigned the molecular formula $C_{28}H_{46}O$ consistent with the mass spectral data m/z(%): 398 (M⁺, 40), 380 ([M- H₂0]⁺, 10), 365 ([M- H₂0- CH₃]⁺, 100).



Figure 3.22: Mass spectrum of ergosta- 5,8-dien-3-ol

Ergosta-7- en-3-ol was assigned the molecular formula $C_{28}H_{48}O$ consistent with the mass spectral data m/z(%): 400 (M⁺, 80), 385, 365,273, 255, 55(100).



Figure 3.23: Mass spectrum of ergosta-7- en-3-ol

Compounds Name	Retention Time (minutes)	Percentage (%)	Molecular Weight	Molecular Formula
Tetradecanoic acid (myristic acid)	7.359	0.30	228.21	$C_{14}H_{28}O_2$
Pentadecanoic acid	8.998	0.83	242.22	$C_{15}H_{30}O_2$
<i>n</i> -Hexadecanoic (palmitic acid)	11.225	21.95	256.24	$C_{16}H_{32}O_2$
9,12- Octadecanoic acid (Z,Z) (linoleic acid)	14.234	71.76	208.24	$C_{18}H_{32}O_2$
5,6-Dihydroergosterol	29.365	0.95	398.36	$C_{28}H_{46}O$
Ergosta-5,8(14)-dien-3-ol	32.111	0.76	398.36	$C_{28}H_{46}O$
Ergosta -5-en-3-ol	33.944	0.41	400.37	$C_{28}H_{48}O$
Total amount : 96.01%				

Table 3.5: Compounds in EP5 identified using GC-MS

Myristic acid was assigned the molecular formula $C_{14}H_{28}O_2$ consistent with the mass spectral data m/z(%): 228 (M⁺, 15), 199 ([M-CHO]⁺, 7), 185 ([M-CHO- CH₂]⁺, 25), 171([M-CHO- (CH₂)₂]⁺, 20), 157 ([M-CHO- (CH₂)₃]⁺, 20), 143 ([M-CHO- (CH₂)₄]⁺, 20), 129 ([M-CHO- (CH₂)₅]⁺, 50), 115, 107, 97, 85, 73(100), 60.



Figure 3.24: Mass spectrum of myristic acid

Pentadecanoic acid was assigned the molecular formula $C_{15}H_{30}O_2$ consistent with the mass spectral data m/z(%): 242 (M⁺,30), 225 ([M-OH]+, 5), 213, 199 ([M-OH-CH₂]⁺, 15), 185 ([M-OH-CH²-CH²]⁺, 10), 171 ([M-OH-CH²-CH²-CH²]⁺, 8), 157 ([M-OH-CH²-CH²-CH²]⁺, 8), 143 ([M-OH-CH²-CH₂-CH₂-CH₂]⁺, 16), 129 ([M-OH-CH₂-CH₂-CH₂-CH₂-CH₂-CH₂]⁺, 50), 73 (M-CH₂-CH₂-CH₂-CH₂-CH₂-CH₂-CH₂-CH₂-CH₂)⁺, 100)



Figure 3.25: Mass spectrum of pentadecanoic acid

Linoleic acid was assigned the molecular formula $C_{18}H_{32}O_{2}$, [m/z 280, M⁺]. The mass spectral data is m/z (%): 280 ([M]⁺,6), 195, 182,123, 109, 95, 81, 67 (100),



Figure 3.26: Mass spectrum of linoleic acid

Ergosta- 5,8(14)-dien-3-ol was assigned the molecular formula $C_{28}H_{46}O$ consistent with the mass spectral data m/z(%): 398 (M⁺, 15), 383 ([M- CH₃]⁺, 10), 355, 337, 300, 285, 271, 255, 241, 229, 213, 199,187, 173, 161, 147, 133, 119, 107, 95, 81, 69,55(100).



Figure 3.27: Mass spectrum of ergosta- 5,8(14)-dien-3-ol

Ergost- 5-en-3-ol was assigned the molecular formula $C_{28}H_{48}O$ consistent with the mass spectral data m/z(%): 400 (M⁺, 80), 385, 367,273, 255, 55(100).



