ANTIOXIDANT ACTIVITY OF FRESH FRUITING BODIES OF *PLEUROTUS SAJOR-CAJU* EXTRACTS

4.1 Introduction

A large variety of methods have been developed to evaluate total antioxidant capacity of food and dietary supplements, herbal extracts or pure compounds, but none of them is an ideal reference method (Elmastas et al., 2007). Nevertheless, a few of them have been used widely in spite of the limitations associated with methodological issues and free radical sources.

Besides that, the number of different antioxidants present in the biological samples makes it difficult to measure each antioxidant separately. Therefore, several methods have been developed and used to determine the total antioxidant capacities of various biological samples. Each of these methods differs in its principles, characteristics and applications. Antioxidative capacity and activity in different assays may be traced back to several factors namely, the physical structure of the test system, the nature of the substrate for oxidation, the presence of interacting components, the mode of initiating oxidation and the analytical method for measuring oxidation (Frankel & Meyer, 2000; Kochhar & Rossell, 1990).

The aims of this part of study were to assess the effects of *P. sajor-caju* extracts (EP1, EP2, EP3, EP4, EE, EAE, BE and AE) on antioxidant activities using four different assays, namely, β - carotene-linoleate bleaching, ferric-reducing antioxidant power (FRAP), trolox equivalent antioxidant capacity (TEAC) and lipid peroxidation and the correlation between the antioxidant properties and total phenolic content was evaluated.

4.2 Materials and Methods

4.2.1 Materials

AppliChem, Denmark

- Thiobarbituric acid

Bio-Tek Instruments Inc, United States of America

- Power Wave $_X$ 340 spectrophotometer

Boehringer Mannheim, Germany

- ABTS

Flow Lab, Australia

- Phosphate buffer saline (PBS) tablet

Merck, Germany

- Chloroform, $CHCl_3$
- Ferric chloride, $FeCl_3$
- Folin-Ciocalteu's reagent
- Hydrochloric acid, HCl (37%)
- Methanol, CH_3OH
- Sodium acetate trihydrate, $CH_3COONa.3H_2O$
- Potassium persulfate, $K_2O_8S_2$
- Trichloroacetic acid

Sigma-Aldrich, United States of America

- Ascorbic acid
- β- Carotene
- Butylated hydroxyanisole (BHA)
- Butylated hydroxytoluene (BHT)
- Gallic acid
- Glacial acetic acid, CH_3COOH
- Ferric chloride, $FeCl_3.6H_2O$
- Ferrous sulphate, $FeSO_4.7H_2O$
- Linoleic acid
- Sodium carbonate, Na_2CO_3
- Sodium acetate trihydrate
- Tween 80
- 1,1,3,3- Tetraethoxypropane, $C_{11}H_{24}O_4$
- Tripyridyltriazine, TPTZ
- Trolox

4.2.2 Methodology

4.2.2.1 Extracts

AE, BE, EE, EAE, EP2, EP3, EP4, EP5 and GE were obtained as stated in Chapter Three. These extracts/compounds were assessed for antioxidant properties using the antioxidant assays described below.

4.2.2.2 β-carotene bleaching assay

Procedure

The antioxidant activity of mushroom extracts was determined according to the β-carotene bleaching method described by Cheung et al., (2003). A reagent mixture, containing 1 ml of β-carotene solution (0.2 mg/ml dissolved in chloroform), 0.02 ml of linoleic acid, and 0.2 ml of Tween 80 was pipetted into a round-bottom flask. After removing the chloroform by using a rotary evaporator, fifty mililitres of oxygenated distilled water was added to the flask with vigorous stirring to yield an emulsified solution. Aliquots (5 ml) of the prepared emulsion were transferred to a series of tubes containing 0.2 ml of mushroom extracts dissolved in either methanol or water with concentrations ranging between from 4 - 20 mg/ml. Pure methanol or water (0.2 ml) was used as the control, and the blank contained all the components of reagent mixture except β-carotene. The tubes containing reagent mixture were placed in a water bath at 50^{o}C and the absorbance of each sample was measured at 470 nm immediately (t=0 min) and at intervals of 20 min for a total of 120 min. All samples were assayed in triplicates. Butylated hydroxyanisole (BHA) was used as the positive reference standard. The antioxidant activity of extracts was expressed as antioxidant activity (A_A) and the oxidation rate ratio (R_{OR}).

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

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

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:

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

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 (R_{OR}) was calculated as:

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

4.2.2.3 Ferric reducing antioxidant power (FRAP) assay

Preparation of reagents

Sodium acetate trihyd**r**ate (0.0155 g - 300 mmol/l, pH 3.6) salt was mixed with 800 µl of glacial acetic acid. The final volume was made up to a total volume of 50 ml with distilled water. TPTZ (0.0156 g - 10 mmol/l in 40 mmol HCl) was dissolved in 0.2 ml of 1 M of HCl. $FeCl_3.6H_2O$ salt (0.0541 g - 20 mmol/l) was dissolved in 10ml of distilled water and the $FeSO_4.7H_20$ stock solution used was 1000 µM (i.e 0.0028 g of $FeSO_4.7H_2O$ salt dissolved in 10 ml of distilled water). Finally, the working FRAP reagent comprised of 50 ml of 300 mmol/l acetate buffer, 1 ml of 10 mmol/l TPTZ in 40 m mol/l HCl and 5 ml of 20 mmol/l of $FeCl_3.6H_20$.

Procedure

The FRAP assay was carried out according to the method by Benzie & Strain (1996) with modifications that enabled the assay to be performed in a 96 well microplates. The extracts (10 µl) with concentrations ranging from 4 - 20 mg/ml and freshly prepared FRAP reagent (300 µl) were added to 96-well microtiter plate wells in triplicates and the absorbance was measured immediately (0 min) and after 4 min at 593 nm. FRAP reagent (without sample) was used as negative control and BHA was used as positive control. The change in absorbance after 4 min ($A_{sample} - A_{control}$) was calculated. A calibration curve, using ferrous sulphate with concentrations ranging from 0 – 1000

µM $FeSO_4.7H_2O$ was prepared as standard for comparison (Appendix B; pg 238). The results are mean values and were expressed as µmol $FeSO_4.7H_2O$ equivalents/ g of fresh mushroom

4.2.2.4 Trolox equivalents antioxidant capacity (TEAC) assay

Preparation of reagents

ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (0.0192 g - 7 mM) was dissolved in 5 ml of distilled water. Potassium persulfate (0.0281 g - 104 mM) was dissolved in 1 ml of distilled water and Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) (0.0063 g - 2.5 mM) was dissolved in 10 ml of ethanol.

