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ANTIOXIDANT ACTIVITY OF CULTIVATED MUSHROOMS (*PLEUROTUS FLORIDA, PLEUROTUS SAJUR-CAJO,* AND *LENTINUS EDODES*) IN SABAH

CHOONG JIAN MING

THESIS SUBMITTED FOR FULFILMENT FOR THE DEGREE OF BACHELOR OF FOOD SCIENCE WITH HONOURS (FOOD TECHNOLOGY AND BIOPROCESS)

SCHOOL OF FOOD SCIENCE AND NUTRITION
UNIVERSITI MALAYSIA SABAH
2010

DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

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ABSTRACT

Objective of this study is to determine antioxidant activity of various species of mushroom extracted using different solvents. Besides, it also evaluate the ability of SPE to elevate the antioxidant activity of mushroom extract. Cultivated mushrooms in Sabah including *Pleurotus florida, Pleurotus sajur-cajo,* and *Lentinus edodes* were analyzed for their antioxidant activity as measured by DPPH radical scavenging activity assay, Beta-carotene bleaching inhibition assay, ABTS Trolox equivalent assay, FRAP ion reduction assay and ABTS Trolox Equivalent assay. *L. edodes* water extract with the yield of 26.16%, 2.55mg/ml of EC_{50} value of DPPH Scavenging activity,5.46mg/ml of EC_{50} value for β-Carotene Bleaching Inhibition, 253.13 μmol Trolox/mg extract and 0.2093 mmol Fe (II)/mg extract. Among the methanolic and water extracts of the mushrooms, *L.edodes* water extract was selected to underwent solid phase extraction (SPE) due to it consistent antioxidant activity for the four antioxidant assays. After SPE, three fractions were obtained and they were underwent the four antioxidant activity assays. From the three fractions, the 5% methanol of the water extract of *L.edodes,* shown the best antioxidant activity among other fractions with , 350.42 μmol Trolox/mg extract and 0.7947 mmol Fe (II)/mg extrac and the antioxidant activity of the fraction is higher than the crude water extract of *L.edodes.*

ABSTRAK

AKTIVITI ANTIOXIDAN CENDAWAN YANG DITUMBUH (PLEUROTUS FLORIDA, PLEUROTUS SAJUR-CAJO DAN LENTINUS EDODES) DI SABAH

Objektif kajian ini adalah untuk menentukan aktiviti antioxidan pelbagai spesis cendawan yang diekstrak dengan pelarut yang berbeza. Selain itu, penilaian terhadap keupayaan Solid Phase Extraction (SPE) dalam peningkatan aktiviti antioxidan ekstrak cendawan juga dijalankan. Cendawan yang tumbuh di Sabah yang termasuk Pleurotus florida, Pleurotus sajur-cajo, *and* Lentinus edodes *telah dianalisis untuk aktiviti antioxidan dengan Kaedah penurunan radikal DPPH, Kaedah perintang mengelantangan B-carotene, Kaedah tahap penyamaan Trolox ABTS dan Keadah penurunan ion FRAP.* L.edodes *ekstrak air mempunyai peratusan hasil sebanyak 26.16%,nilai* EC_{50} *2.55mg/ml untuk of Kaedah penurunan radikal DPPH, nilai* EC_{50} *5.46mg/ml untuk Kaedah perintang mengelantangan B-carotene, dan 253.13 µmol Trolox/mg ekstrak dan 0.2093 mmol Fe (II)/mg ekstrak. Maka, ekstrak air* L.edodes *telah dipilih untuk proses SPE kerana nilai untuk keempat-empat kaedah penentuan activiti antioxidant adalah tinggi. Dengan melalui SPE, tiga pecahan telah didapati. pecahan 5% methanol dari ekstrak air* L.edodes *mempunyai activity antioxidant yang paling baik dan aktiviti antioxidan lebih tinggi daripada ekstrak air asal* L.edodes.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ET	Electron Ion Transfer
EC_{50}	Half Maximal Effective Concentration
GAE	Gallic Acid Equivalence
HAT	Hydrogen Atom Transfer
SPE	Solid Phase Extraction
SPSS	Statistical Package for Social Sciences
TLC	Thin Layer Chromatography
UV	Ultraviolet

LIST OF SYMBOLS

cm	-	Centimetre
°C	-	Degree Celsius
g	-	Gram
λ	-	Lamda
<	-	Less than
µl	-	Microlitre
µm	-	Micrometre
mg	-	Milligram
ml	-	Millilitre
mmol	-	Milimolar
min	-	Minutes
>	-	More than
nm	-	Nanometre
%	-	Percentage
±	-	Plus minus
pH	-	Power of hydrogen
rpm	-	Round per minute