Figure 3.28: Mass spectrum of ergosta- 5-en-3-ol

3.3.4 Identification of ergosterol

Ergosterol was obtained as colourless needles and was identified based on its GC-MS analysis and the ¹H, ¹³C, DEPT, COSY, HMBC and HMQC NMR (Appendix A; pg 232-234) spectral data. Ergosterol showed a parent ion in the EI-MS at m/z 396 consistent with a molecular formula of $C_{28}H_{44}O$. The mass spectral data is EI-MS m/z(%): 396 ($[M]^+$, 35), 378 ($[M - H_2O]^{+}$, 10), 363 ($[M - H_2O - CH_3]^+$, 44). The proton NMR data shown in Table 3.6 is consistent with the structure of ergosterol. Two singlets at $\delta 0.14$ and 0.45 were assigned to the 18- and 19-methyl protons respectively whilst doublets at $\delta 0.31(^{1}\text{H}, \text{d}, \text{J}=2.7 \text{ Hz})$ and $\delta 0.35(^{1}\text{H}, \text{d}, \text{J}=2.7 \text{ Hz})$ were consistent with the 26- and 27- methyl protons. The 21- and 28-methyl protons were expected to resonate at lower fields ($\delta 0.43$, 0.55) as they were in close proximity to the double bond at C-22. Both methyl protons appeared as doublet with coupling constant of 8.1Hz each. The two olefinic protons at $\delta 5.09$ (¹H, dd, J=2.7, 5.4 Hz) and $\delta 4.89$ (¹H, m) were assigned to H-6 and H-7 respectively whilst superimposed dd at 84.68-4.73 were assigned to the olefinic protons H-22 and H-23. Multiplets centred at 83.15 were consistent with H-3. Figure 3.29 shows the structure of ergosterol. The NMR data were consistent with that reported by Reich et al. (1969) and Kang et al. (2003).

Position	δ _C	δ_{H}
	(Reich et al., 1969)	(Kang et al., 2003)
3	70.47	3.62, m
4	40.81	2.48, broad d
5	141.33	-
6	119.58	5.56, dd
7	116.29	5.36, m
8	139.78	-
9	40.37	2.30, broad t
10	42.84	-
13	37.04	2.00, m
14	54.56	-
18	12.03	0.61, s
19	16.28	0.92, s
21	17.58	0.90, s
22	132.00	5.15-5.26, superimposed m
23	135.56	5.15-5.26, superimposed m
26	19.62 ^{<i>a</i>}	0.78,dd
27	19.32 ^{<i>a</i>}	0.82,d
28	21.08	1.02,d

Table 3.6: Proton assignments for ergosterol (Appendix A)

s is singlet; d is doublet; dd is doublet-doublet; m is multiplet and t is triplet.

^aassignment maybe interchangeable



Figure 3.29: Structure of ergosterol identified using GC-MS and NMR analysis

3.3.5 Identification of compounds in BE using GC-MS analysis

The compounds in BE identified using GCMS is shown in Table 3.7. The compounds identified in BE was 4-hydroxybenzaldhyde, benzeneacetamide, nicotinamide and cinnamic acid. Figure 3.30 -3.33 shows the fragmentation patterns of each identified compounds in BE.

Compounds Name	Retention Time (minutes)	Percentage (%)	Molecular Weight	Molecular Formula
4-Hydroxybenzaldyhde	3.356	20.06	122.04	$C_7H_6O_2$
Benzeneacetamide	3.781	8.14	135.62	C ₈ H ₉ NO
3-Pyridinecarboxamide (nicotinamide)	3.719	31.95	122.05	$C_6H_6N_2O$
Cinnamic acid	3.919	39.02	148.17	$C_9H_8O_2$
Total amount : 99.17%				

Table 3.7: Compounds in BE identified using GC-MS

4-Hydroxybenzaldhyde was assigned the molecular formula $C_7H_6O_2$ consistent with the mass spectral data m/z(%): 122 (M⁺, 100), 109, 97, 93 ([M-CHO]⁺, 45), 65 ([M-CHO-





Figure 3.30: Mass spectrum of 4-hydroxybenzaldyhde

Benzeneacetamide was assigned the molecular formula $C_8H_9NO_2$ consistent with the mass spectral data m/z(%): 135 (M^{+,} 30), 91 ([M-CH₃CHNH₂]⁺, 100)



Figure 3.31: Mass spectrum of benzeneacetamide

Nicotinamide was assigned the molecular formula $C_6H_6N_2O$ consistent with the mass spectral data m/z(%): 122 (M^{+,} 100), 106 ([M-O]⁺, 60), 78 ([M-O-CH₂N], 80), 51 ([M-O-CH₂N-C₂H₃]+, 40)



Figure 3.32: Mass spectrum of nicotinamide

Cinnamic acid was assigned the molecular formula $C_9H_8O_2$ consistent with the mass spectral data m/z(%): 148 (M^{+,} 100), 131 ([M-OH]⁺, 25), 103 ([M-OH-C2H₄], 50), 51.



