EVALUATION OF ANTIOXIDANT AND FIBRINOLYTIC ACTIVITIES OF HOT AQUEOUS EXTRACTS OF *Pleurotus pulmonarius* (FRIES) QUÉLET HYBRIDS

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ABSTRACT

In Malaysia, there is a high market demand for oyster mushroom due to its nutritional value, therapeutic properties and biotechnological applications. *Pleurotus pulmonarius* commonly known as grey oyster mushroom is one of the most popular mushroom and has gained demand worldwide. Studies have demonstrated that regular consumption of *P. pulmonarius* in human diet may be beneficial for health and act as protective agents to help the human body reduce oxidative damage. Due to high nutritional value and fast growing nature, *P. pulmonarius* hybrid was introduced to produce high quality mushrooms. Besides, *P. pulmonarius* has been used more as a food than as medicinal purpose. Therefore, in this study, antioxidant activity of the *P. pulmonarius* was evaluated to compare antioxidant activity of the hybrids (UMH001, UMH002 and UMH004) and the parents (UMP001, UMP002 and UMP003). Hot aqueous extracts were prepared to mimics cooking methods. This is typical way how edible mushrooms are consumed. Four different antioxidant assays were carried out viz., DPPH free scavenging activity, ferric reducing antioxidant power, inhibition of lipid peroxidation of buffered egg yolk and Trolox Equivalent Antioxidant Activity (TEAC) assay. The total phenolic content was determined by Folin-Ciocalteu reagent by using gallic acid for comparison. The hybrid UMH001 exhibited significant ($P<0.05$) scavenging activity (IC_{50} of 11.86 ± 0.42mg/ml), followed by UMH004 and UMH002 having IC_{50} 15.84 ± 0.41 and 16.04 ± 3.81 mg/ml, respectively. The weakest scavenging activity with an IC_{50} of 26.35 ± 2.30mg/ml was recorded in the parent UMP002. On the other hand, UMH001 had 32% higher ability to inhibit lipid peroxidation (IC_{50} of 29.57 ± 0.89mg/ml) compared to other mushroom extracts. All the hybrids showed lower ferric reducing power (absorbance ranging from 0.120-0.144) when compared to parents (absorbance 0.132-0.162) at 1.0 mg/ml. Further, UMP003 showed higher TEAC value (70.41 µmol TE/100 g dry weight) when compared to the other hybrid and parent

extracts. The hybrids of *P. pulmonarius* lost their quality compared to parents. However, there was no correlation between total phenolic content and antioxidant activities. There could be other bioactive compounds such as flavonoid, ascorbic acid and tocopherol present in the mushroom extracts. In addition, antioxidant potential of each mushroom was calculated based on the average percentage relative to butylated hydroxyanisole (BHA) and summarised as antioxidant index (AI). UMP002 (7.68%), UMH004 (6.85%) and UMP001 (6.38%) showed relatively high AI. Meanwhile, all the extracts tested were not cytotoxic to RAW264.7 cells. All the mushroom extracts also showed no fibrinolytic activity in fibrin plate assay. The enzyme might be denatured during the extract preparation by using hot aqueous to mimic the typical way how edible mushrooms are prepared for consumption. In conclusion, UMP002 exhibited significantly ($P<0.05$) higher antioxidant activities and could serve as a natural antioxidant. There was no significant difference ($p>0.05$) in antioxidant activity between the hybrids and parent of UMP002.

ABSTRAK

Di Malaysia, permintaan pasaran terhadap cendawan tiram adalah amat tinggi disebabkan oleh nilai pemakanan, sifat-sifat terapeutik dan aplikasi bioteknologi yang tinngi. *Pleurotus pulmonarius* biasanya dipanggil sebagai cendawan tiram kelabu adalah cendawan yang amat dikenali dan menjadi permintaan yang besar diseluruh dunia. Kajian telah menunjukkan bahawa penggunaan *P. pulmonarius* didalam diet manusia boleh memberi manfaat kepada kesihatan dan bertindak sebagai agen perlindungan untuk membantu tubuh manusia mengurangkan kerosakkan oksidatif. Disebabkan nilai pemakanan yang tinggi dan pertumbuhan yang cepat, hibrid *P. pulmonarius* telah diperkenalkan untuk menghasilkan cendawan yang berkualiti tinggi. Selain itu, *P. pulmonarius* kebiasaannya digunakan sebagai sumber makanan daripada tujuan perubatan. Oleh itu, dalam kajian ini, aktiviti antioksidan dinilai untuk menentukan sama ada hybrid (UMH001, UMH002 dan UMH004) atau baka induk (UMP001, UMP002 dan UMP003) menunjukkan aktiviti antioksidan yang lebih baik. Ekstrak akueus panas telah digunakan bagi meniru kaedah memasak. Ini adalah cara yang biasa digunakan bagi memasak cendawan.Empat asai antioksidan telah dijalankan iaitu aktiviti DPPH radikal bebas, pengurangan kuasa antioksidan terhadap ferik, perencatan peroksidaan lipid menggunakan kuning telur dan persamaan Trolox dengan aktiviti antioksidan (TEAC). Jumlah kandungan fenolik ditentukan dengan menngunakan Folin-Ciocalteu reagen dengan menggunakan asid gallic sebagai perbandingan. Hibrid UMH001 menunjukkan aktiviti memerangkap radikal bebas yang tinggi ($p<0.05$) dengan mempunyai nilai IC_{50} terendah iaitu 11.86 ± 0.42mg/ml diikuti oleh UMH004 dan UMH002 yang masing-masing mempunyai IC_{50} 15.84 ± 0.41 dan 16.04 ± 3.81 mg/ml. Aktiviti memerangkap yang paling lemah adalah baka induk UMP002 dengan IC_{50} 26.35 ± 2.30mg/ml. Sebaliknya, UMH001 mempunyai keupayaan yang kuat untuk menghalang peroksidaan lipid dengan 32% lebih tinggi (IC_{50} 29.57 ±

0.89mg/ml) berbanding dengan ekstrak cendawan yang lain. Semua hibrid menunjukkan kuasa pengurangan yang rendah (kadar penyerapan 0.120-0.144) terhadap ferik berbanding dengan baka induk (penyerapan 0.132-0.162) pada 1.0 mg/ml. Selain itu, UMP003 telah menunjukkan nilai TEAC yang lebih tinggi iaitu 70.41 μmol TE/100 g berat kering jika dibandingkan ekstrak hybrid dan baka induk. Hibrid *P. pulmonarius* hilang kualitinya berbanding baka induk. Walaubagaimanapun, tiada korelasi diantara jumlah kandungan fenolik dan aktiviti antioksidaan. Ini berkemungkinan ada sebatian bioaktif lain didalam ekstrak cendawan seperti flavonoid, asid askorbik dan tokoferol. Tambahan lagi, potensi antioksidan bagi setiap cendawan dikira berdasarkan peratusan purata berbanding dengan butylated hydroxyanisole (BHA) dan diringkaskan sebagai Indeks Antioksidan (AI). UMP002 (7.68%), UMH004 (6.85%) dan UMP001 (6.38%) menunjukkan indeks antioksidan yang tinggi. Sementara itu, semua ekstrak cendawan tidak menunjukkan sebarang toksid kepada sel RAW264.7. Semua ekstrak cendawan juga menunjukkan tiada aktiviti fibrinolitik di dalam asai plat fibrin . Enzim mungkin ternyahasli semasa penyediaan ekstrak yang menggunakan air panas sebagaimana cara cendawan dimakan. Kesimpulannya, UMP002 menunjukkan aktiviti antioksidan yang tinggi ($P<0.05$) dan boleh bertindak sebagai antioksidan semulajadi. Tiada perbezaan aktiviti antioksidan ($p>0.05$) di antara hibrid dan baka induk UMP002.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percent
<	Less than
>	Larger than
=	Equal to
±	Plus-minus
^{O}C	Degree celcius
ABTS	2,2-azino-di[3-ethlybenzthiazoline sulfonate]
AI	Antioxidant Index
ANOVA	One way analysis of variance
ATCC	American Type Culture Collection
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CO_2	Carbon dioxide
Cm	Centimeter
Dcm	Dichloromethane
DMEM	Dulbecco's Modified Eagle's Medium
DMRT	Duncan's Multiple Range Test
DMSO	Dimethyl-sulfoxide
DNA	Deoxybonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazy
GAE	Gallic Acid Equivalent
g	Gram
h	Hour
IC_{50}	Inhibition concentration at 50 percent
MDA	Malondialdehyde
µg	Microgram
µl	Microliter
mM	Micromolar
min	Minute/minutes
mg	Milligram
ml	Millilitre
M	Molarity
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]
nm	Nanometer
PBS	Potassium Buffer Solution
$K_2S_2O_8$	Potassium persulphate
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
RSS	Reactive Sulphur Species
R^2	Regression

rpm	Revolutions per minute
s	Second
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reaction
TBHQ	Tert-butylatedhydroquinone
TCA	Trichloroacetic acid
TE	Trolox Equivalent
U/mg	Units per miligran

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CHAPTER 1

INTRODUCTION

1.1 Background and problem statement

Oxygen is one of the most abundant elements in our world. It is essential for the oxidation of organic compounds, a process in which the mammalian cells generates the energy needed to sustain life. As consequence of this activity, highly reactive molecules such as free radicals and other reactive oxygen species (ROS) are produced. A free radical is a species that contain one or more unpaired electrons and is capable of independent existence (Halliwell, 1994). Normally, free radicals are produced internally during metabolic processes within the mitochondria. Moreover, external sources such as heat exposure, tobacco smoke, ionization radiation, certain pollutants, organic solvents and pesticides may promote the production of free radicals. Free radicals will interact with other molecules within cells and cause oxidative damage to proteins, membranes and genes (Lobo et al., 2010). Continuously produced free radicals and other reactive oxygen species (ROS) in vivo may result in cell death and tissue damage. Oxidative damage caused by free radicals may implicate food deterioration that loses the colour, flavour and nutrition value (Vidović et al., 2010).

Furthermore, oxidative stress may occur in the presence of oxidation agents, decrease of antioxidant or both factors. Oxidative stress may be linked to hypertension that is imbalance in superoxide and nitric oxide and it will lead to reduced vasodilation. A long period of hypertension may lead to stroke, heart disease and lead to chronic renal failure (Abdullah et al., 2011). Therefore, to protect the cells and organ systems of the body against reactive oxygen species,

human is gifted by a highly sophisticated and complex antioxidants protection system that functions interactively to neutralize free radicals. Antioxidants are capable to stabilizing or deactivating free radicals before they attack the healthy cells. The main characteristic of an antioxidant is its ability to trap free radicals (Prakash et al., 2001). Naturally, there is a dynamics balance between the amount of free radicals produced in the body and antioxidants to scavenge or quench them to protect the body against deleterious effects. However, in the harsh environment, the amount of antioxidants that present under normal physiological condition may be insufficient to neutralize free radicals generated. Therefore, it is obvious to enrich our diet with antioxidant such as fruits, whole grains, vegetable as well as mushroom to protect against harmful diseases (Adebayo et al., 2012). Antioxidant containing food may be useful in helping human body to reduce oxidative damage that may contribute to the development of several diseases.