CHAPTER 1

INTRODUCTION

Antioxidants are generally classified as primary or secondary, based on their mechanism of action (Reische *et al.*, 1998). Primary antioxidants or chain breaking antioxidants are free radical scavengers that delay or inhibit the initiation step, or interrupt the propagation of autooxidation. The commonly used primary antioxidants in foods include tocopherols, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propylgallate (PG) and tertiary-butyl hydroquinone (TBHQ). Secondary antioxidants slow down autooxidation by chelating prooxidant metals, furnish hydrogens to primary oxidants and decompose hydroperoxides formed during the propagation step. They also deactivate singlet oxygen and absorb ultraviolet radiation. Secondary antioxidants may act synergistically with primary antioxidants to augment the latter's antioxidant activity (Reische *et al.*, 1998).

The mechanism(s) of oxidative reactions leading to decreased quality of processed foods are the same mechanisms described for lipid oxidation in general chemistry. Lipid oxidation is a multistep, multifactorial process and in foods the variables encompassed include individual fatty acid susceptibility, molecular structure of lipids, physical state of lipids, initiation reactions, hydroperoxide (ROOH), decomposition catalysts (eg., metals), presence of oxidized lipids and the amounts and selectives of antioxidants present. Lipids are important functional components of foods and have a significant effect on the quality of foods even though they constitute a minor component. Lipids are the food molecules most susceptible to oxidative free radical reactions. This instability is due to their content of polyunsaturated fatty acids (PUFA) and includes the esters of glycerol with fatty acids, triacylglycerols and phospholipids. They are not only contributed to flavor, odor, color and texture, but also confer a feeling of satiety and palatability to foods. However, the major problem in these oils lies in lipid oxidation during storage or food processing (Frankel, 1998), which can lead to the rancidity and defective nutrition due to degradation products such as reactive oxygen species, resulting in harmful effects on human health (Esterbauer *et al.*,1991; Guardiola *et al.*, 2002).

Rancidity development is an oxidative process that can be blocked by antioxidants, by preventing the formation of free radicals, through the donation of electrons or hydrogen ions. Antioxidants can be effectively used to prevent lipid oxidation (Abdalla and Roozen, 1999). Synthetic antioxidants are widely used to prevent the oxidation of oils and fats and extend the shelf-life of lipid-containing foods. However, some synthetic antioxidants, namely, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have been suspected to be responsible for liver damage and carcinogenesis in laboratory animals (Hwang *et al.*, 2001) . Besides, the use of synthetic antioxidants in foods has suffered severe criticism, as consumers are becoming increasingly conscious of the safety of synthetic chemical additives (Ozcan, 2003). The use of these synthetic antioxidants, however, is restricted because of their toxicity (Kahl and Kappus, 1993; Walton *et al.*, 1999; WHO, 1996). Consumer preferences are for the use of 'natural products' in foods, thus the addition of synthetic antioxidants such as BHT, BHA, TBHQ to food products has become a very sensitive marketing issue even though they are considered as safe for food use due to their toxicity.

Mushroom is broadly defined as a macrofungus with a distinctive fruiting body which can be either epigeous (above ground) or hypogeous (under ground) and large enough to be seen with the naked eye and to be picked by hand. Fruit bodies of mushrooms are appreciated, not only for texture and flavour but also for their chemical and nutritional properties. Mushrooms have also been reported as therapeutic foods that are useful in preventing diseases such as hypertension, hypercholesterolemia and cancer. These functional characteristics are mainly due to their chemical composition (Manzi *et al.*, 2001). Mushrooms accumulate a variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroids. A mushroom phenolic compound has been found to be an excellent antioxidant and synergist that is not mutagenic (Ishikawa *et al.*, 1984). In most countries, there is a well-established consumer acceptance for cultivated mushrooms like *Agaricus bisporus* (button mushroom), *Lentinus edodes* (shiitake), *Pleurotus spp (*oyster mushrooms), *Auricula auricula* (wood ear mushroom), *Flamulina velutipes* (winter mushroom) and *Volvariella volvacea* (straw mushroom). Mushrooms have long been appreciated for their flavor and texture. Now they are recognized as a nutritious food as well as an important source of biologically active compounds of medicinal value (Breene, 1990).

Cultivated mushrooms that are easily available in Sabah including *Pleurotus florida (Kulat Putih), Pleurotus sajur-cajo (Kulat Kelabu)* and *Lentinus edodes (Shiitake)*. All the three species of the mushroom are high in demand and used widely in restaurant, café and even household for cooking. This study was carried out to determine the antioxidant activities of different species of cultivated mushroom in Sabah.