Procedure

The antioxidant activity was assessed according to the method described by Re et al., (1999). ABTS was dissolved in water to yield 7 mM concentration. ABTS radical cation ($ABTS^{\cdot+}$) 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 hours before use. The $ABTS^{\cdot+}$ solution was further diluted with ethanol, to an appropriate concentration to yield an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 37^{o}C. The $ABTS^{\cdot+}$ solution (100µl) was added to 10 µl of sample and the absorbance was measured after 1 min. $ABTS^{\cdot+}$ diluted in ethanol was used as negative control and BHA was used as positive control. A calibration curve, using trolox with concentrations ranging from 0 – 200 µM was prepared as standard for comparison (Appendix B; pg 239). The results are mean values of triplicate assays and were expressed as µmol trolox equivalents/g of fresh mushroom.

4.2.2.5 Lipid peroxidation assay

Preparations of reagents

Ferrous dehydrate sulfate (0.667 g - 24 mM) was dissolved in 50 ml of distilled water. Thricholoroacetic acid (20 g - 20%) was dissolved in 100 ml of distilled water and thiobarbituric acid (0.8 g - 0.8%) was dissolved in 100 ml of hot-distilled water.

Procedure

The lipid peroxidation assay was carried out based on a method reported by Daker et al., (2003) 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 comprising 0.5 ml of yolk suspension, one ml of *P. sajor-caju* extract and 0.5 ml of 24 mM $FeSO_4.7H_2O$ in PBS was incubated at 37^oC for 15 min. After the addition of 0.5 ml trichloroacetic acid (20% v/v) and one ml thiobarbituric acid (0.8% v/v) the incubation mixture was heated at 100^oC for 15 min. The reaction mixture was centrifuged at 2900 × g for 30 min and absorbance of the resulting supernatant was measured at 532 nm using a spectrophotometer. BHA was used as positive control. All determinations were carried out in triplicates. A calibration curve, using 1,1,3,3-tetraetoxypropane (TEP) with concentrations ranging from 2.5 – 20 µM TEP was prepared as standard for comparison (Appendix B; pg 240). The results are expressed as µmol of TEP equivalents /g of fresh mushroom.

4.2.2.6 Total phenolic assay

Preparation of reagents

In a 1000 ml volumetric flask, 0.5 g of AR grade dry gallic acid was dissolved in 10 ml of ethanol. The final volume was made up to 100 ml with methanol. Anhydrous

sodium carbonate (200 g) was dissolved in 800 ml water and boiled. After cooling, few crystals of sodium carbonate were added, and after 24 hr, the mixture was filter. Water was then added to a final volume of 1 L.

Procedure

The concentrations of phenolic compounds were determined according to the method described by Cheung et al., (2003). Aliquots (0.02 ml) of *P. sajor-caju* extracts at different concentrations ranging from 4 - 20 mg/ml or methanol (negative control) were mixed with 1.58 ml of distilled water and 0.1 ml of Folin-Ciocalteu's reagent. After 3 minutes, 0.3 ml of saturated sodium carbonate (Na_2CO_3) (≈35 %) solution was added to the mixture. The contents were vortex-mixed for 15 seconds and then left to stand at 40°C for 30 min. Then the absorbance was determined at 765 nm using a spectrophotometer. A calibration curve, using gallic acid with concentrations ranging from 25 - 1000 mg/L gallic acid was prepared as standard for comparison (Appendix B; pg 240). Estimation of the phenolic compounds was carried out in triplicate. The results were expressed as µmol GAEs (gallic acid equivalents)/g of fresh mushroom.

4.2.2.7 Inhibition of lipid peroxidation in cooking oil

Preparation of reagents

One gram of thiobarbituric acid (1%) was dissolved in 100 ml of hot-distilled water and thricholoroacetic acid (15 g - 15%) was dissolved in 100 ml of distilled water.

Procedure

The lipid peroxidation assay was carried out based on the method reported by Daker et al., (2003) but with minor modifications. The extracts were dissolved in distilled water to yield a final concentration of 0.2, 1.0 and 5.0 mg/ml. Extracts were added to 12 ml of palm cooking oil in Erlenmeyer flasks and heated on a heating block

until the temperature of the extracts reached 180^{o}C to stimulate cooking or frying condition. Lipid peroxidation assay was performed in reaction tubes containing 250 µl of 15% TCA and 500 µl 1% TBA and 500 µl of heated cooking oil with and without the addition of *P. sajor-caju* extracts. Each assay was carried out in triplicate. The reaction tubes were incubated in boiling water bath for 10 minutes. The tubes were centrifuged at 1200 × g for 10 minutes, to separate the aqueous phase from the oil phase. The absorbance was read at 532 nm using a spectrophotometer.

The oil mixtures were kept in Erlenmeyer flasks, sealed with paraffin and stored in dark. The lipid peroxidation assay was carried out, on aliquots of the used oil over a period of 15 days at an interval of two days. BHT was used as positive control. The percentage of inhibition (%) was calculated as shown below:

Percentage of inhibition (%) = $[(Abs_{control} - Abs_{sample\ or\ standard})/ Abs_{control}] \times 100$

where $Abs_{control}$ and $Abs_{sample\ or\ standard}$ represent the lipid peroxidation without and with the addition of extract, respectively.

4.2.2.8 Statistical Analysis

Data are shown as mean ± SD. One-way analysis of variance was used to determine the significant differences between groups. Duncan's multiple range tests (DMRT) was used to determine the significant differences between groups. STATGRAPHICS Plus software (version 3.0, Statistical Graphics Corp., Princeton, NJ, USA) was used for all statistical analysis. Statistical significance was accepted at $p < 0.05$. All figures were drawn using GraphPad Prism 5 (GraphPad Software Inc., California, USA).