Mushrooms have been used in Asia as food and in folk medicine since ancient times (Wasser and Weis, 1999). In traditional Chinese medicine, mushrooms have been use as a therapeutic food that is useful in preventing disease. They usually use hot water to extract soluble components from the fruit bodies. Mushrooms cannot be consumed raw or uncooked and usually need various food processing procedures. Therefore, preparation of hot aqueous extracts will mimic cooking methods. To date, mushrooms have been recognised as sources of antioxidant by several studies. Tsai et al. (2009) discovered antioxidant properties of *Pleurotus ostreatus, Pleurotus ferulae* and *Clitocybe maxima*. Mushrooms accumulate a variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroid. Mushrooms also contain various polyphenolic, non-polyphenolic and flavonoid compounds recognized as

good antioxidants. Farreira et al. (2009) reported that antioxidant found in mushrooms are mainly phenolic compounds that possess protective role to prevent human body against chronic diseases related to oxidative stress. Moreover, several important components including polysaccharides (β-glucan), ergosterol, vitamins, α-tocopherol and β-carotene have been isolated from the mushroom. Generally, mushrooms are rich in dietary fibers, minerals, vitamins and low in calories, sodium, fat and cholesterol. This nutrient properties make mushrooms a very good dietary food which can contribute to the formulation of well-balanced diet (Manzi et al., 1999). Further, people have turned to consume natural food source such as plants and herbs rather than artificial substances. The increase in consumer demand lead to an emergence of various health promoting products in the market. There are actually referred to foods that have special beneficial effects on the human (Childs, 1997). Mushrooms are known to contain antioxidant such as selenium, ergothioneine and phenolic that is beneficial to our body (Abdullah et al., 2011; Estrada et al., 2009). Besides that, mushrooms are highly nutritious and environmental friendly crops that carry numerous medicinal benefits. In industry, mushroom production is more important every year because mushrooms possess an efficient low-fat protein sources (Gharehaghaji et al., 2007). The genus *Pleurotus* commonly known as oyster mushrooms are the dominant cultivated mushrooms nowadays due to their delicate taste, chewy texture and unique aroma. They are highly nutritive as they contain good quality proteins, vitamins and minerals. They are also low calories food with little fat and highly suitable for obese person (Adebayo et al., 2012). *Pleurotus* mushrooms also have ability to protect major organs such as the liver, heart and brain against oxidative stress (Jayakumar et al., 2007). In addition, fungi of the *Pleurotus* genus have important place among the

commercially employed basidiomycetes because they can be easily cultivated on a large range of substrates.

On the other hand, in the 1980s, the hybrid mushrooms were well received and popular in large scale cultivation to develop products. Impartial aspect, quality of the strains is one way to obtain good strains or hybrids for mating. Hybrids strains have not only showed resistance to diseases and pest but also reduced the dependence and risk of environmental and cultural stresses (Chakravarty, 2011). To our knowledge, there is no information available on the antioxidant activities of the *Pleurotus* hybrids. In this study, the *Pleurotus pulmonarius* (Fries) Quélet hybrids and the parents were evaluated for their antioxidant activities, fibrinolytic activities, cell viability and nutritional profiles.

1.2 Objectives of the study

The main objectives of the study were:

i. to evaluate antioxidant activities of hot aqueous extracts of *Pleurotus pulmonarius* hybrids and their parents.
ii. to screen fibrinolytic activities in *Pleurotus pulmonarius* hybrids and parents
iii. to measure cell viability of *Pleurotus pulmonarius* hybrids and parents
iv. to determine nutritional components of *Pleurotus pulmonarius* hybrids and parents.

CHAPTER 2

LITERATURE REVIEW

2.1 Free Radicals (oxidants)

Electrons usually associate in pairs in the structure of atoms and molecules. Each pairs move within a defined region of space (an atomic or molecular orbital) around the nucleus (Halliwell and Cross, 1994). Any molecule species capable of independent existence that contain unpaired electron in an atomic orbital is defined as free radical (Lobo et al., 2010). The presence of unpaired electron may give a chance to most radicals to share its electron in the orbital. Many radicals are highly reactive and unstable. They are capable of donating or accepting an electron from other molecules and may cause oxidants or reductants. They can react with most molecules including proteins, lipids, carbohydrates and DNA. The simplest free radical is an atom of the element hydrogen in which has one proton and a single electron (Halliwell, 1994). Besides, they also trying to gain its stability by capturing the needed electron that they do not survive in their original state for long and quickly react with their surroundings. The free radicals attack the nearest stable molecule by stealing its electron. The 'attacked' molecule will lose their electron and it becomes free radicals itself. Finally, it will result to the disruption of living cells. Furthermore, free radical may be derived from three elements which are oxygen, nitrogen and sulphur and thus creating reaction oxygen species (ROS), reaction nitrogen species (RNS) and reaction sulphur species (RSS). Cheeseman and Slater (1993) have reported that the free radicals are produced continuously in cells either as a by-product of metabolism or deliberately as a phagocytosis. The structure of free radicals is given below:

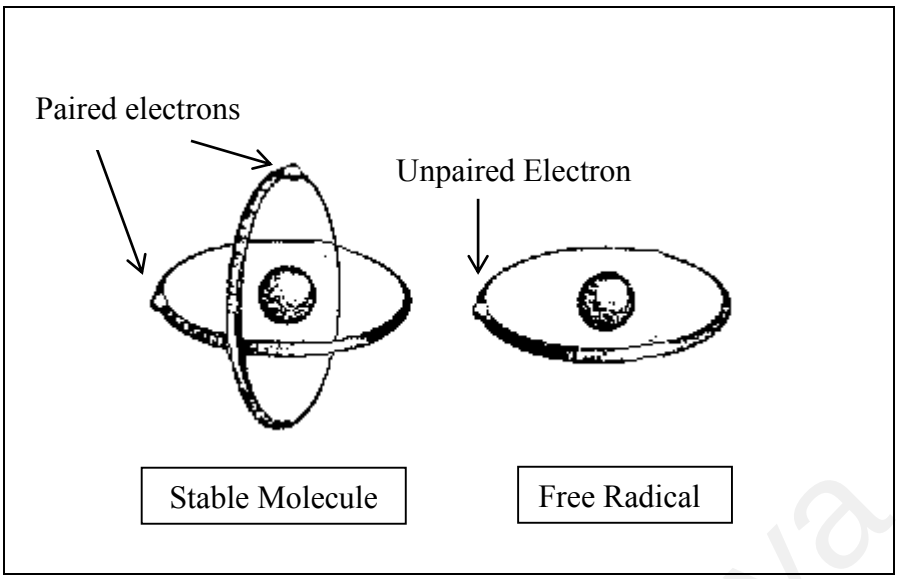


Figure 2.1: The structure of free radicals (Reither and Jo Robenson, 1995).

2.1.1 Production of free radical in human body

Free radical production in the body is a continuous and natural process. They are formed as by-products of chemical and enzymatic processes. An excessive amount of free radicals damage the body cells. Studies have found that free radicals play a role behind deadly disease like cancer, inflammatory, atheroschelerosis and the process of aging. Moreover, free radical and other ROS are also derived from external sources such as exposure to x-rays, ozone, cigarette smoking, air pollutants and industrial chemical (Bagchi and Puri, 1998). Free radical formation occurs continuously in cells both enzymatic and non-enzymatic reactions. Enzymatic reaction occurs in respiratory chain, in phagocytosis, in prostaglandin synthesis and in the cytochrome P-450 system (Liu et al., 1999). Meanwhile, free radical can be formed in non-enzymatic reactions of oxygen with organic compound by ionising reactions. Some internally and externally generated sources of free radicals are shown below:

Table 2.1: Internally and externally generated sources of free radicals. Adopted from Bugchi and Puri (1998).

Internally sources	Externally sources
Mitochondria	Cigarette smoke
Xanthine oxidase	Environmental pollutants
Peroxisomes	Radiation
Inflammation	Ultraviolet light
Phagocytosis	Certain drugs, pesticides, anaesthetics and industrial solvent
Arachidonate pathway	
Exercise	
Ischemia/reperfusion injury	

2.1.2 Types of free radical in the body

The most important free radicals in the body is the radical derivative of oxygen, also known as reactive oxygen species (ROS).

i. Superoxide

The superoxide free radical anion is formed when oxygen is reduced by the transfer of a single electron to its outer shells. The major source of superoxide in-vivo is the electron leakage that results from the electron transfer chain of the mitochondria. Superoxide ion also be detoxified to hydrogen peroxide through a dismutation reaction with the enzyme superoxide dismutase through the Haber-Weiss reaction and finally to water by the enzyme catalase.

ii. Hydrogen peroxide

Hydrogen peroxide is non-radical molecule generated by the same sources that produce superoxide anion. Hydrogen peroxide and superoxide anion can occur both inside and outside cells. In

the presence of a transition cation such as iron or copper, superoxide anion can give rise to the highly reactive hydroxyl radical species by the Haber-Weiss reaction.

iii. Hydroxyl radical

Hydroxyl radical is an extremely reactive oxidising radical that will react to most biomolecules at diffusion controlled rates. It is considered to be a principal actor in the toxicity of partially reduced oxygen species since it is very reactive with all kinds of biological macromolecules that produce the product that cannot be regenerated by cell metabolism. It is most dangerous free radical because it is involved in reactions such as lipid peroxidation and generation of other toxic radicals.

iv. Singlet oxygen

Singlet oxygen is non-radical often associated with oxygen free radical that has strong oxidising activity. It is an electronically excited and mutagenic form of oxygen.

v. Nitric oxide

Nitric oxide is a common gaseous free radical. It plays a role in vascular physiology and also known as endothelium derived relaxing factor. Besides, vascular endothelium produces nitric oxide.

vi. Peroxynitrite

Peroxynitrite is produced by the reaction of nitric oxide with superoxide.

2.2 Oxidative stress

Oxygen is essential to our planet's life force and consisting 21% of the air we breathe (Halliwell, 1994). Without it, our organ would not function well and live. Besides, oxygen is the primary oxidant in metabolic reaction designed to obtain energy from the oxidation of a variety of organic molecules. It is essential for the oxidation in the organic compounds, which is the process where mammalian cells generate the energy needed to sustain life. However, oxygen also plays a vital role in the breakdown of our body functionality. Although we need oxygen to live but high concentrations of it are actually corrosive and toxic to our body. Oxidation is essential to many organisms for the production of energy for biological process. However, free radicals, also known as reactive oxygen species (ROS) are produced during numerous physiological processes. An imbalance between oxidants and antioxidants in favour of the oxidants will potentially lead to oxidative stress (Seis, 1997). Normally, reactive oxygen species (ROS) which notably include free radicals like superoxide anion and the hydroxyl radical OH- will generated during mitochondrial respiratory chain which is modulated by the rate of electron flow throughout the respiratory chain complex (Chan et al., 2013). When the production of ROS is excessive, they have harmful effect because they induced apoptosis in healthy cells or activate various genes coding for pro-inflammatory cytokines or adhesion proteins. In addition, their unstable nature makes them reactive and capable to damaging the healthy cell by causing break and mutation in DNA. Radicals of oxygen (superoxide anion, hydroxyl radicals and peroxyl radicals) and reactive non-radical of oxygen species such as hydrogen peroxides and singlet oxygen, as well as carbon, nitrogen and sulphur radicals comprise the variety of reactive molecules that can constitute an oxidative stress to cells. It has been estimated

that 5% of the total oxygen metabolism of liver tissue results in the production of partially reduced oxygen species (Thomas, 1999).