The specific objectives of the study are:

1. To determine the antioxidant activities of extract of the *Pleurotus florida, Pleurotus sajur-cajo* and *Lentinus edodes* extracted using different solvents.
2. To evaluate the antioxidant activities of the fractionated fractions by using Solid Phase Extraction (SPE) of the selected extract.

CHAPTER 2

LITERATURE REVIEW

2.1 Mushroom Cultivation and Consumption

2.1.1 Mushroom Production

Approximately 14,000 described species of the 1.5 million fungi estimated in the world produce fruiting bodies that are large enough to be considered as mushrooms. The world market for the mushroom industry in 2005 was valued at over $45 billion. The mushroom industry can be divided into three main categories: edible mushrooms, medicinal mushroom products, and wild mushrooms (Chang, 2006). According to Sanchez (2004), over 200 species have been collected from the wild and used for various traditional medical purposes, mostly in Far East. About 35 species have been cultivated commercially and 20 are cultivated on an industrial scale. The most cultivated mushroom worldwide is *A. bisporus* (button mushroom), followed by *Lentinus edodes* (shiitake), *Pleurotus spp (*oyster mushrooms), *Auricula auricula* (wood ear mushroom), *Flamulina velutipes* (winter mushroom) and *Volvariella volvacea* (straw mushroom) (Aida *et al.*, 2009).

Table 2.1 summarizes worldwide production of mushroom as updated by the Food and Agriculture Organization of the United Nations (2009). China was found to be the biggest producers for mushrooms, as they produced more than 1.5 million metric tons in the year 2007 which showed an increment of about 65% in 10 years times. This was followed by United States and Canada as the world second and third biggest producer of mushroom. Israel and India showed drastic increased in the number of metric tons produced in 10 years, while Singapore and Kazakhstan can be regarded as new beginners as they are producing the least mushrooms since 1997. Production of mushrooms seems to continuously increase over time. This might due to high consumer demand and increase in consumer awareness on the health benefits of mushrooms.

China was the largest producer of mushrooms accounting for 45 percent of world production (Horen, 2008). Domestic consumption in China is growing and there is still potential for growth in production. Mushroom production in Europe accounts for 35 percent of world figures but, unlike China, consumption is stable and there is little opportunity for increases in total production. The dramatic 100 percent increase in production in Poland in recent years has had a significant impact on the industries in most European producer countries. North America, the third largest producer at 15 percent of the world total is in a similar situation to Europe in that the market and consumption is fairly stable. Growth areas are in the area of specialty mushrooms and added value products.

Table 2.1: Worldwide production of mushroom

Country	**Production (tonnes)**		**Percentage (%)**
	1997	**2007**	
China	562,194*	1,605,000	65.0*
United States	366,810	390,000	5.9*
Canada	68,020	81,500	16.5*
India	9000*	48,000	81.3*
Indonesia	19,000*	30,000	36.7*
Republic of Korea	13,181	28,500	53.8*
Islamic Republic of Iran	10,000*	28,000	64.3
Vietnam	10,000*	18,000	44.4*
Thailand	9000*	10,000	10*
Israel	1260	9500	86.7*
Jordan	500	700	28.6*
Kazakhstan	–	500	100*
Singapore	–	10	100*

* FAO estimate.

Source: Food and Agriculture Organization of United State, 2009.

The most popular mushroom variety in Canada is the White Button (*Agaricus bisporus*), also known as Stuffers, Paris, and Grillers. Brown mushrooms such as Crimini and Portabella belong to the same species. White and brown mushrooms make up 90% of the mushroom crop in Canada. The balance is specialty mushrooms such as Shiitake, Oyster, King Oyster, and Enoki. Total mushroom production in Canada is approximately 105 million kg , with Ontario producing 57%, British Columbia 27%, the Prairies 12% and eastern Canada 4%. In 2006, approximately 25 million Kg of Canadian Mushrooms were exported to the USA. That is approximately one-quarter of the crop (Chang *et al.*, 2006).

2.1.2 Mushroom Consumption

In 2006, Canada imported 25 million lb. (11 m kg.) of canned mushrooms, 80% of those came from China. In some instances, mushrooms are imported in bulk and canned in Canada. According to Mushroom Association of Malaysia, in year 2008, the consumer demand in Malaysia is more than 50 000 kg/day. Moreover, according to information from Food Beat 2008, a market research firm based in Wheaton, IL, as supplied by the San lose, Ca-based Mushroom Council, 77 percent of the Top 200 restaurant chains now have mushrooms on their menus. These references highlight mushroom's versatility and cross every food service segment from Italian to Mexican and from every part of the menu from burgers and pizzas to entrees and salads.