Figure 3.33: Mass spectrum of cinnamic acid

3.3.6 Isolation of crude polysaccharides from hot-water extraction.

The fresh fruiting bodies of *P. sajor-caju* were boiled for 8 hr to extract the polar compounds such as polysaccharides and proteins. Then the extracted sample was further purified to obtain the crude polysaccharides (**GE**). Figure 3.34 shows the yield of crude polysaccharides obtained after the isolation and purification process.





3.3.7 Importance and medicinal properties of some identified compounds

3.3.7.1 Ergosterol

Ergosterol was the major component in *P. sajor-caju* and in this study, the percentage of ergosterol present in EAE of *P. sajor-caju* is 50.80 %. Many reports have revealed that ergosterol acts as a biological precursor to vitamin D_2 . Firstly, ergosterol is turned into viosterol and then it can be converted into ergocalciferol (which is a form of Vitamin D_2) by ultraviolet light, temperature of irradiation and moisture content (Jasinghe et al., 2007) that can be used for pharmaceutical applications and food supplements. Ergosterol is a component of fungal cell membranes, serving the same function as cholestrol in animal cells. The presence of ergosterol in fungal cell membranes coupled with its absence in animal cell membranes makes it a useful target for antifungal drug (Perera et al., 2003). Kuo et al., (2011) reported that, ergosterol was able to inhibit LPS-induced inflammation in RAW 264.7 macrophages by suppressing the NF- κ B signaling pathway.

Besides that, ergosterol is present in two forms, as free ergosterol and esterified ergosterol. Ergosterol and its isomers were reported in this investigation and these compounds present the characteristic template of tetracyclic triterpenes, mostly sterols, with structural characteristics that vary in number and position of the unsaturations in the rings and in the side chains and the keto and hydroxyl groups located in different positions of the triterpenic template. These compounds are reported for the first time in fruiting bodies of *P. sajor-caju*. However, there are other ergosterol isomers that have been identified by Nieto et al., (2008) but were not detected in this study. These compounds were ergosta-2,5,7,9(11),22-pentaene, ergosta- 5,7,9(11),22-tetraen-3β-ol, ergosta-5 α ,8 α -epidioxy-6,22-dien-3 β -ol (ergosteryl peroxide), ergosta-7,22-dien-3 β -ol,

ergosta-7-en-3 β -ol, ergosta-3 β -5 α ,6 β -trihydroxy-7,22-diene (cerevisterol), ergosta-4,6,8(14),22-tetraen-3-one and ergosta-4,6,15(16),22-tetraen-3-one.

Ergosterol and its peroxidation products may contribute to potential health benefits (Yuan et al., 2007). Free ergosterol is important for cell integrity and contributes to a variety of cellular functions. The ergosteryl ester sequestered in cytosolic lipid particles is an inert storage form of sterol and may serve as intermediates for the supply of ergosterol (Yuan et al., 2007). Hitherto, ergosterol peroxides have been isolated from various species of edible and medicinal mushrooms – e.g., *H. erinaceum* (Krzyczkowski et al., 2008), *Agaricus Blazei* Murill (Takaku et al., 2001), *Lactarius hatsudake* (Gao et al., 2007) and *V. volvacea* (Mallavadhani et al., 2004). Ergosterol peroxide has been demonstrated to have a wide spectrum of biological activities. This compound was shown to have antitumor activity on various cell lines (Krzyczkowski et al., 2008). However, ergosterol peroxide was not detected in *P. sajor-caju*. This could be due to the difference in the extraction methods applied.