2.2.1 Effects of oxidative stress

Oxidative stress describes a state of physiological stress in the body that arises from exposure to high level of reaction oxygen species (ROS) relative to the level of neutralising antioxidant. The imbalance between the ROS and antioxidant will damage a wide range of molecules species such as lipids, proteins and nucleic acids. Tissue injured by trauma, heat injury, hypertoxia, toxin and excessive exercise may occur in short-term oxidative stress (Lobo et al., 2010). It would tend the cells start to suffer the consequences of oxidative stress (Wiernsperger, 2003). Oxidative damage caused by free radicals may be related to the aging and disease such as atherosclerosis, cancer and rheumatoid arthritis (Mau et al., 2002). Basically, the main target of ROS, RNS and RSS are proteins, DNA (Deoxyribonucleic acid) and RNA (Ribonucleic acid) molecules, sugars, carbohydrate and lipids that are displayed in Figure 2.2:

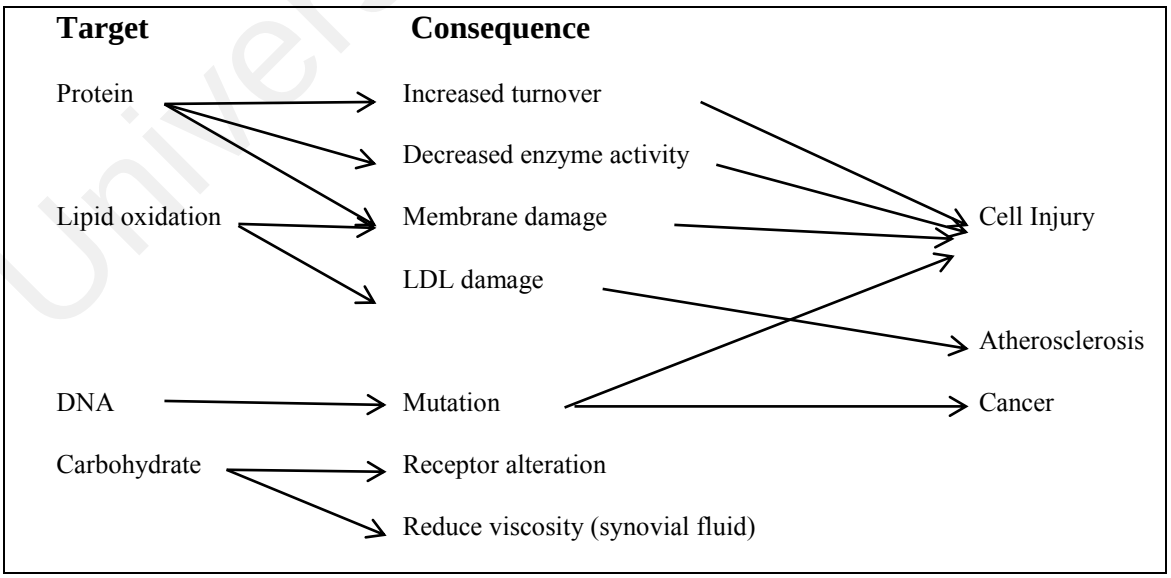


Figure 2.2 : Target of free radicals. Adopted from Bagchi and Puri (1998).

2.3 Antioxidant

Antioxidants are stable enough molecules that are capable of donating an electron to neutralise free radicals and thus reducing damage of healthy cell in our body. Halliwell (2007) stated that antioxidant is 'any substances that can delay, prevent or removes oxidative damage to a target molecule'. On the other hand, Huang et al. (2005) defined antioxidant as a substance that opposes oxidation or inhibits reactions promoted by oxygen or peroxides. Antioxidants have ability to trap or scavenge free radicals and protect other chemicals of the body from damaging oxidation reactions by reacting with free radicals and other reactive oxygen species within the body. Antioxidant acts as radical scavenger, hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor, synergist and metal-chelating agent (Lobo et al., 2010). Even though human and other organisms possess their own antioxidant defence and repair system to protect them against oxidative damage, these systems are insufficient to totally prevent the damage. Thus, antioxidants in human diets are most interest as a protective agent to help human body to reduce oxidative damage. Many researchers suggest that an adequate antioxidant defense may control free radicals damage and that optimal intake of antioxidant nutrient may contribute to enhance quality of life. A diet rich in antioxidant seems to be delaying age-related disorder such as atherosclerosis. The most antioxidant nutrients usually derived from phytochemicals (plant-derived chemicals) and include polyphenols such as flavonoids, anthocyanidins, catechins or stilbenes (Obrenovich et al., 2011). Generally, antioxidant can be classified into natural and synthetic antioxidant.

2.3.1 Synthetic antioxidant

Synthetic antioxidants have been developed in order to have a standard measurement antioxidant activity system to compare with natural antioxidants and to be incorporated in food. In 1940s, synthetic antioxidants were first introduced into packaged foods. It is primarily added to edible fats and fat-containing foods for their ability to prevent food from becoming rancid, developing unpleasant odour as well as prolong shelf life. The most widely used of synthetic antioxidant is butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). BHA was first introduced in 1947 that primarily used as a food preservatives. It began to appear in food packaging, animal food, rubber and petroleum products. Besides, tert-butylatedhydroquinone (TBHQ) also the one of synthetic antioxidant and it will stabilise and preserve the freshness, nutritive value, flavour and colour of animal food product. Synthetic antioxidants have been used in personal product such as antiaging formulation and acne treatment. It also incorporated in statins which is a class of drug that used to treat high cholesterol and prevent cardiovascular disease.

Today, many researchers and health critics are questioning the safe of synthetic antioxidants. The use of these common synthetic alternatives has increasingly become viewed as a threat to human health around the world. Recently, Botterweck et al. (2000) reported that synthetic antioxidants have to be carcinogenic and thus their use has been restricted. Although, studies by Naveena et al. (2008) revealed that long using of synthetic antioxidant by food industries to prevent lipid oxidation tend to be carcinogenic. A study by National Library of Medicine demonstrated that BHT will promote the development of lung tumor from previously initiated cells. Thus, synthetic antioxidants tend to be dangerous to human health. Due to, nontoxic natural compounds with

antioxidant activity such as soybean, green or black tea, coffee, red wine, citrus and other fruits, onions, olives, mustard and turmeric as well as mushrooms have been intensified in recent years.

2.3.2 Natural antioxidant

In human, antioxidant system can be divided into two groups which are enzymatic and non-enzymatic antioxidants. Further, the enzymatic antioxidants are divided into primary and secondary enzymatic defences. Primary enzymatic defence includes superoxide dismutase, catalase and glutathione peroxidase. Secondary enzymatic defence includes glutathione reductase and glucose-6-phosphate dehydrogenase. Meanwhile, there is a quite number of non-enzymatic antioxidant such as vitamin A, coenzyme Q10, uric acid, glutathione, vitamin C and E, flavonoid, phenolic acids, carotenoids and minerals (Carocho and Ferreira, 2013). However, our antioxidant defences is insufficient to protect our body from oxidative damage and adequate amounts of antioxidants are needed to fight off damaging free radicals.

Recently, natural antioxidants are widely used to prevent or delay some types of cell damage. Studies by Ramarathnam et al. (1995) showed a multitude of natural antioxidants have already isolated from the different kinds of plant materials such as oilseed, cereal crops, vegetable, fruit, leaves, roots, spices and herbs. Based on their studies, all the different kinds of plant materials will inhibit lipid peroxidation and offer protection against oxidative damage to membrane functions. Fruits, vegetables and red wine play a role in disease prevention due to its antioxidant properties of their constituent polyphenols (vitamin E and C and carotenoids) (Rice-Evans et al., 1997). Several plants have been studied as sources of potentially safe natural antioxidants for the food industry. Research done by Shobana and Naidu (2000) showed that Indian

spices such as clove, cinnamon, ginger, garlic, pepper and onion might act as a good antioxidant and thus prevent oxidation of lipids and adverse effects of lipid peroxidation instead of impart flavour and taste to the foods. A study by Lin et al. (1998) showed that green and black tea has strong antioxidant activities that contain up to 30% of the dry weight as phenolic compound, which green tea contains higher amounts of simple polyphenols like cathecins however black tea contains larger amounts of gallic acids.

Many studies have shown that natural sources are widely used as an ingredient in dietary supplement for protective agents in human health. Not only in food, natural antioxidant also used in cosmetic and therapeutic industry rather than synthetic antioxidant in respect to low cost, highly compatible with dietary intake and no harmful effects inside the human body. Notably, the uses of antioxidants are important in industrial processes such as in prevention of metal corrosive, the vulcanisation of rubber and the polymerisation of fuel in the fouling of internal combustion engine.

2.4 Mushroom

Mushrooms have been used as folk medicine throughout the world such as Korea, China, Japan and eastern Russia since ancient times. They are used as nutritionally functional food and beneficial nontoxic medicines. Mushrooms comprise an untapped source of powerful new pharmaceutical products (Menaga et al., 2013). The term ‘mushroom’ was defined by Miles and Chang (1997) as macrofungi with distinctive fruit bodies that may grow hypogeous (below ground) or epigeous (above ground) which large enough to be seen with the naked eye and to be picked by hand. Mostly, these fungi is belong to class Basidiomycetes (fungi producing basidiospores) and some fungi of class Ascomycetes (fungi producing ascospores). The fruit bodies of the mushroom

are formally called basidocarp. Basically, there are three parts in a mushroom fruiting body, which is cap, stipe and veil. A fruiting body is formed from spacious underground mycelia (hypae) by the process of fructification. Normally, the life time of the bulk of fruit bodies is only about 10-14 days. The basic terminology of fruiting body is given below in Figure 2.3:

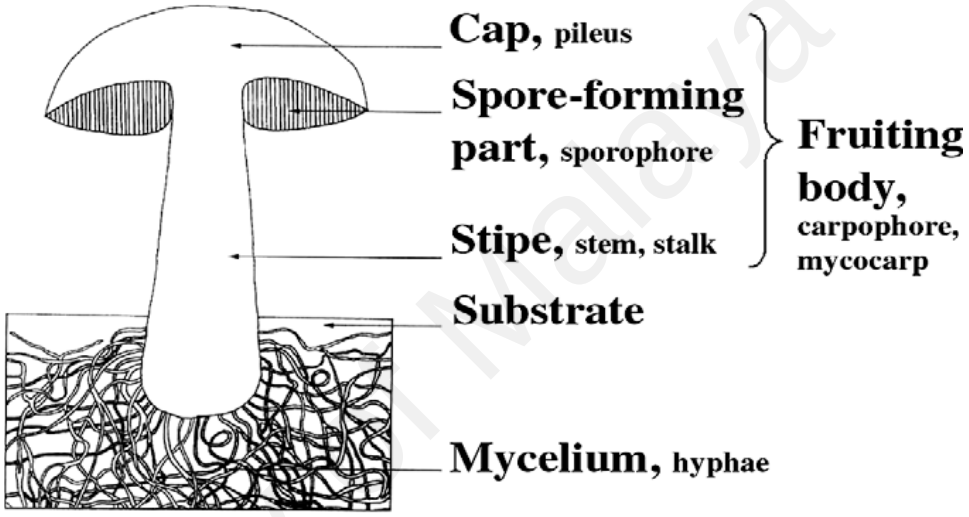


Figure 2.3: Mycology terms of mushroom. Adopted from Kalač (2009).