4.3 Results and Discussion

4.3.1 Effects of *P. sajor-caju* extracts on antioxidants activities.

The antioxidant activity of different extracts of *P. sajor-caju* by the β-carotene-linoleate bleaching method was evaluated because β-carotene shows strong biological activity and is a physiologically important compound (Kubola et al., 2008). The presence of antioxidant extracts can prevent the extent of β - carotene bleaching, neutralizing the linoleate free radical and other free radicals formed within the system (Suja et al., 2005). There was an inverse correlation between degradation rate and the bleaching rate of β-carotene; the extract that caused the lowest β-carotene degradation rate corresponds to the highest antioxidant activity. The AE, BE, EE and EAE extract showed 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 4.1. The oxidation rate ratio exhibited an inverse relationship with antioxidant activity. Table 4.2 shows the oxidation rate of *P. sajor-caju* extracts at concentrations ranging from 4 - 20mg/ml.

BE exhibited the highest antioxidant activities in this assay and this could be due to the presence of 4-hydroxybenzaldhyde which is a phenolic compound that was identified in the extract. Phenolic compounds are considered to be major contributor of antioxidant activities in fungi and plants. Meanwhile, EP5 exhibited a low antioxidant activity because of the high percentage of linoleic acid content in that sub – fraction (see section 3.3.3). In this assay, antioxidant activity was determined by measuring the inhibition of the volatile organic compounds and the conjugated diene - hydroperoxides arising from linoleic acid oxidation, since EP5 consist mainly of linoleic acid thus, this

promotes the degradation of β – carotene higher compared to other sub - factions tested in this assay.

When comparing the antioxidant activities of *P. sajor- caju* extracts with other edible mushrooms, AE and BE of *P. sajor-caju* extracts showed better antioxidant activity than the crude extracts of fresh fruiting bodies of *L. edodes*, *H. erinaceus* (Wong et al., 2009), *Volvorella volvacea* (Cheung et al., 2003), *Boletus edulis*, *Clitocybe geotropa* and *Lactarius deterrimus* (Sarikurkcu et al., 2008). Furthermore, *P. sajor-caju* extracts also showed a comparable antioxidant activity with the fruiting bodies of *P. citrinopileatus*, *A. bisporus*, *P. energii*, *P. ferulae* and *P. ostreatus* (Lee et al., 2007). These results are in agreement with Tsai et al., (2009) where the hot water extracts of *P. ostreatus* and *P. ferulae* exhibited higher antioxidant activity than the ethanol extract in the β–carotene bleaching assay. Moreover, α-tocopherol and quercetin are antioxidants that are currently being used for various medicinal purposes and the antioxidant activities of these compounds were 96.4 % and 98.4 % respectively and were comparable to the antioxidant activities of *P. sajor-caju* extracts (Sarikurkcu et al., 2010)

Table 4.1: Effects of *P. sajor-caju* extracts and BHA on β-carotene bleaching assay

Extracts (mg/ml)	Antioxidant activity, A_A (%)			
	4	**8**	**16**	**20**
EE	90.16 ± 1.82^{as}	91.72 ± 4.97^{as}	92.66 ± 2.77^{as}	92.62 ± 2.61^{as}
EAE	36.07 ± 4.98^{aq}	41.28 ± 7.44^{aq}	43.21 ± 5.81^{apq}	81.06 ± 9.87^{br}
AE	90.91 ± 3.58^{as}	91.49 ± 1.23^{as}	93.84 ± 1.99^{as}	97.03 ± 1.18^{as}
BE	92.11 ± 2.87^{as}	91.05 ± 0.50^{as}	95.39 ± 3.47^{as}	95.74 ± 2.23^{as}
EP2	36.04 ± 6.09^{aq}	35.68 ± 8.75^{aq}	50.05 ± 4.50^{abq}	56.60 ± 0.37^{abq}
EP3	39.35 ± 9.39^{aq}	47.67 ± 7.59^{aq}	53.22 ± 1.13^{bq}	51.54 ± 3.10^{bq}
EP4	36.26 ± 1.18^{aq}	34.12 ± 7.44^{aq}	35.39 ± 9.37^{ap}	48.91 ± 9.46^{bpq}
EP5	15.27 ± 3.30^{ap}	22.45 ± 6.08^{abp}	37.18 ± 8.16^{bp}	35.16 ± 3.40^{bp}
BHA	72.96 ± 3.60^{ar}	74.56 ± 3.95^{ar}	87.48 ± 2.08^{br}	91.97 ± 0.12^{bs}

Values expressed are means ± S.D of triplicate measurements. For the extracts with the different concentrations, means in the same column with different letters (a-d) were significantly different ($p<0.05$). For different extracts with same concentrations, means in the same row with different letters (p-t) were significantly different ($p<0.05$). A_A is inhibition of β-carotene bleaching; AE is aqueous extract; BE is butanol extract; EE is ethanol aqueous extract; EAE is ethyl acetate extract; EP1-EP4 is sub-fractions of the ethyl acetate extract.

Table 4.2: The oxidation rate of *P. sajor-caju* extracts and BHA at different concentrations measured by β-carotene bleaching assay

Extracts (mg/ml)	Oxidation rate ratio (R_{OR})			
	4	**8**	**16**	**20**
EE	0.097^{a}	0.082^{a}	0.071^{a}	0.072^{a}
EAE	0.639^{bc}	0.587^{bc}	0.568^{b}	0.555^{b}
AE	0.091^{a}	0.085^{a}	0.062^{a}	0.03^{a}
BE	0.079^{a}	0.089^{a}	0.046^{a}	0.043^{a}
EP2	1.36^{c}	1.357^{c}	1.5^{c}	1.566^{c}
EP3	1.153^{c}	1.224^{c}	1.372^{c}	1.551^{c}
EP4	1.568^{cd}	1.742^{cd}	1.758^{cd}	1.789^{cd}
EP5	1.692^{cd}	1.776^{cd}	1.831^{cd}	1.914^{cd}
BHA	0.27^{b}	0.25^{b}	0.12^{a}	0.08^{a}

Values expressed are means ± S.D of triplicate measurements. For different extracts with same concentrations, means in the different row with different letters (a-d) were significantly different ($p<0.05$). AE is aqueous extract; BE is butanol extract; EE is ethanol aqueous extract; EAE is ethyl acetate extract; EP1-EP4 is sub-fractions of the ethyl acetate extract.