3.3.7.2 Fatty acids composition

In this study, fatty acid analysis was done to evaluate the percentages of fatty acids composition in EAE of *P. sajor- caju*. Linoleic acid, palmitic acid, ethyl linolenate, ethyl linoleate and oleic acid are some of the fatty acids identified in *P. sajor-caju* and the composition of these fatty acids from EAE of *P. sajor-caju* was 22.30 %, 12.01 %, 6.65 %, 5.82 % and 1.74 % respectively. Amongst the polyunsaturated fatty acids, linoleic acid was the predominat fatty acid in *P. sajor-caju*. Linoleic acid was also found to be predominant in *P. djamor*, *T. heimii*, *Lentinus sajor-caju* and *Boletus edulis* (Kavishree et al., 2008). However the percentages of linoleic acid vary between these mushrooms. Thus, the linoleic: oleic acid ratio can be useful to

determine the taxonomical differentiation between species of the same genus. Linoleic acid is a member of the group of essential fatty acids (EFAs) called omega-6 fatty acids, so called because they are essential dietary requirement for all mammals. Essential fatty acids are fatty acids that cannot be synthesized by humans and therefore must be obtained from the diet (Ruthig et al., 1999). Linoleic acid is the precursor for a number of compounds vital for health (e.g. arachidonic acid which is involved inflammation response). It produces compounds called prostaglandins and prostaglandins are substances that regulate a wide range of functions, including blood pressure, blood clotting, blood lipid levels, immune response and inflammation response to injury or infection and are found in every cell for the body's health maintenance (Ghoreishi et al., 2011).

Palmitic acid (16 C) is the predominant saturated fatty acid in *P. sajor-caju*. However, in several mushrooms such as *T. heimii*, *T. microcarpus* and *Auricularia polytricha*, stearic acid was the predominant saturated fatty acid but it was not detected in *P. sajor-caju*. Palmitic acid is the first fatty acid produced during lipogenesis (fatty acid synthesis) and from which longer fatty acids can be produced. Meanwhile, oleic acid was the major unsaturated fatty acid in mushrooms such as *P. ostreatus* and *Clitocybe odora* (Vaz et al., 2011). These mushrooms have been reported to have good nutritive values and antioxidant potential (Orhan & Ostun, 2011). Low calorie and low fat diets are recommended for people with high blood cholesterol because high linoleic and oleic acids levels are known to help preventing atherosclerosis (Barros et al., 2007).

It is an interesting observation that an odd carbon number fatty acid such as pentadecanoic acid was observed in *P. sajor-caju*. The presence of pentadecanoic acid in *P. sajor-caju* was also reported by Kavishree et al., (2008). Besides *P. sajor-caju*, pentadecanoic acid was also detected in *Termitomyces microcarpus* and *Termitomyces*

tylerance. Another odd carbon number fatty acid have been identified in several edible mushrooms is heptadecanoic acid. This compound was noticed in *Lactarius deliciosus, Lactarius sanguifluus* and in traces in *Helvella crispa, Hydnum repandum, Lentinus squarrulosus, Russula brevepis, Sparassis crispa* and *Termitomyces tylerance* (Kavishree et al., 2008).

3.3.7.3 Phenolic compounds

Natural phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups and can range from simple molecules for instance phenolic acids, phenylpropanoids, flavonoids to highly polymerised compounds such as lignins, melanins, tannins (Barros et al., 2009). In this study, two phenolic compounds were identified in *P. sajor-caju*. They were 2,4-ditertbutylphenol from fractions EP2 and EP3 and 4-hydroxybenzaldeyhde from fraction BE. 2,4-Ditertbutylphenol is structurally related to the well known antioxidant buthylated hydroxyanisole (BHA). This compound exhibited cytotoxic activity against KB cell lines (human mouth epidermal carcinoma cell line) with IC₅₀ value of 0.81 μ g/ml (Sri Nurestri et al., 2008). There are not many reports of this compound in literature; furthermore this is the first information of this compound being identified in *P. sajor-caju*.

4-Hydroxybenzaldeyhde is an analogue to *p*-hydroxybenzyl alcohol, has shown inhibitory effect on GABA (γ -aminobutyric acid) transminase levels thus indicates this compound can be served as anticonvulsive and antiepileptic agents. This could be due to the ability of 4-hydroxybenzaldeyhde to inhibit lipid peroxidation in the brain that degrades the enzyme for GABA which is a major inhibitory neurotransmitter in the brain (Hu et al., 2001). In recent investigations, condensation and synthesis of long chain alkyl hyroxycinnamates from 4-hydroxybenzaldeyhde showed a powerful antioxidant activities in DPPH and ABTS radicals scavenging, crocin bleaching and rancimat assays (Bountagkidou et al., 2010; Menezes et al., 2011). Besides that, 4-hydroxybenzaldeyhde was also able to inhibit the growth of several bacteria such as *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aeruginosa,* MRSA, *Klebsiella pneumonia* and *Vibrio parahemolyticus* (Chang et al., 2001). Lo et al., (2001) had investigated the effect of phenolic monomers on the growth of fungus. They have reported that 4-hydroxybenzaldeyhde showed the highest inhibitory to fungal growth thus making 4-hydroxybenzaldeyhde an excellent antioxidant, antimicrobial and antifungal agent.