Mushrooms have been valued by humankind as an edible and medicine source. Nowadays, cultivated and wild edible mushrooms are widely used directly or indirectly as food or ingredients that have been clearly separated from medical mushroom by the industry. Mushroom is suitable to consume for all age groups, child to aged people. Recently, the most cultivated mushrooms are *Agaricus bisporus, Lentinula edodes* and *Pleurotus* spp. Mushrooms are appreciated worldwide for their attractive taste, aroma and nutritional values. Guillamon et al. (2010) reported that an appropriate nutritional value in edible mushroom such as high protein, low amounts of fat, high dietary fiber and rich in bioactive compounds may promote human well-being as well as preventing and treating cardiovascular diseases. However, the awareness of mushrooms as

an important source of biological active substances with medicinal value has only recently emerged.

2.5 Mushroom as a source of antioxidant

Antioxidant present in dietary mushrooms act as a protective agent to help human to reduce oxidative damage. Sudha et al. (2012) reported that an edible mushroom could serve as natural antioxidant owing to their significant antioxidant activity. Various activities of the mushrooms have been study which includes antimicrobial, antiviral, antitumor, antiallergic, anti-inflammatory, immunodulating, hypoglycaemia, antioxidant and hepatoprotective activity (Lindequist et al., 2005). Puspha et al. (2012) stated that antioxidant components including total phenols, tocopherols, flavonoids and polysaccharides that found in mushroom extracts are naturally source of antioxidants. Recent research carried out showed that medicinal mushrooms occurring in South India namely *Ganoderma lucidum, Phellinus rimosus, Pleurotus florida* and *Pleurotus pulmonarius* possessed antioxidant and antitumor activities and showed significant antimutagenic and anticarcinogenic activities. Studies done by Yang et al. (2002) reported that tree oyster mushrooms showed better antioxidant activity, reducing power, scavenging activities and high total phenolic content compared to winter, shiitake and abalone mushrooms. Their research also showed scavenging effects of commercial mushrooms on DPPH radicals increased with the increased concentrations and showed moderate to high (42.9%-81.8% at 6.4 mg/ml).

Moreover, study done by Tsai et al. (2009) reported several cultivated mushrooms that exhibit antioxidant properties apart from their high intense umami taste. Their research indicated that the three mushrooms were relatively effective by EC_{50} values were less than 14 and 30mg/ml for ethanolic and hot

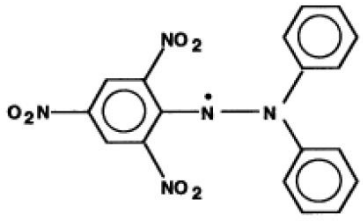
water extracts, respectively. Wong and Chye (2009) showed petroleum ether extract of *Pleurotus porrigens* (angle's wing) has excellent antioxidant activity with 85% DPPH radical scavenge abilities and methanolic extract of *Hygrocybe conica* (witch's hat mushroom) has 94% chelating effect at 20mg/ml. Mau et al. (2002) reported that four specialty mushrooms *Dictyohora indusiata* (basket stinkhorn), *Grifola frondosa* (maitake), *Hericium erinaceus* (lion's mane) and *Tricholoma giganteum* (white matsutake) are excellent sources of antioxidants. Basket stinkhorn at 6.4 mg/ml showed 92.1% scavenging percentage. Rest of the mushrooms the scavenging activity was within the range 63.2-67.8%. Another study by Mau et al. (2005) showed hot water extract from mature and baby ling chih possess high antioxidant activities; 78.5% and 78.2% at 20 mg/ml which had EC_{50} value of 7.25 and 5.89 mg/ml, respectively. Therefore, these mushrooms are potential sources of antioxidants.

2.6 Principles of methods for antioxidant determination

2.6.1 DPPH free radical scavenging

2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging is the most popular method to determine antioxidant activity. This is a rapid, simple and inexpensive method to measure antioxidant capacity in food. DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors instead of evaluate antioxidant activity of foods and beverages (Prakash, 2001). The original DPPH method was introduced by Blois in 1958. The Blois method was used by several researchers like Brand William et al. (1995) and also used in this study. Mechanism of DPPH free radical scavenging was modified by Molyneux (2004) based on Blois (1958). This study showed the delocalisation of the spare electron in the DPPH molecule and the reaction mixture became deep violet in colour. DPPH is characterised as a stable

molecule and thus the molecule do not dimerise (Figure 2.4). When the solution of DPPH is mixed with the substance that can donate a hydrogen atom, the molecule will reduced from radical to nonradical which change the deep violet colour to pale yellow colour (Figure 2.5).



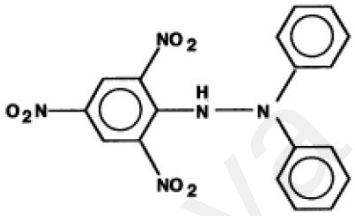


Figure 2.4 Diphenylpicrylhydrazyl (free radical)	Figure 2.5 Diphenylpicryldrazine (nonradical)

2.6.2 Inhibition of lipid peroxidation

Lipid peroxidation is a process in which free radicals attack lipids containing carbon-carbon double bond especially polyunsaturated fatty acids (PUFAs) (Ayala et al., 2014) becoming oxidised into lipid peroxides. Lipid peroxides are toxic and capable to damaging most cells in the body (Halliwell and Chirico, 1993). Lipid peroxidation may disrupt biological membranes that decrease membrane fluidity, change membrane permeability and decrease electrical resistance (Devasgayam et al., 2003). Lipid peroxidation process consists of three steps: initiation, propagation and termination. In initiation step, an unsaturated fatty acid (LH) is attacked by any species such as hydroxyl radical (OH•) and hydroperoxyl ($OH•_2$) that abstract hydrogen atom to form carbon-centered lipid radical (L•). Then, lipid radical (L•) rapidly react with oxygen molecule forming lipid peroxyl radical (LOO•) which abstract hydrogen atom from other lipid molecule to become lipid hydroperoxide (LOOH). When the lipid radical (L•) is formed from second lipid molecule (LH), lipid

peroxidation is propagated. Lipid peroxides (LOOH) can be further degraded by a Fenton's reaction in presence of Fe^{2+}. Lipid peroxyl radical (LOO•) is unstable and breakdown to form various products including malondialdehyde (MDA) and 4-hydroxyl-nonenal. In the termination step, antioxidant will donate the hydrogen atom that react with lipid hydroxyl radical (LOO•) to produce nonradical products. Once lipid peroxidation is initiated, a propagation of chain reaction will take place until the termination products are produced.

MDA and other related aldehydes are the most commonly products by the lipid peroxidation. MDA is a secondary by-product of polyunsaturated fatty acid peroxidation and arachidonic acid decomposition that consist of three-carbon dialdehyde (Fernández et al., 1997). The most commonly method used for lipid peroxidation is Thiobarbituric Acid Reactive Substances (TBARS) assay by colorimetric reaction between the thiobarbituric acid (TBA) and MDA yielding pink chomagen. TBARS assay remain popular due to its simplicity, inexpensive and rapidity with which large number of sample (Hodges et al., 1999).

2.6.3 Ferric Reducing Antioxidant Power

Ferric Reducing Antioxidant Power (FRAP) assay is a quick and simple method to measuring antioxidant power. The FRAP assay was first performed by Iris Benzie and J.J Strain (1996). The reaction takes advantage of electron-transfer reactions. The presence of reducers (antioxidants) may causes the reduction of Fe^{3+}-ferricyanide complex to form ferrous (Fe^{2+}) which turn the yellow colour to Pearl's Prussian blue (Barros et al., 2007). Reducing capacity of the compounds could serve as an indicator of potential antioxidant properties. Öztürk et al. (2007) reported that the higher absorbance indicates greater reducing power.

2.6.4 Trolox Equivalent Antioxidant Capacity

Trolox Equivalent Antioxidant Capacity (TEAC) is a simple method to measuring antioxidant capacity in a sample. Re et al. (1999) reported that TEAC assay is a decolourisation assay that applicable for both lipophilic and hydrophilic antioxidant such as flavonoids, hydroxycinnamates, carotenoids and plasma antioxidant. TEAC assay is based on scavenging of the $ABTS^{+}$ radical cation by the antioxidant present in a sample compared with a reference antioxidant standard (Trolox). When there is antioxidant presence in the reaction medium, they may capture the fee radicals and decolourise the blue-green colour of $ABTS^{\bullet+}$ radical. $ABTS^{\bullet+}$ radical is generated by oxidation of ABTS with potassium persulfate and may reduce in the presence of such hydrogen-donating antioxidant. Besides, due to its operational simplicity, TEAC assay has been used in many research laboratories for studying antioxidant capacity.

2.6.5 Comparison of four assays system

There are various mechanisms of antioxidant activities. In order to provide the more reliable and accurate antioxidant capacity, multiple assays based on different antioxidant mechanism are used. A single assay is not sufficient to measure the total antioxidant capacity in mushrooms. Each assay has its own principle and mechanisms. DPPH is widely used to determine antioxidant activity that act as free radical scavengers or hydrogen donors. This is a simple, rapid and inexpensive method. However, DPPH reacted very slowly with the sample and sensitive to acidic pH. On the other hand, lipid peroxidation is the most common method used to determine aldehydic products by their ability to react with thiobarbituric acid (TBA) that yield 'thiobarbituric acid reactive substance' (TBARS). Though, this method is sensitive, it is not specific

and TBA can react with a number of components present in biological sample (Devasagayam et al., 2003).

Ferric Reducing Antioxidant Power (FRAP) is a simple and quick method to determine antioxidant activity. The reaction is reproducible and linearly related to molar concentration of the antioxidants. However, study from Guo et al. (2003) found that FRAP assay does not react fast with some antioxidants such as gluthathione. Furthermore, TEAC assay measures the relative ability of antioxidant to scavenge the ABTS generated in aqueous phase. This method has flexibility that can be used at different pH levels. Additionally, it is useful to measuring antioxidant activity of samples extracted in acidic solvents. Hence, numerous assays based on different antioxidant mechanisms are crucial in order to provide more reliable approach to assess the antioxidant capacity of the mushrooms.

2.7 *Pleurotus* species (oyster mushroom)

The genus *Pleurotus* (Jacq.: Fr.) Kumm. (pleurotaceae, higher basidiomycetes) is the most commonly cultivated edible mushroom worldwide especially in south east Asia, India, Europe and Africa. *Pleurotus* species are commonly called oyster mushroom due to its oyster shaped basidiocarp (Patel et al., 2012). Most of the *Pleurotus* species are found in both tropical and temperate climates throughout the world in the range 20-32^{O}C. Besides, it is a lignocellulolytic fungus that grows naturally on dead and decaying matters and it is a second grade among the important cultivated mushrooms due to easiest, fastest and cheapest to grow apart of require less preparation time and production technology (Mandeel et al., 2005). China is the major producer and contributes to about 88% of the total world production of *Pleurotus* species. There are about 40 species are coming under *Pleurotus* mushrooms (Jeena et al.,

2014) but only 25 species are commercially cultivated in different parts of the world, to mention a few; *P.ostreatus, P.florida, P.eryngii, P.cornucopiae, P.sajor-caju, P.flabellatus, P.sapidus, P.cystidiosus* and so on (Singh et al., 2011). The basidiocarps or fruiting bodies of the oyster mushrooms have three distinct parts, a fleshy shell or spatula shape cap (pileus), a short or long lateral and central stalk called stipe, long ridges and furrows underneath the pileus called gills or lamellae. The gills stretch from the edge of the cap down to the stalk and bear the spores. Visually, there are different shades of white, grey, yellow, pink or light brown depending upon species.