Table 4.3 shows the ferric reducing antioxidant power (FRAP), trolox equivalent antioxidant capacity (TEAC), lipid peroxidation activity and total phenol content of *P. sajor-caju* extracts. The FRAP and TEAC assays give fast and reproducible results, are easy to use, have high sensitivity and allow rapid analysis for large number of samples (Benzie & Strain, 1999). The ferric reducing potency of the extracts (per gram of fresh mushroom) in descending order was AE > BE > EE > EAE > EP5 > EP3 > EP4 > EP2. The reducing activity in EE and EAE could probably be due to methyl esters such as methyl linoleate, methyl palmitate and metyl stearate (see section 3.3.3). The lone pairs of electron on the carbonyl oxygen of the fatty esters can be easily donated to the ferric ions in the reducing assay (Sim et al., 2010). Meanwhile, the ABTS radical scavenging potency of the extracts (per gram of fresh mushroom) in descending order was AE > BE > EE > EAE > EP5 > EP3 > EP4 > EP2. In these two assays, the fractions EP5 and EP3 showed higher antioxidant activity than fractions EP2 and EP4 and this corresponds to the high linoleic content (71.76%) in EP5 (Table 3.5) and high phenolic content in EP3. In summary, phenolic compounds, linoleic acid (Jung et al., 2010) and cinnamic acid (Kim et al., 2008a) are known to be responsible for the antioxidant capacities in mushrooms.

Studies have reported that oyster mushrooms exhibited better reducing power ability than shiitake and golden mushrooms (Chirinang & Intarapichet, 2009). *Pleurotus sajor-caju* extracts showed better scavenging potency but poorer reducing activity than *P. ostreatus* extracts. Nevertheless, the fresh fruiting bodies of *Auricularia auricularia-judae* also exerted better reducing activity than *P. sajor-caju* extracts (Kho et al., 2009).

Table 4.3: Ferric reducing antioxidant power (FRAP), trolox equivalent antioxidant capacity (TEAC), lipid peroxidation activity and total phenol content of *P. sajor-caju* extracts.

Extracts	FRAP (µmol of $FeSO_4.7H_2O$ equivalents/g)	TEAC (µmol of Trolox equivalents/g)	Lipid peroxidation (µmol TEPs equivalents/g)	Total phenolic content (µmol GAEs equivalents/g)
AE	35.06 ± 0.86^{d}	29.45 ± 3.07^{d}	0.16 ± 0.01^{a}	10.48 ± 1.37^{c}
BE	30.23 ± 3.36^{cd}	25.67 ± 2.70^{cd}	0.35 ± 0.01^{a}	15.23 ± 1.31^{d}
EE	26.29 ± 2.82^{c}	16.55 ± 3.09^{bc}	1.35 ± 0.01^{ab}	9.48 ± 0.85^{c}
EAE	10.26 ± 2.33^{b}	11.19 ± 1.22^{ab}	1.7 ± 0.11^{ab}	2.20 ± 0.43^{a}
EP2	1.01 ± 0.31^{a}	4.59 ± 1.54^{a}	5.63 ± 0.04^{de}	5.63 ± 1.39^{ab}
EP3	3.44 ± 0.79^{a}	13.61 ± 1.70^{ab}	5.11 ± 0.11^{cd}	6.14 ± 0.66^{ab}
EP4	2.87 ± 0.81^{a}	8.49 ± 3.02^{ab}	2.89 ± 0.06^{bc}	2.52 ± 0.33^{a}
EP5	1.07 ± 0.58^{a}	13.96 ± 0.32^{ab}	7.78 ± 0.16^{e}	4.29 ± 0.54^{ab}
BHA	100.09 ± 0.08^{e}	74.48 ± 0.14^{e}	1.04 ± 0.04^{ab}	178.58 ± 1.07^{e}

Values expressed are means ± S.D of triplicate measurements for each sample. Different letters (a-f) within rows indicate significant differences between samples ($p<0.05$). AE is aqueous extract; BE is butanol extract; EE is ethanol aqueous extract; EAE is ethyl acetate extract; EP1-EP4 is sub-fractions of the ethyl acetate extract; TEPs is 1,1,3,3-tetraetoxypropane; GAEs is gallic acid

In the lipid peroxidation assay, fowl egg yolk was used as an alternative to rat liver microsomes because it consists mainly of phospholipids, triacylglycerols and proteins. Furthermore, it is cost effective and required non-tedious preparation. Lecithin a major component of egg yolk is suitable for lipid peroxidation assay because the unsaturated lipids are more prone to oxidation (Daker et al., 2008). Lipid peroxidation results in a wide range of secondary aldehydic products, which are predominantly alkanals, alkenals, 4-hydroxy-2-alkenals, and 4-oxo-2-alkenals and they may exert toxicological effects in biological systems (Saito et al., 2011). Thus finding antioxidants that can remove these aldehydes from the biological system are essential.

The inhibitory potency of the extracts (per gram of fresh mushroom) against lipid peroxidation in descending order was AE > BE > EE > EAE > EP3 > EP2 > EP1 > EP4. AE and BE were able to significantly prevent the lipid peroxidation efficiently compared to the other extracts. BE contained 4-hydroxybenzaldehyde and phenolic compounds were found to inhibit low density lipoproteins (LDL) oxidation (Cheung & Cheung, 2005). Besides that, the amide structure in nicotinamide also plays an important role in eliminating singlet oxygen as well as other reactive species that occur in the system (Kamat & Devasagayam, 1999). However, the lipid peroxidation values for the sub- fractions of EAE was significantly higher because the composition of these extracts were mainly unsaturated fatty acids thus these fatty acids generates TBARS upon decomposition. Besides that, these fractions were not assayed immediately upon isolation thus these unsaturated fatty acids could have undergone auto-oxidation to form aldehydes. The lipid peroxidation value was high in EP5 compared to other sub-fractions and this could be due to high content of linoleic acid which is a polyunsaturated fatty acid which can also be a substrate for the assay system to generate TBARS. Similarly, EP2 also demonstrated high lipid peroxidation value and this could

be due to the presence of higher percentage of methyl linoleate (Table 3.2). It has been reported that the oxidation of methyl linoleate can be converted to 4-oxo-2-nonanel which is capable of acting as a pro-oxidant in the biological system (Saito et al., 2011).