There are many phenolic compounds identified in edible and medicinal mushrooms such as gallic acid, pyrogallol, vanillic acid, ferulic acid, caffeic acid, syringic acid and p-hydrobenzoic acid (Kim et al., 2008a). However in this study, only two phenolic compounds were identified using GC-MS. Nevertheless, further studies are required to conclude about this point, as it is known that the levels of phenolic compounds depend on several factors such as cultivation techniques, cultivar, growing conditions, ripening process, processing and storage conditions, as well as stress conditions such as UV radiation, infection by pathogens and parasites, wounding air pollution and exposure to extreme temperatures.

3.3.7.4 Cinnamic acid

Even though cinammic acid is not categorized as a phenolic compound, it is structurally related to several phenolic acids such as *p*-coumaric, *p*-hydroxybenzoic and *p*-protocatechuic acids (Vaz et al., 2011). Thus many studies are 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 has also been proven to be effective for antimutagenecity, anticarcinogenecity, anticytotoxicity (Letizia et al., 2005), antiviral against *equid herpes 1* (Gravina et al., 2010) and antifungal properties against *Neurospora crassa* (Said et al., 2004). Besides that, tyrosinase inhibitors have recently attracted a lot of interest due to decrease of the hyper-pigmentation resulting from the enzyme action. Hence, tyrosinase inhibitors are supposed to have broad applications in medicinal and cosmetic products. Shi et al., (2005) reported that cinnamic acid strongly inhibited the diphenolase activity of mushroom tyrosinase. The activity is comparable to the inhibitory effects shown by 4-hydroxycinnamic and 4-methoxycinammic acids which are phenolic acids.

Cinnamic acids have been detected in either edible or wild or medicinal mushrooms namely, *Armillaria mellea*, *Calocybe gambosa*, *Clitocybe odora*, *Coprinus comatus* (Vaz et al., 2011), *Chantharellus cibarius*, *Lycoperdon perlatum*, *Macrolepiota procera*, *Agaricus arvensis*, and *Agaricus silvicola* (Barros et al., 2009). These investigations also reported that cinnamic acids were good antioxidants.

3.3.7.5 Nicotinamide

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. Investigations on the medicinal properties of nicotinamide have been carried out for more than a decade. Nicotinamide was reported to inhibit lipid peroxidation in rat brain mitochondria. It is stated that, the percentage of lipid peroxidation inhibition exerted by nicotinamide was comparable to tryptophan and isonicotinic acid and higher than ascorbic acid. This is due to the amide group in nicotinamide that plays an important role as 1O_2 and other reactive species quenchers (Kamat & Devasagayam, 1999). Hence nicotinamide can be considered as a potent antioxidant capable of protecting the cellular membranes in brain, which is highly susceptible to pro-oxidants, against oxidative damage induced by ROS. 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 (Suave & Xu, 2010). Thus nicotinamide could also provide new approaches in treating some of the most common disorders of human memory: those associated with age.

3.3.7.6 Polysaccharides (GE)

Mushrooms polysaccharides have been studied for many years because they are known to posses many medicinal properties. Ikekawa (1969) made one of the first scientific reports about the medicinal activities obtained from the fruiting bodies of mushrooms from the family Polyporaceace. Soon thereafter, three major drugs were developed from medicinal mushrooms and all of them were polysaccharides especially β -glucans. They are Krestin (PSK) from *Trametes versicolor*, Lentinan from *Lentinus edodes* and Schizophyllan from *Schizopyllum commune*. These polysaccharides are used to treat tumor effectively. Currently, there are extensive reports on the medicinal potential of polysaccharides from various mushrooms. However different strains from the same mushrooms can produce polysaccharides with different properties due to the variations in protein molecules bound to the polysaccharide (Wasser, 2002).