In Malaysia, there is high market demand for oyster mushroom due to high nutritional value, therapeutic properties and biotechnological applications. Several studies have been reported that *Pleurotus* species will generate beneficial effects for health and in the treatment of diseases. Mattila et al. (2002) reported that mushrooms proved to be a good source of almost all essential amino acids such as cysteine, methionine and aspartic acid compared to common vegetables. In addition, in the world of medicinal mushrooms, *Ganoderma* has been considered as a king of medicinal mushrooms followed by *Lentinula* but recently, *Pleurotus* also has a potential as a good medicinal properties (Patel et al., 2012). *Pleurotus* has significant antioxidant activity that could serve as an easily accessible item of natural antioxidant food which may enhance immune system against oxidative damage (Menaga et al., 2013). Khatun et al. (2014) excessively studies an antioxidant activity in *Pleurotus* species reported that oyster mushrooms are comparable to the best antioxidant which is attributed to their catalase, phenolics and peroxidase contents.

2.7.1 *Pleurotus pulmonarius* (Fries) Quélet

In Malaysia, genus *Pleurotus* is widely cultivated. Currently, *Pleurotus pulmonarius* is cultivated throughout the world and it is the second most important mushroom in production due to its definite nutritive (high quality proteins, vitamins and very low lipids or starch) and medicinal value (Kanagasabapathy et al., 2013). *Pleurotus pulmonarius* (Fries) Quélet has been called by the other name which is *Pleurotus sajor-caju.* Nutritive values of different mushroom are given in Table 2.2. *Pleurotus sajor-caju* showed high nutritional value compared to other edible mushrooms.

Generally, *P. pulmonarius* can be characterised by spathulate-shaped, brown pileus, a lateral to subcentral stipe, cream to buff spore print and a monomitic hypae system (Bao et al., 2004). Research done by Pramanik et al. (2005) reported that *P. sajor-caju* contain vitamins (B1, B2, B12, C, D, folate and niacin), minerals, lipids and carbohydrates that may reduce the cholesterols level in blood. Besides that, *P. sajor-caju* also has good antioxidant activities that contain linoleic acid, cinnamic acid and nicotinamide (Kanagasabapathy et al., 2011). Extensively study by Kanagasabapathy et al. (2012) revealed that low fat and high fibres of the *P. sajor-caju* have been useful in the prevention of diabetes mellitus.

Table 2.2: Nutritive values of different mushrooms (dry weight basis g/100g). Adopted from Singh et al. (2011).

Mushroom	Carbohydrate	Fibre	Protein	Fat	Ash	Energy k cal
Agaricus bisporus	46.17	20.90	33.48	3.10	5.70	499
Pleurotus sajor-caju	63.40	48.60	19.23	2.70	6.32	412
Lentinula edodes	47.60	28.80	32.93	3.73	5.20	387
Pleurotus ostreatus	57.60	8.70	30.40	2.20	9.80	265
Volvariella volvaceae	54.80	5.50	37.50	2.60	1.10	305
Calocybe indica	64.26	3.40	17.69	4.10	7.43	391
Flammulina velutipes	73.10	3.70	17.60	1.90	7.40	378
Auricularia auricula	82.80	19.80	4.20	8.30	4.70	351

2.7.2 *Pleurotus pulmonarius* hybrids

To date, people woke up to the beneficial effects of mushrooms (Chakravarty, 2011). Due to, several researcher have used mating test to clarifying the biological species in basidiomycetous mushrooms instead of improving the genetic of cultivated mushrooms (Bao et al., 2004). In Malaysia, oyster mushrooms are widely consumed. Several researchers are excessively study the cross-mating between the *Pleurotus* species (oyster mushroom) due to its nutritious potential and medical properties. The study done by Avin et al. (2012) reported that the production of the new high yield hybrid especially *Pleurotus* species could improve the market, reduce the cost in cultivating and increase the farmer's revenue. University Putra Malaysia (UPM), National University of Malaysia (UKM), and Malaysia Research Agriculture Research

and Development Institute (MARDI) in collaboration with Department of Agriculture (DOA) were early conducted research on mushroom cultivation in Malaysia. *Pleurotus pulmonarius* was introduced due to its fast growing mushroom. Actually, the strain of *P. pulmonarius* which is PL-27 in India was released by University Putra Malaysia in 1963. The mating between *P. pulmonarius* produced the hybrids that are still in the research by University of Malaya. Several hybrids have been generated by the University of Malaya. Besides that, the grower also obtained the mushroom from China, Thailand and also American Type Culture Collection (ATCC). The phylogenetic tree of the *Pleurotus pulmonarius* hybrids are showed in Figure 2.6. The hybrids are produced from the mating between the parents from India (PL-27), Thailand and local.

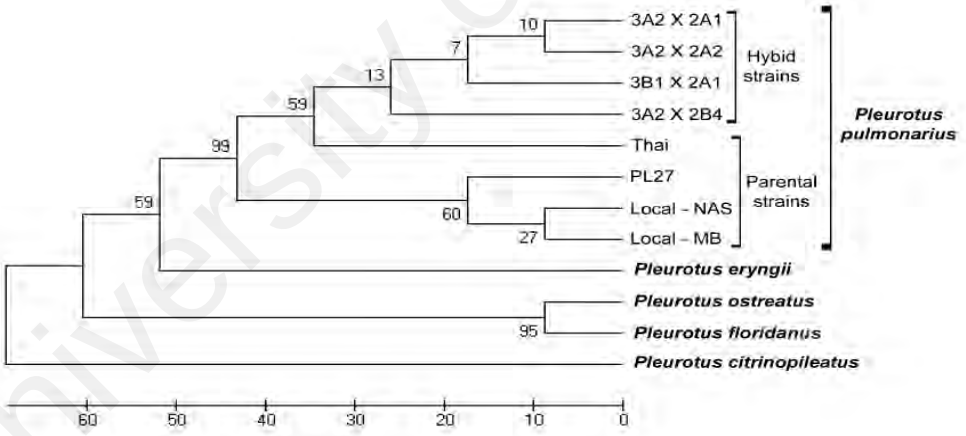


Figure 2.6: Phylogenetic tree of *Pleurotus pulmonarius* hybrids and their parents. Adopted by Avin et al. (2012).

Up to date, many agricultural breeders desired high quality, high yield performance and resistance to disease mushroom. The breeding of economically important mushrooms especially the white button mushroom, *Agaricus bisporous* and the oyster mushroom was done by Kothe (2001) in order to increase the yield and quality of the mushroom production. The desired

characteristics from different varieties of mushrooms can be selected during mating programme. However, due to more often use of mushroom as a food, an antioxidant activity of the hybrid mushroom could be evaluated instead of the production yield. Research done by Gorinstein et al. (2004) revealed that the total phenol content and antioxidant potential are significantly higher in grapefruit hybrids than in white grapefruits. The level of polyphenol and antioxidant activity has significantly higher in the extract of soybean hybrids (Malenčić et al., 2008). On the other hand, study by Maksimović et al. (2005) reported that antioxidant activity of the maize hybrids were higher due to high level of polyphenol. Therefore, that is why in this study, antioxidant activity of the *P. pulmonarius* hybrids were evaluated in order to determine either the hybrid or the parent showed a better antioxidant activity as the study has never been done before.

2.8 Fibrinolytic enzymes

Recently, fibrinolytic enzymes have been discovered in both food and non-food sources. Fibrinolytic enzymes are mostly extracted from snake venom, earthworm secretion and bacteria. Further, it also can be found in a variety of selected traditional foods such as Japanase natto, tofuyo, Korean Chungkook-Jung soy sauce as well as edible mushroom (Mine et al., 2005). The ability of fibrinolytic enzyme such as nattokinase, streptokinase or urikinase to degrade fibrin and inhibit clot formation may have a great potential for antithrombotic therapy (Lu and Chen, 2012). The use of edible mushroom as a fibrinolytic agent was successfully done by Kim and Kim (1999). Their study revealed that an edible mushroom *Armillariella mellea* has a potential for fibrinolytic therapy. Besides, Cui et al. (2008) revealed that the medicinal and edible mushroom, *Cordyceps militaris* showed a strong fibrinolytic enzyme and become a source

of theombolytic agents. The fruiting body of *Pleurotus eryngii* also produces fibrinolytic enzyme that could be useful in thrombolytic therapy (Cha et al., 2010).

CHAPTER 3

MATERIALS AND METHODS

3.1 Mushroom species

All fruit bodies were obtained from Mushroom Research Centre, University of Malaya. The code of the *Pleurotus pulmonarius* hybrids and parents used is illustrated in Table 3.1.

Table 3.1: The code of the *Pleurotus pulmonarius* hybrids and parents

Sample	Code
Hybrids	• UMH001 • UMH002 • UMH004
Parents	• UMP001 (Thailand) • UMP002 (India) • UMP003 (Unknown source and considered as commonly grown)

3.2 Chemicals

Chemicals and reagents were of analytical grade. Gallic acid, 2,2-diphenyl-1-picrylhydrazle (DPPH), trichloroacetic acid (TCA), thiobarbituric acid (TBA), phosphate buffer tablet, butylated hydroxyanisole (BHA), ferrous sulphate, potassium ferricyanide, ferric chloride, 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid (Trolox), 2,2-azino-di[3-ethylbenzthiazoline sulfonate] (ABTS), Tris-hydrochloride and Dulbecco's Modified Eagle's Medium (DMEM) were obtained from Sigma-Aldrich ® Inc (USA). MTS reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium was obtained from Promega. potassium persulfate was obtained from Acros organics. Folin Ciocalteu's phenol reagent, sodium carbonate and

dimethyl-sulfoxide (DMSO) were purchased from Merck. Absolute methanol was purchase from Fisher Scientific ® UK Ltd (UK).

3.3 Preparation of mushroom hot aqueous extracts

The mushroom hot aqueous extracts were prepared according to the method Abdullah et al. (2011) with some modification. The preparation of hot aqueous extracts was done to mimic the cooking methods. *Pleurotus pulmonarius* hybrids and the parents were obtained from the Mushroom Research Centre (MRC), University Malaya. The fruit bodies were cleaned, cut into small pieces and boiled in the distilled water at the ratio 1:10 (w/v) at 100 ^{O}C for 30 minutes. After boiling, all the mushrooms were cooled at room temperature and remove using thin transparent fabric. Then, the hot aqueous extracts were then freeze-dried (Christ Beta 2-8 LD Plus) and kept in the desiccator at 3^{O}C for further analysis. In all experiments, the hot aqueous extract was used, unless otherwise stated.

3.4 Estimation of Antioxidant Activity

3.4.1 DPPH Free Radical Scavenging Activity

The ability of the extracts to donate hydrogen atom was evaluated using 2,2-diphenyl-1- picrylhydrazle (DPPH) according to a modified method described by Brand-William et al. (1997). The DPPH was dissolved in methanol. Then, 195 µL of 0.06 mM DPPH radical was added to 5 µL of *Pleurotus* extract at different concentrations (5, 10, 15, 20 and 25 mg/ml). The mixture was incubated for 3 hours in the dark and the absorbance was measured at 515 nm, against a blank using an ELISA plate reader machine (Sunrise Basic Tacan). The scavenging activity was calculated as follows:

$$\text{Scavenging activity} = \frac{(\text{Absorbance of control} - \text{absorbance of sample})}{\text{Absorbance of control}} \times 100$$

The IC_{50} values of the extracts were then calculated. The IC_{50} value is the inhibition concentration at which the DPPH radicals were scavenged by 50%. Butylated hydroxyanisole (BHA) was used as a positive standard and methanol was used as a negative control. All the determinations were performed in triplicates.