Pleurotus ostreatus exhibited *in-vitro* inhibition of lipid peroxidation in a dose dependent–manner against Fe (II)–ascorbate induced peroxidation in rat liver homogenate and the percentage of inhibition was 56.20% at 10 mg/ml (Jayakumar et al., 2007). In this study, AE and BE showed better lipid peroxidation than EAE, however, Cheung & Cheung, (2005) reported that, the ethyl acetate fraction of *L. edodes* and *V. volvacea* showed better lipid peroxidation inhibition activity compared to the aqueous and butanol extracts respectively.

A sufficiently wide range of interest has been given to phenolics or polyphenols because of their antioxidant, antimutagenic, antitumor (Pellegrini et al., 2003) antibacterial, anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, anticarcinogenic and vasodilatory actions (Soobratte et al., 2005) and are therefore an integral part of the diet. Phenolics are one of the major groups of non-essential dietary components that have been associated with the inhibition of diabetes type 2, atherosclerosis and cancer. The reasons for recent renewed interest in phenolics are that most phenolics possess stronger antioxidant capacity than vitamin C and E (Guo et al., 2003). Phenolic compounds in plants are powerful free radical-scavengers which can inhibit lipid peroxidation by neutralizing peroxyl radicals generated during the oxidation of lipids and an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides. Since mushrooms also possess phenolic compounds (Cheung et al., 2003), it is important to evaluate the role of phenolic compounds in the antioxidant activity of mushroom extracts. Furthermore, molecular studies have revealed that phenolics can exert modulatory actions in the cell

by interacting with a wide spectrum of molecular targets central to the cell signaling machinery. Thus, in addition to their antioxidant capacity, phenolics may exert protective effect by selectively inhibiting or stimulating key protein in the cell signaling cascades (Soobrattee et al., 2005).

The total phenolic (TPC) concentration (per gram of fresh mushroom) in descending order was BE > AE > EE > EP3 > EP2 > EP5 > EP4 > EAE. BE exhibited highest total phenolic content predominantly attributed to the presence of 4-hydroxybenzaldyhe (phenolic compound) and EP3 also showed high total phenolic content attributed to the presence of 2,4-ditertbutylphenol. EP4 and EP5 mainly consisted of sterol isomers and mono/polyunsaturated fatty acids thus, the total phenolic contents were low.

It was reported that the antioxidant activity by phenolics was mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donars and single oxygen quenchers (Saltarelli et al., 2009). Flavonoids are phenolic compounds with antioxidant activity (Mau et al., 2002) but were not detected in *P. sajor-caju*. Our observation is in agreement with findings of Matilla et al., (2001) and according to U.S Department of Agriculture, mushrooms are regarded as non-sources of flavonoids. The absence of flavonoids in mushrooms maybe of biological advantage in their various ecological niches since these bioactive compounds inhibit enzyme activities involved in their pigmentation, growth and development. 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.

Total phenolic contents of *P. sajor-caju* extracts obtained in this study were much higher than methanol extracts of mycelium of *G. frondosa*, *T. albuminosus* and *Morchella* (Mau et al., 2004). Besides that, *P. sajor-caju* extracts also exhibited higher

total phenolics contents than longan flesh, mango flesh and apple juice (Soong & Barlow, 2004) and tomato juice (Schleisier et al., 2002). The total phenolic content in mushrooms may differ because medicinal mushrooms are reported to have higher phenolic content than edible mushrooms and different mushroom species contained different types of phenolic compounds in varying numbers. For example, three phenolic compounds were detected in *P. eryngii* and *L. edodes* each, while 15 phenolic compounds were reported in *Sparassis crispa* (Kim et al., 2008a).

The method of Folin–Ciocalteu's is largely used to evaluate total phenolics despite all the interferences of this assay since the reagent (mixture of phosphotungstic acid and phosphomolibdic acid) also reacts with other non-phenolic reducing compounds leading to an overvaluation of the phenolic content. For instance, ascorbic acid is a widespread reducing agent that can interfere in the Folin–Ciocalteu reaction and that was, in fact, reported to be present in this studied species (Barros et al., 2009). Besides that, other reducing substances such as some sugars and amino acids could also interfere in this assay. In addition, the results have to be expressed in equivalents of a particular standard compound such as catechin, gallic acid or tannin acid. All these aspects make the results obtained from different investigations are difficult to be compared.

4.3.2 Correlation between antioxidant activities and total phenolic content of *P. sajor-caju* extracts.

To elucidate the relationship between the phenolic content and antioxidant activity, correlation analysis between total phenolic content and antioxidant activity determined by different assays were done. The total phenol content was shown to provide the highest association with FRAP assay in the present study (R^2 = 0.79). This

result was also in agreement with Kubola et al., 2008, who found strong positive correlation between total phenolic content and FRAP assay. Similar results were also found for TEAC assay (R^2 = 0.72). However, there was no significant correlation between total phenol content and β-carotene-linoleate bleaching and lipid peroxidation assay. Figures 4.1 shows the correlation between TPC and FRAP assay and between TPC and TEAC assay respectively.

These results suggest that approximately 70% of the antioxidant activity of *P. sajor-caju* results from the contribution of phenolic compounds. Also it can be concluded that antioxidant activity of mushrooms is not limited to phenolics. The antioxidant activity may also come from the presence of other antioxidant secondary metabolites which contributed to 30% of the antioxidant activity.