Australia Mushroom Growers Association (2008) stated that the consumption and marketing pattern of mushroom in Australia 2006-2007 is that 3kg/head (the per capita consumption of all mushrooms in Australia). The consumption of Agaricus mushroom is 2.69kg/head and for other mushroom consumption is 0.31kg/head. 86% of Australian household buy mushrooms and 55% of them buy at least once a week. 38% always include mushrooms in the shopping list and 83% of Australians love mushrooms in salad.

2.2 Medicinal and Pharmaceutical Properties of Mushroom

2.2.1 Antitumor properties of mushroom

According to Mahajna *et al.* (2009), fungi from the Basidiomycota received great interest because it contains large number of biologically active compounds such as polysaccharides. However, among all bioactive compounds, polysaccharide has been extensively studied. Several glycans have been isolated from the fruit bodies, spores and the mycelium of "Reishi" or "Mannentake" (*Ganoderma lucidum*) (Bao *et al.*, 2001; Liu *et al.*, 2002). Previous studies suggested that these polysaccharides had immunomodulating properties, including the enhancement of lymphocyte proliferation and antibody production (Bao *et al.*, 2001) as well as producing both anti-genotoxic and antitumor promoting activities (Kim *et al.*, 1999; Wasser, 2002). Antitumor activity of mushroom polysaccharides (sclerotia of Pleurotus tuber-regium) against human hepatic cancer cell has been observed by Tao *et al.* (2006). Other than that, polysaccharides isolated from the fruiting bodies of *Pleurotus ostreatus* have also been proven to exert antitumor activity against Hela tumor cell (Tong *et al.*, 2009). The sporoderm-broken germinating spores (SBGS) of Reishi were also found to show a significant antitumor effect, especially in the prevention of the recrudescence or metastasis of cancerous cells. It mitigates the toxic and side effects of radiotherapy and chemotherapy in some patients (Bao *et al.*, 2001). These polysaccharides are of different chemical composition, with most belonging to the group of β-glucans. In order to exhibit their antitumor activity, the main chain of the glucan have to be β-(1→3) linkages with additional β-(1→6) branch points (Wasser, 2002). The antitumor activities of polysaccharides were exhibited mainly by the branched (1→3) -β-d-glucan moiety (Sone *et al.*, 1985). However, the antitumor activities also depend on several factors solubility in water, size of the molecules, branching rate and its form. The antitumor activity of polysaccharides and their clinical quality can be improved by chemical modification such as Smith degradation (oxydo-reducto-hydrolysis), formolysis and carboxymethylation (Wasser, 2002).

In other study, the crude extracts of Reishi exhibited anticancer activity in in vitro systems against a variety of cancer cells including leukemia, lymphoma, breast, human bladder (Lu *et al.*, 2004), prostate, liver, lung and myeloma cell lines. The mechanism of action include the inhibition of proliferation, induction of apoptosis, induction of cell cycle arrest, inhibition of invasive behavior and suppression of tumor angiogenesis in many experimental systems including prostate cancer (Mahajna *et al.*, 2009). Daba and Ezeronye (2003) had reviewed the anticancer effect of polysaccharides isolated from various higher basidiomycetes mushrooms including *Lentinus edodes, Schizophyllum commune* and *Grifola frondosa.*

Wu and Lanier (2003) stated that activated macrophages and dendrites secrete IL-12, and helper T cells (whose activation is contingent of APCs) produce IL-2; each of these cytokines boosts the cytotoxic activity of Natural Killer (NK) cells for tumors but cannot be produced by NK cells themselves. In particular, certain cell wall polysaccharides produced by mushrooms or fungus can interact with receptors expressed on macrophages to activate these cells; these receptors no doubt evolved as part of our innate immune defenses against pathogens that produce polysaccharides not produced by humans. Moreover, although such polysaccharides are typically more effective as immune adjuvants when administered parenterally, some preparations of these agents are sufficiently absorbable to modulate immune mechanisms when fed orally. A number of polysaccharide-rich preparations derived from various types of mushrooms—some of which have long been used in folk medicine—have been studied with respect to their ability to act as orally effective immunostimulants; preparations derived from *Agaricus blazei* Murr (Agaricaceae; Brazilian sun mushroom), *Ganoderma lucidum* P Karst (Ganodermataceae; reishi), and *Lentinula (Lentinus) edodes* (Berk) Pegler (Marasmiaceae; shiitake), among others, have shown potential in this regard. (Oshiman *et al.*, 2002; Gao *et al.*, 2003; Kohguchi *et al.*, 2004; Gao *et al.*,2005). The polysaccharides derived from *Agaricus* and *Ganoderma* mycelia—β-D-glucans with 1 →3 and 1 →6 linkages—have been shown to activate macrophages via toll-like receptor 4 and CD14 (the receptor system activated by lipopolysaccharides (Kasai *et al.*, 2004;Shao *et al.*, 2004).