3.4.2 Inhibition of Lipid Peroxidation of Buffered Egg yolk

The lipid peroxidation was measured according to the method of Kuppusamy et al. (2002) and Daker et al. (2008) with some modification. One millilitre of egg yolk buffer (0.1 M phosphate buffer, pH 7.4) was added into 100 µL of mushroom extract at different concentration (10-100 mg/ml). Then, 100 µL of ferrous sulphate was added to all tubes. The mixture was incubated at 37 ^{O}C for one hour at room temperature. After incubation, 500 µL of 15% trichloroacetic acid and 1000 µL of 1% thiobarbituric acid (TBA) were added into the mixture. The mixture was boiled in water bath for 15 minutes at 100 ^{O}C and left to cool down. Then, the mixture was centrifuged for 10 minutes at 3500 rpm. Further, 100 µl of the supernatant was pipetted out into an ELISA plate and the absorbance was taken at 532 nm using ELISA plate reader machine (Sunrise Basic Tecan) to measure the formation of thiobarbituric acid reactive substances (TBARS). Buffered egg with Fe^{2+} only was used as negative control and Butylated hydroxyanisole (BHA) was used as positive control in this assay. All determinations were performed in triplicates. The percentage of the inhibition was calculated using the equation:

$$\text{Inhibition (\%)} = \frac{\text{absorbance of the control} - \text{absorbance of the sample}}{\text{Absorbance of the control}} \times 100$$

3.4.3 Ferric Reducing Antioxidant Power

The reducing power was determined according to the method Öztürk et al. (2007) with some modification. In tis assay, 2.5 mL of mushroom extracts were mixed with 2.5 mL of 0.04 M phosphate buffer (pH 7.49) and 2.5 mL of 1% potassium ferricyanide. The reaction mixture was incubated at 50 ^{O}C for 20 minutes. After incubation, 2.5 mL of 10% trichloroacetic acid (TCA) was added. Then, the mixture was centrifuged for 10 minutes at 1000 rpm. Then, 2.5 mL of the supernatant was mixed with 2.5mL of distilled water and 0.5 mL of 0.1% ferric chloride. The absorbance was measured at 700 nm against blank. The higher absorbance indicates a higher reducing power. Butylated hydroxyanisole (BHA) was used as a positive control and distilled water was used as negative control. All determinations were performed in triplicates.

3.4.4 Trolox Equivalent Antioxidant Activity (TEAC) assay

The TEAC assay is an electron-based assay which involving the ability of antioxidant to quench the long-live $ABTS^{\bullet+}$. The assay was determined according to the method Re et al. (1999) with some modification. The $ABTS^{\bullet+}$ reagents were prepared by mixing 5 mL of 7mM $ABTS^{\bullet+}$ with 89 µl of 140 mM $K_2S_2O_8$. The mixture was added and kept in the dark at room temperature for 16 h. After 16 h, PBS buffer (pH 7.4) was used to adjusted the absorbance of the $ABTS^{\bullet+}$ reagent to 0.7±0.05 at 734 nm. One millilitre of $ABTS^{\bullet+}$ reagent was added to 100 µL of mushroom extract. Then, the mixture was allowed to stand for I minute and the absorbance was measured at 734 nm. Trolox (water-soluble Vitamin E analogue) was used as a standard. Butylated hydroxyanisole (BHA)

was used as a positive control and distilled water was used as negative control. The TEAC values were calculated based on final concentration and expressed as mmol Trolox equiv/100g of dry weight. All determinations were performed in triplicates.

3.5 Determination of Total Phenolic Content

The total phenolics of mushroom extracts were measured according to the method Slinkard and Singleton (1977) with some modifications. 250 µL of 10% Folin Ciocalteu's phenol reagent was added into 250 µL of mushroom extract (5 mg/ml). The mixture was incubated for three minutes and 500 µL of 10% sodium carbonate was added. Then, the mixture was incubated for one hour in the dark. The absorbance was measured at 750 nm against a blank using UV-vis spectrophotometer (Shimadzu). Gallic acid was used as the reference standard. Butylated hydroxyanisole (BHA) was used as positive control and distilled water was used as negative control. The total phenolic content is expressed as mg of gallic acid equivalents (GAE)/100g of dry weight. All determinations were performed in triplicates.

3.6 Antioxidant Index (AI)

Antioxidant Index (AI) was used to grade the mushroom samples on the basis of their antioxidant potential as proposed by Puttaraju et al. (2006). Mushroom samples were grade in a numerical scale based on Butylated hydroxyanisole (BHA) which is considered to be equivalent to 100. AI represents the average percentage compared to BHA of the results obtained based on the four methodologies for estimation of antioxidant activity.

3.7 Screening of fibrinolytic activities

The fibrin plate assay was performed to screen the fibrinolytic activity according to the method of Kim and Kim (1999) with some modification. The hot aqueous extracts were suspended in 20mM Tris-hydrochloride buffer (pH 8.0) in a 1:20 ratio. Then, the suspended samples were incubated in ice bath for one hour and centrifuged at 7000rpm for 30 min to remove cell debris. The supernatant was used to test fibrinolytic activities. The freeze-dried fibrin powder was dissolved in distilled water (0.6% -w/v) followed by homogenisation at 11000g for 30s. The homogenate was then centrifuged at 10000g for 15 min to spin down undissolved fibrin. The supernatant was collected as a fibrin solution and warmed up to 45±2 ^{O}C whereas the agarose was prepared at 2% (w/v) with distilled water and maintain at 55±2 ^{O}C. The fibrin solution was mixed with 2% agarose in 1:1 ratio and poured onto a Petri-dish to form a thin of fibrin film. The clot was allowed to solidify for one hour at room temperature. After that, 20µL of crude extracts were carefully dropped on the fibrin film in the Petri-dish. The Petri-dish was incubated at 37±2 ^{O}C for 12 hour. The lytic zone was measured. Plasmin at 2.28mg/ml was used as positive control and distilled water was used as a negative control. A clear lytic region was observed when fibrin was hydrolysed and the diameter of lytic zone was directly proportional to the strength of the fibrinolytic activity. All determinations were performed triplicates.

3.8 Measurement of cell viability (MTT assay)

The cell viability was measured according to the method Dudhgaonkar et al. (2009) with some modification. RAW264.7, macrophage cells were obtained from American Type Cell Culture (ATCC). RAW264.7 cells were collected and

100 µL aliquots containing 5 x 10^4 cells were dispensed into each well of a 96-well culture plate. After allowing overnight attachment, 50 µL of Dulbecco's Modified Eagle's Medium (DMEM) and 50 µL of samples were added into the wells at the range (0-100 µg/mL). Then, the culture plates were incubated for 12 h prior to addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent. After that, 10 µL aliquot of MTS reagent was added into each well and continuously incubate at 37^{O}C in 5% CO_2 under humidified 95% air for 2 h. Finally, the absorbance of each well was measured using ELISA plate reader at 490 nm. The percentage of cell viability was calculated using the equation:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of the sample}}{\text{Absorbance of the control}} \text{ X } 100$$

3.9 Nutritional analysis

The samples of hybrid (UMH004) and parents (UMP005 and UMP006) were analysed for protein, total carbohydrates, total fat and total dietary fibre content using AOAC procedures (AOAC, 1995). The estimations were carried out at DXN laboratory.

4.0 Statistical analysis

All analysis was performed in triplicates and data was recorded as means ±standard deviation. Data were analysed using SPSS for Windows (ver. 17) as one way analysis of variance (ANOVA). Significant level was set at $p < 0.05$ using Duncan's multiple range test (DMRT). Pearson's correlation analysis was carried out to determine the correlation between the assays.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Estimation of Antioxidant Activity

4.1.1 Scavenging of DPPH Free Radicals

The scavenging activity of the mushroom extracts towards DPPH free radicals were expressed in IC_{50} value (Table 4.1). The lower IC_{50} value indicates stronger ability of the extract to act as DPPH free radicals scavenger. The DPPH scavenging effects of hybrids and parents of *P. pulmonarius* hot aqueous extract is presented in Table 4.1. The descending order of their activity; BHA > UMH001 > UMH004 > UMH002 > UMP003 > UMP001 > UMP002. The highest DPPH scavenging activity was observed in UMH001 (IC_{50} 11.86±0.42mg/ml) followed by the other two hybrids UMH004 and UMH002 showing an IC_{50} value of 15.84±0.41mg/ml and 16.04 ±3.81mg/ml, respectively. A significant increase ($P<0.05$) in DPPH scavenging activity was observed in UMH001 and UMH002 compared to their respective parents, UMP001 and UMP002. The IC_{50} values of all parents were between the range of 17 and 23 mg/ml. Interestingly, *P. pulmonarius* hybrids showed significantly high ($P<0.05$) scavenging activities compared to parents. Even though, the scavenging activity of the standard BHA is many folds higher than hybrids and parents of *P. pulmonarius*, the activity is better compared to other mushroom species. Our results showed the radical scavenging activity of aqueous extract of *P. pulmonarius* hybrids (11.86-16.04 mg/ml) were better than other *Pleurotus* species (IC_{50} of 15.42-31.50 mg/ml) reported by Abdullah et al. (2011).

Obviously, the extracts that possessed antioxidant components could react rapidly with DPPH free radical and may reduce the DPPH radical molecule. The different kinetic reaction depends on the nature of the antioxidant (Brand-William et al., 1995). Tsai et al. (2006) revealed that mushroom extracts were act as free radical scavenger and possibly act as a primary antioxidant.

Basically, antioxidant activity can be determined by the reduction of methanolic DPPH solution in the presence of hydrogen donating (antioxidant). The resulting decolourisation from deep purple to pale yellow is stoichiometric with respect to number of hydrogen atom captured the radical of DPPH. The reduction capacity of the DPPH free radical scavenging was determined by the decrease in its absorbance at 515nm. Several studies reported that the higher phenolic content correlates with high radical scavenging activity. Cheung et al. (2010) reported that better scavenging activity in mushroom extracts might be due to the higher phenolic contents.

Several studies reported that fruits and vegetables possess higher scavenging activity (Du et al., 2001; Zhou and Yu, 2006). Interestingly, Miller et al. (2000) noticed that mushrooms had higher in antioxidants than most vegetables and fruits. Besides, three edible seaweeds which were *Padina antillarum*, *Caulerpa racemosa* and *Kappaphycus alvarezzi* showed better scavenging activity (IC_{50} in the range 0.337 to 14.3 mg/ml) (Chew et al., 2008). Thus, it can be concluded that *P. pulmonarius* hybrids had a higher antioxidant activity in terms of radical scavenging activity on the DPPH free radical. The compound to higher scavenging activity of the mushroom could be due to the more hydrogen donating components extracted from the mushroom (Sudha et al., 2012). Free radical scavenging seem to be known as an established phenomenon in inhibiting lipid oxidation, which otherwise can have deleterious

effects on cellular component and cellular function (Puttaraju et al., 2006). Hence, with the presence of radical scavenging activity, the consumption of mushrooms may be beneficial in protecting human body from the oxidative damage.