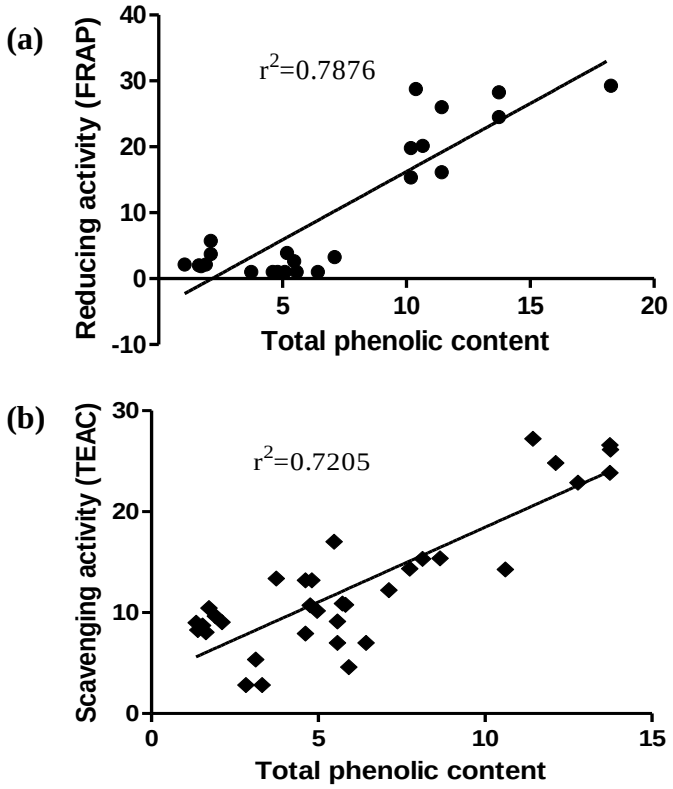

Figure 4.1: Correlation between antioxidant assays and total phenolic content; (a) total phenolic content and ferric reducing activity and (b) total phenolic content and ABTS radical scavenging activity.

4.3.3 Effects of *P. sajor-caju* extracts on lipid peroxidation and storage effect in cooking oil.

Comparing mushrooms antioxidant potential with commercial food preservatives and synthetic products may help to discover new sources of natural antioxidants (Caillet et al., 2007). This study was designed to investigate the ability of *P. sajor-caju* extracts to protect palm cooking oil from peroxidation that occurred during food preparation. Table 4.4 shows the effects of lipid peroxidation values in heated cooking oil with or without the addition of *P. sajor-caju* extracts. On day one, heated cooking oil with the addition of *P. sajor-caju* extracts showed a significantly reduced lipid peroxidation values compared to cooking oil without *P. sajor-caju* extracts. The inhibitory potency (%) of extracts (0.2 mg/ml) against lipid peroxidation in descending order was AE > BHT > BE > EAE > EE. Meanwhile, on days 5, 10 and 15, the percentage of inhibition in lipid peroxidation for all the five combination of oils exhibited a significantly lower peroxidation values than day one.

The method described by Daker et al., (2008) was modified when tested using GE. The oil containing with BHT or GE was heated on day one, five, ten, fifteen, twenty and thirty before being assayed for lipid peroxidation compared to the method described by Daker et al., (2008) where the combination of oils were only heated on day one. On day one and five, the percentages of inhibition of lipid peroxidation were significantly higher in oil containing BHT compared to GE. However on day ten and onwards, GE displayed significantly higher percentage of lipid peroxidation inhibition compared to BHT. Table 4.5 shows the percentages of inhibition of lipid peroxidation of GE in cooking oil

Table 4.4: Percentages of lipid peroxidation inhibition by *P. sajor-caju* extracts and BHT in heated cooking oil.

Extracts (mg/ml)		Percentages of Inhibition (%)			
		0	**5**	**10**	**15**
EE	**0.2**	36.4±1.3^{ap}	61.1±0.4^{aq}	68.5±0.3^{bcq}	61.6±0.6^{bq}
	1	68.1±0.9^{cp}	66.7±0.3^{ap}	67.0±0.4^{bcp}	63.0±0.5^{bp}
	5	79.7±0.4^{dpq}	87.6±0.1^{cq}	70.3±0.1^{cp}	66.3±0.8^{bcp}
BE	**0.2**	73.0±0.8^{cdq}	90.9±0.3^{cdr}	68.5±0.4^{bcq}	49.1±0.8^{abp}
	1	83.5±0.2^{der}	91.5±0.4^{cds}	74.1±0.2^{cq}	61.4±0.3^{bp}
	5	85.4±0.1^{er}	89.7±0.7^{cr}	76.5±0.3^{cdq}	65.6±0.4^{bcp}
EAE	**0.2**	53.2±0.3^{bq}	83.4±0.6^{bcr}	43.0±0.6^{ap}	40.2±0.3^{ap}
	1	53.5±0.2^{bp}	82.2±0.9^{bcr}	59.9±0.5^{bpq}	50.9±0.1^{abp}
	5	65.0±0.4^{cp}	87.6±0.5^{cq}	62.7±0.2^{bp}	62.0±0.2^{bp}
AE	**0.2**	84.6±0.3^{der}	80.6±0.1^{bcr}	68.3±0.4^{bcq}	51.5±0.1^{abp}
	1	82.8±0.6^{der}	86.9±0.2^{cr}	70.9±0.6^{cq}	54.4±0.6^{abp}
	5	78.6±0.3^{dq}	92.8±0.1^{cdr}	75.9±0.7^{cdq}	66.3±0.7^{bcp}
BHT	**0.2**	78.8±0.6^{dr}	77.1±0.3^{br}	60.6±0.7^{bpq}	53.4±0.4^{abp}
	1	91.6±0.1^{er}	90.6±0.4^{cdr}	68.9±0.6^{bcq}	58.3±0.6^{bp}
	5	94.6±0.1^{er}	93.2±0.2^{cdr}	75.6±0.4^{cdq}	44.4±0.9^{ap}

Values expressed are means ± S.D of triplicate measurements. For the different extract on the same days, means in the same row with different letters (a-e) were significantly different ($p<0.05$). For the same extract on different days, means in the same column with different letters (p-r) were significantly different ($p<0.05$). AE is aqueous extract; BE is butanol extract; EE is ethanol aqueous extract; EAE is ethyl acetate extract.