Mushrooms—shiitake (*Lentinula edodes*), maitake (*Grifola frondosa*), and the caterpillar fungus (*Cordyceps sinensis*)—all have immune-enhancing activity. Cordyceps has been shown in animal studies to inhibit malignant melanoma by promoting natural killer (NK) cell activity and inhibiting tumor formation. (Xu *et al*, 1992). Grifolin is a natural biologically active substance isolated from the fresh fruiting bodies of the mushroom *Albatrellus confluens* which has the ability to inhibit the growth of tumor cells by the induction of apoptosis. Grifolin strongly inhibited the growth of tumor cell lines: CNE1, HeLa, MCF7, SW480, K562, Raji and B95-8. (Ye *et al*, 2005). Besides, TML-I and TML-2 were two lectins isolated from the mushroom *Tricholoma mongolicum* that did not differ appreciably in their pH stability and cationic requirement for hem agglutinating activity. They both stimulated the production of nitrite ions and activated the macrophages in mice. The two lectins were able to inhibit the growth of implanted sarcoma 180 cells by 68.84% and 92.39% respectively. The growth of tumor cells in the mouse peritoneal cavity was also inhibited by the two lectins with TML-2 expressing a greater potency. (Wang, 1996). Li *et al.* (2008) found that the lectin from fresh fruiting bodies of the mushroom *Pleurotus citrinopileatus* exerted potent antitumor activity in mice bearing sarcoma 180, and caused approximately 80% inhibition of tumor growth when administered intraperitonealy at 5 mg/kg daily for 20 days. Lectin inhibited HIV-1 reverse transcriptase with an IC_{50} of 0.93 μM. It was devoid of antifungal activity.

Lectin AAL (*Agrocybe aegerita* lectin) from the edible mushroom *A. aegerita* is an antitumor protein that exerts its tumor-suppressing function via apoptosis-inducing activity in cancer cells. The crystal structures of ligand free AAL and its complex with lactose have been determined. The results show that dimerization of AAL is a prerequisite for its tumor cell apoptosis- inducing activity, and both galactose and glucose are basic moieties of functional carbohydrate ligands for lectin bioactivity.

These findings reveal the structural basis for the antitumor property of AAL, which may lead to *de novo* designs of antitumor drugs based on AAL as a prototype model. (Yang *et al.*, 2009)

Table 2.2 lists the source, type and bioactivities of some distinctive fungal polysaccharides with their demonstrated activities. Bioactive polysaccharides can be isolated from mycelium, the fruiting body, and sclerotium, which represent three different forms of a macrofungi in the life cycle. The species listed in Table 2.2 have been extensively studied in the years. Among them, several polysaccharides and polysaccharide conjugates have been commercialized for the clinical treatment of patients undergoing anticancer therapy. For instance, they are schizophyllan, lentinan, grifolan, krestin (polysaccharideepeptide complex) and PSK (polysaccharide protein complex).

2.2.2 Antimicrobial properties of mushroom

Latest finding by Hearst *et al.* (2009) had revealed another benefit of mushrooms. Shiitake (*Lentinula edodes*) and Oyster (*P. ostreatus)* mushrooms were tested for their antibacterial and antifungal properties. Surprisingly, shiitake extract was found to be effectives as an antimicrobial substance and was significantly more antibacterial than ciprofloxacin. This due to the fact that mushrooms are rich sources of natural antibiotics; in these, the cell wall glucans are well known for their immunomodulatory properties, and many of the externalised secondary metabolites (extracellular secretions by the mycelium) combat bacteria and viruses. According to Barros *et al.* (2007) the exudates from mushroom mycelia are active against protozoa such as the parasite that causes malaria, *Plasmodium falciparum* and other microorganisms. The importance of the Chinese Shiitake mushroom (*Lentinus edodes*) is well known; besides its antitumour activity, it has been demonstrated to increase the host resistance to bacterial and viral infections. Several compounds extracted from this mushroom revealed antifungal and antibacterial activity, namely against *Staphylococcus aureus, Bacillus subtilis* and *Escherichia coli.* The chloroform and ethyl

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