Table 4.1: Antioxidant activity of *Pleurotus pulmonarius* hybrids and parents as determined by the DPPH free radical scavenging activity.

Sample	DPPH free radical scavenging activity IC_{50} (mg/ml)
UMH001	11.86 ± 0.42^{b}
UMH002	16.04 ± 3.81^{c}
UMH004	15.84 ± 0.41^{c}
UMP001	22.96 ± 0.77^{d}
UMP002	26.35 ± 2.30^{e}
UMP003	17.93 ± 0.82^{c}
Butylated hydroxyanisole (BHA) (positive control)	0.11 ± 0.02^{a}

* values are mean ± standard deviation of three replicates.

mean values in the column with different lowercase letters (a-e) indicate significant difference ($p<0.05$).

4.1.2 Inhibition of Lipid Peroxidation of Buffered Egg Yolk

The ability of the mushroom extracts to inhibit lipid peroxidation is presented in Table 4.2. Similar to DPPH assay, a low IC_{50} value indicates higher inhibition of lipid peroxidation and vice-versa. The IC_{50} values of the inhibition of lipid peroxidation of the mushroom extracts ranged from 29.57 ± 0.89mg/ml to 92.17 ± 2.55 mg/ml. Among the hybrids and parents, UMH001 exhibited the highest inhibition (IC_{50} 29.57 ± 0.89) followed by UMH002 (IC_{50} 47.68 ± 2.18). The weakest extract to inhibit lipid peroxidation was UMP003 with IC_{50} of 92.17 ± 2.55 mg/mL. Apparently, *P. pulmonarius* hybrids showed a significant increase ($p<0.05$) in inhibition of lipid peroxidation compared to the parents. Although, BHA had excellent ability to inhibit lipid peroxidation with the IC_{50} of 0.09 ± 0.02 mg/ml compared to all mushroom extracts. In this study, the buffered egg yolk was used in the lipid peroxidation assay in order to determine the ability of the mushroom extracts to inhibit lipid peroxidation of

phospholipids in egg yolk. Aldehyde products were estimated by their ability to react with thiobarbituric acid (TBA) that yields thiobarbituric acid reactive substances (TBARS). The formation of pink colour indicates that the product of lipid peroxidation react with TBA that absorbs at 532 nm (Devasagayam et al., 2003).

Table 4.2: Antioxidant activity of *Pleurotus pulmonarius* hybrids and parents as determined by inhibition of lipid peroxidation using buffered egg yolk.

Sample	Lipid peroxidation IC_{50} (mg/ml)
UMH001	29.57 ± 0.89^{b}
UMH002	47.68 ± 2.18^{c}
UMH004	78.14 ± 3.25^{e}
UMP001	66.30 ± 4.48^{d}
UMP002	89.47 ± 9.16^{f}
UMP003	92.17 ± 2.55^{f}
Butylated hydroxyanisole (BHA) (positive control)	0.09 ± 0.02^{a}

*values are mean ± standard deviation of three replicates.

mean values in the column with different lowercase letters (a-f) indicate significant difference ($p<0.05$).

Generally, lipid peroxidation can be defined as a process induced by free radicals that lead to oxidative deterioration in lipids and fats. Hence, it might damage the cell structure and lead to the cell death. To date, the food industry has become a great concern to the effects of lipid peroxidation because it leads to the formation of undesirable flavour and development of rancidity (potentially toxic to reaction products). Deterioration during the storage of fats, lipids and other lipid-rich foods are a major cause by lipid peroxidation (Wang, 2005). Therefore, many synthetic antioxidant such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ) have been efficiently used to retard the oxidation and prevent peroxidation processes. However, the use of this synthetic antioxidant has been reported to cause toxicity problems of (Ansari et al., 2005).

Khan et al. (2011) suggested that *Pleurotus* mushrooms had a potential in lowering the lipid peroxidation due to their nutritional and chemical composition. He and his co-worker found that vitamin E is an important natural antioxidant had capability to protect lipid peroxidation. Moreover, inhibition of lipid peroxidation against rat liver homogenate was studied by Jayakumar et al. (2009) and reported that *P. ostreatus* showed a dose-dependent inhibition of lipid peroxidation and at 10mg/mL the percentage of inhibition of *P. ostreatus* was 56.20%. Another study by Cheung and Cheung (2005) revealed that the mushroom extracts had ability to inhibit lipid peroxidation on rat brain homogenate with dichloromethane subfraction of *Volvariella volvacea* and *Lentinula edodes* that possessed lower IC_{50} values of 0.109 mg/ml and 0.297 mg/ml, respectively. In contrast, our study showed that *P. pulmonarius* hybrids and parents needed high concentration of extracts (29.57 ± 0.89 mg/ml to 92.17 ± 2.55 mg/ml), respectively to inhibit 50% lipid peroxidation. However, study done by Cheung et al. (2003) revealed that in some cases stronger inhibition of lipid oxidation occurring at higher concentrations of mushroom extracts. As a conclusion, *P. pulmonarius* hybrid extracts had better ability to inhibit lipid peroxidation than parents.

4.1.3 Ferric Reducing Antioxidant Power

Reducing power ability of *P. pulmonarius* hybrids and parents were tested in different range of concentration from 0.1 mg/ml to 10 mg/ml. The results showed that the reducing power was dose-dependent which increased with increasing concentrations (Table 4.3). High absorbance values indicate effective ferric reducing power. In the present study, the positive control that was butylated hydroxyanisole (BHA) at 0.1-10mg/mL exhibited the highest reducing power (absorbance 0.67 to 3.73), which was distinctively higher than all mushroom extracts that had been tested. Surprisingly, in contrast to DPPH and Lipid peroxidation assays, all the hybrids showed lower reducing power (absorbance 0.12 to 0.14) as compared to parents (absorbance 0.13 to 0.16) at 1.0 mg/ml. Among the mushrooms, UMP002 exhibited highest reducing power ability of (absorbance 1.17) at the maximum concentration test (10.0 mg/ml). Apparently, the reducing power of UMP002 (absorbance 0.16) was comparable to work done by Abdullah et al. (2011). They mentioned that reducing power of *Pleurotus eryngii* was 0.165 (absorbance) at 1.0 mg/ml.

Fe^{3+}-ferricyanide complex is reduced to form ferrous form Fe^{2+} in the presence of antioxidants which turn the colour from yellow to Perl's Prussian blue (Barros et al., 2007). In the presence of reducing agents as well as reductones such as ascorbic acid may increase reducing properties of an antioxidant (Duh et al., 1999). Reducing capacity of compounds could serve as an indicator of potential antioxidant properties (Adesegun et al., 2009) and higher absorbance indicates greater reducing power (Öztürk et al., 2007). Study done by Abdullah et al. (2011) mentioned that mushroom extracts were showed

variable reducing capacity. Therefore, we can conclude that reducing capacity increased with the increasing concentration of mushroom extracts.

Table 4.3: Antioxidant activity of extracts of *P. pulmonarius* hybrids and parents as determined by the ferric reducing power (FRAP).

Sample	Absorbance values (at 700nm) of *P. pulmonarius* hybrids and the parents of different concentrations (mg/ml)				
	0.1	0.5	1.0	5.0	10.0
UMH001	0.01 ± 0.002^{a}	0.07 ± 0.002^{a}	0.12 ± 0.002^{a}	0.50 ± 0.004^{bc}	0.91 ± 0.043^{a}
UMH002	0.01 ± 0.001^{a}	0.07 ± 0.002^{a}	0.14 ± 0.005^{a}	0.44 ± 0.021^{a}	1.16 ± 0.027^{b}
UMH004	0.02 ± 0.001^{ab}	0.08 ± 0.004^{a}	0.14 ± 0.013^{a}	0.58 ± 0.014^{d}	1.05 ± 0.022^{ab}
UMP001	0.03 ± 0.003^{b}	0.08 ± 0.002^{a}	0.14 ± 0.007^{a}	0.54 ± 0.012^{cd}	0.94 ± 0.033^{a}
UMP002	0.03 ± 0.001^{b}	0.09 ± 0.001^{a}	0.16 ± 0.004^{a}	0.65 ± 0.007^{e}	1.17 ± 0.030^{b}
UMP003	0.02 ± 0.001^{a}	0.07 ± 0.001^{a}	0.13 ± 0.001^{a}	0.46 ± 0.017^{ab}	0.92 ± 0.046^{a}
Butylated hydroxyanisole (BHA) (positive control)	0.67 ± 0.0102^{c}	2.26 ± 0.102^{b}	2.79 ± 0.071^{b}	3.71 ± 0.065^{f}	3.73 ± 0.181^{c}

*values were expressed as mean ± standard deviation of three triplicates.

mean values in the column with different lower case letters (a-f) indicate significant difference (at $p<0.05$).

Furthermore, study done by Lee et al. (2007) revealed that *Pleurotus citrinopileatus* possessed higher reducing power ability than other *Pleurotus* spp. Hot water extracts and hot alkali extracts containing polysaccharides from mycelia of *Ganoderma tsugae* showed a steady increase in the absorbance recorded at 700 nm (absorbance 0.41 to 0.52 and absorbance 0.87 to 1.14 at the concentration 20 mg/ml, respectively) (Tseng et al., 2008). Obviously, in our result, *P. pulmonarius* hybrids and parents showed higher reducing power ability ranging from 0.91 to 1.17 (absorbance) at 10 mg/ml compared to *Ganoderma tsugae*. However, the hot water extract from mature and baby Ling chih, mycelia and filtrate were reported to have a reducing power at absorbance 0.48, 0.44, 0.23 and 0.42 at 1.0 mg/ml (Mau et al., 2005). These values were higher than those obtained in the present study of both *P. pulmonarius* hybrids and parents (absorbance 0.12 to 0.16), respectively at 1.0 mg/ml. Moreover, Oboh (2005) reported that green leafy vegetables also possess higher reducing capacity. The study mentioned that higher reducing power capacity could be due high phenolic content that could compensate the loss of vitamin C and other non-phenolic antioxidant presented in the vegetable. Hence, with regard to reducing power, it can be concluded that higher reducing power ability of mushroom extracts could be due to the presence of components that can react directly with peroxides and also with certain precursor to prevent peroxide formation as well as hydrogen-donating ability that lead to terminate free radical chain reaction.

4.1.4 Trolox Equivalence Antioxidant Capacity

The TEAC value of *P. pulmonarius* hybrids and parents varied from 32.79 to 70.41 µmol TE/100 g dry weight (Table 4.4), which was not significant compared to the standard BHA (4.45 x 10^3 µmol TE/g dry weight). Among the mushrooms, UMP003 showed higher TEAC value (70.41 µmol TE/100 g dry weight). The lower TEAC value of 32.79 µmol TE/100 g dry weight was observed in UMH004. The results showed that there was significantly different in the TEAC values ($p<0.05$) between *P. pulmonarius* hybrids and parents. The parents possess significantly ($P<0.05$) higher TEAC value (42.43 to 70.41 µmol TE/100 g dry weight) than hybrids (32.79 to 46.56 µmol TE/100 g dry weight). Standard linear calibration curve of trolox was used as a reference and the concentration used for all mushroom extracts were 5 mg/ml. Trolox (an analog of vitamin C) was used as a standard because it can be dissolved in aqueous and organic media.