Table 4.5: Percentages of lipid peroxidation inhibition by GE and BHT in heated cooking oil.

Days	Percentages of Inhibition (%)	
	GE (1 mg/ml)	**BHT (0.2 mg/ml)**
1	44.04 ± 2.05^{ap}	51.05 ± 1.31^{aq}
5	48.26 ± 4.82^{bp}	51.02 ± 2.64^{ap}
10	65.94 ± 5.18^{cq}	54.25 ± 2.11^{ap}
15	63.80 ± 0.47^{cq}	55.31 ± 3.08^{ap}
20	68.06 ± 0.76^{cp}	68.57 ± 3.59^{bp}
30	76.22 ± 4.16^{dq}	68.56 ± 6.08^{bp}

Values expressed are means ± S.D of triplicate measurements. For the same extract on different days, means in the same column with different letters (a-d) were significantly different ($p<0.05$). For different extracts on same days, means in the same row with different letters (p-q) were significantly different ($p<0.05$). GE is polysaccharide extract.

Deep fat frying affects the flavor, texture, shelf life and nutrients of fried food. At high temperatures in the presence of air and food, oxidation, hydrolysis and polymerization of oil takes place, leading to the formation of desirable and undesirable secondary products, affecting both oil and finished product qualities (Saito et al., 2011; Daker et al., 2008) and lipid oxidation represents the main factor of edible oils degradation. Lipid peroxyl radicals from oxidized oils such as 4-hydroxy-2-nonenal, 4-hydroxy-2-hexenal and 4-oxo-2-nonenal, reportedly modify DNA bases covalently that can hasten mutagens in food (Kawai et al., 2006). Oxidation of fats and oils during processing and storage of food products worsen the quality of their lipid content and nutritive values. In frying, heat is transferred to the food by oils and fats and oxidation changes are produced in both, food and the oil or fat, when the hot oil contacts with food surface. Moreover, oils can modify fatty acid composition and antioxidant content in food which could therefore affect the oxidation in final products (Broncano et al., 2009). *In vivo* studies have demonstrated that, feeding rats with oils used during deep-frying can cause elevated levels of oxidative stress and altered eicosanoid metabolism (Yen et al., 2010). Meanwhile, several studies have also been carried out to investigate the interaction of hydrolysed compounds of free fatty acids, monoacylglycerols and diacylglycerols during the oxidative phenomena that involve oils during processing and storage (Gomes et al., 2011). Consumption of these potentially toxic products can give rise to several diseases (Sarmadi & Ismail, 2010).

Palm oil is one of the most common frying media and is used extensively, both domestically and on a commercial scale. Furthermore, in Malaysia, palm cooking oil is the most commonly available and economical oil used in the food industry. The rate of auto-oxidation in food can be reduced by freezing, refrigeration, packaging under inert gas in the absence of oxygen and vacuum packaging (Daker et al., 2008). However,

these methods are neither economical nor practical under different circumstances thus, the use of antioxidants is necessary to eliminate the oxidized radicals present in the oils. Besides palm oil, olive oil (Silva et al., 2010) and soyabean oil (Toyosaki et al., 2008; Yen et al., 2010) have also displayed high lipid oxidation during deep-frying process. Understanding oxidative phenomena is necessary for researchers in order to determine effective methods to delay deterioration. Hitherto, great attention has been paid to antioxidant substances present in oils and to their anti-oxidative mechanisms.

AE and BE showed a promising antioxidant properties in inhibiting lipid oxidation that occurred during cooking under high heat in palm cooking oil. U.S Food Drug and Administration (FDA) approved 0.2 mg/ml or 0.02% or 200 ppm as generally recognized as safe (GRAS) concentration and at higher concentrations, BHT may cause side effects to the biological system. AE and BE proved to be a good source of natural antioxidant to prevent lipid oxidation even at low concentrations. This is important because if the extracts were to be used in the industry as an antioxidant, then only small amount of the extracts is required for addition into large amount of palm cooking oil to save the cost of manufacturing and processing. Besides that Daker et al., (2008) have reported that catechin which is a plant derived flavonoid, exerted better inhibitory effect on oil peroxidation compared to BHT supports the use of natural antioxidants as more effective than synthetic antioxidants. AE and BE showed highest inhibitory effect on lipid oxidation and this effect was dose-dependent. Meanwhile EE and EAE exerted moderate inhibitory effects compared to AE, BE and BHT and this could be due to the composition of this fractions. These fractions mainly consists of polyunsaturated and saturated fatty acids that contribute to the oxidized lipids to form volatile γ-keto aldehyde products such as trans-4-oxo-2- nonenal (4-ONE), trans,tans-2,4-decadienal (Toyosaki, et al., 2008), trans-4-oxo-2-hexenal (4-OHE), trans-4-hyroxy-nonenal (HNE)

and trans-4-hyroxy-2-hexenal (HHE) (Long & Picklo, 2010). Furthermore, polyunsaturated fatty acids are easily oxidized compared to unsaturated fatty acids and it is known that approximately 50 % of EE and EAE consist of polyunsaturated fatty acids (Vaz et al., 2011).

In this study, the purified polysaccharide (GE) was extracted using the hot-water extraction method from the fresh fruiting bodies of *P. sajor-caju* as stated in section 3.2.2.4. GE also showed a comparable inhibition of lipid peroxidation with AE and BE. The quality of deep-fried foods during storage deteriorates as a result of oxidation of lipids, which are transferred mainly from frying oil (Toyosaki et al., 2008). Thus, the effect of GE on heated oil storage over one month was examined to assess the protective effects of the GE. GE was able to inhibit lipid peroxidation for the duration of one month and the percentages of inhibition increased from day one to 30. Besides that, GE also showed better inhibitory effects than BHT on day 30, even tough, at day one BHT showed higher percentages of inhibition than GE. This suggests that, GE has prolonged protective effects than BHT in inhibiting lipid oxidation in cooking oil (used oil) making GE a potential natural preservative. Choi et al., (2006) reported that heat treatment can increase the antioxidant activities of mushrooms. The heat treatment might disrupt the cell wall and release antioxidant compounds from insoluble portion of mushroom, which, in turn could increase the pool of bio-accessible antioxidant compounds (Thetsrimuang et al., 2011).