These polysaccharides from mushrooms possess promising antitumor (Ukawa et al., 2000), immune-modulating (Yu et al., 2009), antioxidant (Vaz et al., 2011), cardiovascular, antihypercholestrolemic (Oyetayo, 2008), antiviral, antibacterial, anti-inflammatory (Smiderle et al., 2008), hepatoprotective and anti-diabetic activity (Fukushima et al., 2001). The mushroom polysaccharides are mostly present as glucans

with different types of glycosidic linkages such as $(1\rightarrow 3)(1\rightarrow 6)$ - β -glucans and $(1\rightarrow 3)$ - α -glucans and some are heteroglucans (Jie et al., 2009). Studies *in vitro* and *in vivo* revealed that the immunostimulating activity of polysaccharides depends on the structure, molecular weight and number of branches. It is probably associated with the activation of cytotoxic macrophages and T-helper or natural killer (NK) cells and with the promotion of T lymphocyte differentiation and activation (Mantovani et al., 2008). Polysaccharides together with lignin and other hemicelluloses, contributes to the potential source of dietary fibre in *P. sajor-caju*. Dietary fibres are resistant to hydrolysis of human enzyme, hence helps to speed up the transit of bowel movements and protects the body from diseases (Manzi & Pizzoferrato, 2000).

The medicinal properties of *P. sajor-caju* polysaccharides are still elusive even though, there are many reports on the chemical characterization of the polysaccharides. Pramanik et al., (2005, 2007) have reported that polysaccharides from hot water extract of *P. sajor-caju* consists of soluble glucans as well as heteroglucans. The linkage in the glucans are identified as $(1\rightarrow3),(1\rightarrow6)$ - β -glucans and these linkages are supposed to play a key role in some healthy properties of *P. sajor-caju*. From this study, approximately 0.22% from 5.5 kg of freeze-dried fruiting bodies was crude polysaccharides. There are many ways to extract crude polysaccharides from hot water extracts of mushrooms. The method applied in this study is a new method reported first by Yap & Ng, (2001) to extract Lentinan from *L. edodes*.

Throughout the years, various types of polysaccharides have been identified in other mushrooms such as *T. striatus*, *P. ostreatus*, *P. florida*, *P. geestranus*, *P. eryngii*, *P. citrinopileatus*, *P. pulmonarius*, *H. erinaceus*, *G. frondosa*, *A. blazei*, *G. lucidum*, *Ganoderma tsugae*, *F. velutipes*, *Lentinus squarrosulus*, *Artemisia tripartite*, *Agrocybe*

cylindracea, Sarcodan aspratus, Calocybe indica and *A. auricular-judae* (Dey et al., 2010; Jie et al., 2009; Kiho et al., 1994; Zhang et al., 2007).

3.4 Conclusion

From this part of study, the following conclusions can be arrived at:

- Twenty- seven compounds were identified from the fruiting bodies of *P. sajor-caju*. The compounds mainly consisted of fatty acids (polyunsaturated and saturated fatty acids), phenolic compounds and sterols.
- 2) The predominant fatty acids were linoleic acid, palmitic acid, ethyl linoleate, ethyl linolenate and oleic acid. The composition to these fatty acids contributed to poor calories and low fat diet creating *P. sajor-caju* useful as health foods. In addition, the high content of polyunsaturated fatty acids, particularly the essential fatty acid linoleic acid, contributes to the recommendations of mushrooms in the diets of people with high blood cholesterol to protect against coronary disease.
- It is also very interesting that an odd carbon number fatty acid was detected in *P. sajor-caju*. The compound was identified as pentadecanoic acid.
- 4) The major component in *P. sajor-caju* is ergosterol and in this study, ergosterol was detected as free ergosterol as well as isomers of ergosterol. Eventough ergosterol is not known for medicinal properties but studies have revealed that ergosterol can be converted to vitamin D using UV light. This supports the finding that sun-dried *P. sajor-caju* showed better antioxidant activity than oven and freeze-dried fruiting bodies.

- 5) In BE, four compounds were identified where the major compound was cinnamic acids followed by nicotinamide, 4-hyroxybenzaldehyde and benzeneacetamide. 4-hyroxybenzaldeyde is a phenolic compound meanwhile cinnamic acid is reported to have structure similar to phenolic compounds.
- 6) The polysaccharides (GE) from *P. sajor-caju* were extracted from hot water extracts of *P. sajor-caju*. It is reported that polysaccharides in *P. sajor-caju* were mainly β-glucans and hetero glucans.

The compounds identified in *P. sajor-caju* possess medicinal properties and have been reported in many literatures throughout the years. However the compounds studied have not been isolated from the fruiting bodies of *P. sajor-caju* thus, it is very important to validate the bioactivity of the identified compounds from *P. sajor-caju*. Thus the antioxidant activities of these extracts were investigated in Chapter Four. The findings can be used to develop food functional supplements and pharmaceutical products.