Extensive studies have been done on the isolation and identification of compounds in the aqueous extract of *P. sajor-caju.* The 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 with molecular weights of 2.4×10^5 Dalton and 3.5×10^4

Dalton respectively (Pramanik et al., 2005; Pramanik et al., 2007). The polysaccharides from *P. sajor-caju* extracts have also showed potent antioxidant activities in the total antioxidant capacity, superoxide radical scavenging, reducing power and ferric chelating assays (Telles et al., 2011). Vaz et al., (2011) reported that the polysaccharide fraction revealed higher antioxidant activity than ethanol fraction of three wild mushrooms (*Armillaria mellea*, *Calocybe gambosa* and *Clitocybe odora*) when investigating the lipid peroxidation inhibition. Another study revealed that the crude polysaccharides of *Lentinus* spp. are not only known for the ability as lipid peroxidation inhibitors but are good radical scavengers and reducing agents (Thetsrimuang et al., 2011). Finally, mushroom polysaccharides from *Geastrum saccatum* also showed a good inhibition of microsomal lipid peroxidation as well as inhibition on superoxide and hydroxyl radical (Dore et al., 2007).

The formation of *n*-3 derived lipid peroxidation products is of concern in the food industry owing to supplementation of eicosapentaneonic (EPA) or docosahexaeonic (DHA) in foods such as eggs and fish. Thus, it can be recommended that, supplementation of GE while cooking/ deep-frying, can help to reduce the lipid peroxidation at the same time increase the oxidative stability of food. Similar result was obtained when chicken hamburgers were cooked with adding grape fruit dietary fiber (Ayerdi et al, 2009). In a very recent study by Gomes et al., (2011), the lipid oxidation in cooking oil can be attenuated by purifying the oils by preparative gel permeation chromatography to obtain the oil, free of oxidation products. However the limitations of this method include the removal of antioxidants during the tedious purification process and the high cost.

4.3.4 Comparison of the antioxidant activities assessed by the different methods

No single testing method was sufficient to estimate the antioxidant activity of a sample accurately. Therefore, four different methods were used in this study. This could give valuable information to evaluate the antioxidant activity of *P. sajor-caju.* The TEAC and FRAP assays are more rapid methods compared to β-carotene bleaching method and are very useful in providing preliminary information on antioxidant levels. These methods are sensitive, require small sample amounts and allow testing of both lipophilic and hydrophilic substances (Kulisic et al., 2004). The β-carotene bleaching assay utilizes an emulsified lipid as substrate and this may introduce a number of variables influencing the antioxidant activity of examined sample. This assay is useful for assessment of lipophilic antioxidants but not polar compounds such as ascorbic acid, rosmarinic acid, caffeic acid (Kulisic et al., 2004).

The correct estimation of an antioxidant activity of *P. sajor-caju* required the evaluation of its optimal concentration because the specificity and sensitivity are different for each method used. The antioxidant power depends on the concentration and on the nature and physiochemical properties of studied antioxidants (Koleva et al., 2002). In the present study, the same antioxidant samples exhibited different antioxidative values depending on the concentration and the measured antioxidant parameter. It is important to achieve a multiple different concentration measurements to avoid incorrect conclusion in these cases.

Antioxidant activity estimation is testing-system dependent. The specificity and sensitivity of one method does not lead to complete examination of all bioactive compounds in the extract. A combination of several *in vivo* and *in vitro* tests could provide a more reliable assessment of the antioxidant activity, such as six methods were applied to measure the antioxidant capacity in *Actinidia* fruits (Du et al., 2009) and four

methods were applied to estimate the antioxidant activity from guava fruit extracts (Thaipong et al., 2006) depending on the antioxidant potential expected and perhaps on the origin of the substances (Schleisier et al., 2002). Therefore, the four different methods used in this study may confirm that *P. sajor-caju* possess remarkable antioxidant properties.

4.4 Conclusion

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

1) AE and BE fractions of *P. sajor-caju* have the highest antioxidant levels based on the four types of antioxidant assays namely β-carotene bleaching assay, ferric reducing antioxidant power assay, trolox equivalent antioxidant capacity assay and lipid peroxidation assay.
2) GE from the hot water extract also exhibited good antioxidant activity. It is proposed that, the polysaccharides in aqueous extract of *P. sajor-caju* consist of soluble β-glucans and hetero polysaccharides.
3) BE displayed a potential antioxidant activity due to the presence of 4-hydroxylbenzaldehyde (phenolic compound), cinnamic acid and nicotinamide, that showed good radical scavengers, reducing agents as well as inhibitors of lipid oxidation.
4) EP5 demonstrated the lowest antioxidant activity in β-carotene bleaching assay and lipid peroxidation assay but a moderate antioxidant activity in ferric reducing antioxidant power assay and trolox equivalents antioxidant capacity assay. This could be as result of high content of polyunsaturated (PUFAs) that can be easily oxidized to form lipid peroxyl radicals.

5) The use of hot water to extract soluble components from *P. sajor-caju* mushroom (GE) was to stimulate the preparation of traditional medicine and the brewing of herbal tea. Therefore, as compared to other solvent extraction, the information obtained by use of hot water would be more valuable for human consumption.

6) Although BHA showed good inhibitory ability on lipid oxidation, reducing capability and scavenging ability on ABTS radicals, it is a chemical additive and can only be used is a small level in foods. However, *P. sajor- caju* extracts may be used in larger quantities as food or as food ingredient without causing any adverse effects to the body.

Antioxidants obtained through diet are taking on major significance as possible protector agents to diminish oxidative damage, and edible mushrooms might be used as nutraceutical/nutriceutical to maintain good health. Since the *P. sajor-caju* extracts showed good antioxidant activities, their potential effects against lipid accumulation and glucose mobilisation was assessed using an in vitro cellular model (3T3-L1 cell lines) in Chapter Five.