TEAC assay involve the reaction between antioxidant and oxidant ($ABTS^{\bullet+}$ radical) in the mixture that may reduce the radical cation (Re et al., 1999). When there is antioxidant compounds presence in the reaction medium, they may capture the free radical and decolourise the blue-green colour of $ABTS^{\bullet+}$ radical which is measured by the decrease in absorbance at 734 nm (Zulueta et al., 2009). The $ABTS^{\bullet+}$ radical was generated by the reaction between ABTS reagent and potassium persulfate that presented as excellent tool for determining antioxidant activity of hydrogen- or electron-donating antioxidant (scavenger of aqueous phase radicals) and of chain breaking (scavenger of lipid peroxyl radicals) (Leong and Shui, 2002). Arnao et al. (2001) suggested that this method is more accurate and rigorous to determine antioxidant activity because it may avoid interference due to endogenous

peroxidase activity in samples. Besides, Re et al. (1999) and Rice-Evans et al. (1996) reported that ABTS•$^{+}$ assay can be applied in the measurement of antioxidant activity of a broad diversity of substances such as aqueous mixtures as well as beverages.

Table 4.4: Antioxidant activity of *Pleurotus pulmonarius* hybrids and parents as determined by trolox equivalence antioxidant capacity (TEAC).

Sample	TEAC (µmol TE/100 g dry weight)
UMH001	46.56 ± 0.26^{b}
UMH002	53.08 ± 0.45^{c}
UMH004	32.79 ± 0.09^{a}
UMP001	42.43 ± 0.92^{b}
UMP002	66.79 ± 1.49^{d}
UMP003	70.41 ± 0.19^{d}
Butylated hydroxyanisole (BHA) (positive control)	TEAC (µmol TE/g BHA) $4.44 \times 10^{3} \pm 8.88^{e}$

*values are mean ± standard deviation of three replicates.

mean values in the column with different lowercase letters (a-e) indicate significant difference ($p<0.05$).

Moreover, study done by Kanagasabapathy et al. (2011) reported that an aqueous extract of *P. sajor-caju* had higher TEAC value (29.45± 0.87 Mm TE/ g of fresh mushroom) compared to other mushrooms. Further, study done by Tan et al. (2015) revealed that *P. pulmonarius* (117.24±2.45µmol TE/150g) possess higher TEAC value than *P. eryngii* (73.30±5.50 µmol TE/150g). Hagerman et al. (1998) was observed that high ability to quench ABTS•$^{+}$ radical might be due to high molecular phenolic such as tannins and their effectiveness depend on the molecular weight, number of aromatic rings and nature of hydroxyl group's substitution than the specific functional group. On the other hand, Manian et al. (2008) reported that green tea, *Ficus racemosa* and *Ficus bengalensis* exhibited higher ability to quench ABTS•$^{+}$ radical due to the presence of high molecular phenolic such as catechins, pelargonin and leucopelargonin derivatives and flavonoid. Therefore, we can conclude that hot aqueous extract of *P.*

pulmonarius hybrids and parents may contain secondary metabolites that act as a good scavenger of radical cations.

4.2 Estimation of Total Phenolic Content

Total phenolic content of the *P. pulmonarius* hybrids and the parents is illustrated in Table 4.5. All the determinations were carried out in three replicates and were expressed as mg gallic acid equivalents (GAEs)/100g dry weight. By referring to Table 4.5, total phenolic content of *P. pulmonarius* hybrids and the parents tested varied from 88.59 to 118.25 mg GAE/100g dry weight. The hybrids UMH001 and UMH002 showed higher phenolic content (118.25 ± 0.04 and 115.65 ± 0.02 mg GAE/100g dry weight, respectively) compared to the reported values in other mushrooms, for instance *P. ostreatus* (5.49 mg GAE/100g) (Jayakumar et al., 2009). However, there was no significant difference ($P>0.05$) between the total phenolic content of *P. pulmonarius* hybrids and parents. In this study, a standard linear calibration curve of gallic acid was used as a reference. Gallic acid (3,4,5-trihydroxy benzoic acid) is a phenolic compound that found to be a strong antioxidant and widely used in processed food, cosmetics and food packaging to prevent rancidity induced by lipid peroxidation and spoilage (Yen et al., 2002). Zheng et al. (2001) reported that high content of rosmarinic acid (91.8 mg/100g fresh weight) and luteolin (39.5 mg/100g fresh weight) were found in the extracts of thyme. BHA, used as the positive control showed the highest phenolic content ($1.44 \times 10^4 \pm 0.06$ mg GAE/g extract) compared to all the hybrid and parent extracts.

Table 4.5: Total phenolic content of extracts of *Pleurotus pulmonarius* hybrids and the parents.

Sample	Total phenolic content (mg GAE/100g dry weight)
UMH001	118.25 ± 4.98^{a}
UMH002	115.65 ± 2.86^{a}
UMH004	88.59 ± 8.04^{a}
UMP001	100.33 ± 0.48^{a}
UMP002	104.42 ± 2.19^{a}
UMP003	97.41 ± 6.12^{a}
Butylated hydroxyanisole (BHA) (mg GAE/g BHA) (positive control)	$1.44 \times 10^{4} \pm 257.27^{b}$

*values were represented as mean ± standard deviation of three replicates.

mean values in a column with different lowercase letters (a-b) indicate significant difference ($p < 0.05$).

Phenolic compounds are widely distributed in plant and food plants that may sustain antioxidant and protect against free radical damage (Rice-Evans et al., 1997). A diet containing a major source of antioxidant and polyphenol is recommended for prevention of disease such as anti-carcinogenic, anti-atherogenic, anti-thrombotic, anti-inflammatory, immune modulating, anti-microbial, vasodilatory and analgesic effects (Jonfia-Essien et al., 2008). Phenolics are important constituents with scavenging ability due to their hydroxyl group and hence may contribute to the antioxidant action. Owing to the importance of total phenolic compounds and their contribution towards antioxidant activity, the colorimetric method, Folin-Ciocalteu was used to estimate the total phenolic content of mushroom extracts which is a rapid oxidation reaction of phenols by using alkali generally sodium carbonate that yield phenolate ions. The phenolates reduce the Folin-Ciocalteu reactives and change the yellow colour to blue pigment and spectrophotometrically measured

at 750 nm. However, it must be noted that Folin-Ciocalteu reagent does not exclusively react with phenolic substance but other reducing agents such as ascorbic acid and tocopherol (Yang et al., 2006; Meda et al., 2005). Previous literatures reported that genus *Pleurotus* contained several types of phenolic compounds such as gallic acid, homogentisic acid, protocatechuic acid, naringin (Kim et al., 2008), and also includes ergosterol, linoleic acid, nicotinamide, cinnamic acid, and fatty acid (Kanagasabapathy et al., 2011).

Furthermore, Study done by Poudel et al. (2008) showed the total phenolic content and antioxidant activity of hybrid grapes were higher compared to the parent. From the study, he expected that the changes in the content of total phenolics and other metabolites may inherited in the hybrids and their progenies during the cross mating. Hence, we can conclude that the cross mating between the gene could not totally change the content of total phenolics and others metabolites that inherited in the *P. pulmonarius* hybrids. In addition, the antioxidant activity of the *P. pulmonarius* hybrids was determined for the first time in this study and therefore, no data are available in the literature to compare with our results.

4.3 Correlation between antioxidant activity and total phenolic content

The results revealed that there was a weak correlation (R^2=0.611) between total phenolic content and inhibition of lipid peroxidation of hot aqueous extract of *P. pulmonarius* hybrids and parents. Hot aqueous extracts were prepared in this study to simulate cooking conditions. Besides that, there was no correlation between DPPH free radical scavenging activity and total phenolic content. The results were supported by the study of Chan et al. (2013) reported that there was no correlation between the antioxidant activities of *A.*

rugosum extracts and total phenolic content due to other non-phenolic compound that may exhibit antioxidant activities. The other non-phenolic compound that soluble in water and alcohol such as polysaccharide might influence antioxidant activity (Othman et al., 2007; Zeng et al., 2010). According to Chye et al. (2008) reported that no correlation between DPPH radical scavenging activity and metal chelating activity with total phenolics content for both petroleum ether (R^2=0.308) and methanolic extracts (R^2=0.069) of selected edible wild mushroom. Other studies (Jayakumar et al., 2009; Abdullah et al., 2011) reported presence of ascorbic acids, α-tecopherol, β-carotene and flavonoid compounds (rutin and chrysin) could be some of the naturally occurring antioxidant compounds in mushrooms responsible for the antioxidant activity.

Several studies revealed that mushrooms have phenolic compounds that may contribute to the antioxidant activity. A correlation of higher antioxidant activity and larger amount of total phenolics in mushroom was found by Cheung et al. (2003). According to Tsai et al. (2006) the phenolics are the major naturally occurring antioxidant components found in hot water extract of *Agrocybe cylindracea*. Excessively study by Tsai et al. (2007) revealed that tocopherol and total phenol were found as an antioxidant component in the extracts of several mushroom species. Total phenolic content of mushroom extracts can be related to the antioxidant activities as discussed earlier. Several studies found correlation between the total phenolic content (TPC) in hot water extracts and antioxidant activities. Abdullah et al. (2011) reported the strong correlation (R^2 = 0.8181) between the TPC and DPPH free radical scavenging activity of the selected culinary-medicinal mushrooms. Besides, finding by

Kanagasabapathy et al. (2011) reported strong correlation (R^2=0.754) between TPC and FRAP of the *P. sajor-caju*.

However, some studies showed no correlation between the total phenolic content and antioxidant activity. It might be due to other compounds conferring antioxidant activities for example ß- carotene, polysaccharides and flavanoids that may exhibit higher activity. On the other hand, Kähkönen et al. (1999) reported there was no significant correlation between the total phenolic content and antioxidant activities in plant extract. In their study, they explained that the molecular antioxidant response of phenolic compound depends on their chemical structure. It must be noted that the efficiency of the antioxidant depends strongly on the oxidation condition and lipid structure. Hence, we can conclude that the antioxidant activity does not necessarily correlate well with the amount of phenolic compound. The antioxidant activity of the mushroom extracts cannot be predicted on the basis of its total phenolic content as supported by Kähkönen et al. (1999). Individual phenolics should be further quantified to establish a better correlation between the effects of phenolics on antioxidant activity of the mushroom extracts that suggested by Chye et al. (2008).

4.4 Antioxidant Index (AI)

The result of antioxidant index (AI) of hybrid and parent extracts of *P. pulmonarius* is presented in Table 4.6. It is clear from the results that UMP002 had highest relative antioxidant potential (7.68%) followed by UMH004 (6.85%) and UMP001 (6.38%). The antioxidant potential of other three mushrooms did not show any significant difference. The antioxidant potential of UMP003 was 5.65% followed by UMH001 (5.54%) and UMH002 (5.37%). Studies done by Abdullah et al. (2011) reported that *Ganoderma lucidum*

(30.1%) had higher antioxidant potential as compared to *P. sajor-caju* (14.6%). Besides, Puttaraju et al. (2006) revealed that *Termitomyces heimii* and *Termitomyces mummiformis* had excellent antioxidant potential whereas *P. sajor-caju* had moderate activity.

Most mushroom species have various antioxidant mechanisms. Multiple assays based on different antioxidant mechanisms are used in order to provide a more reliable and accurate antioxidant capacity of the mushroom. Mushroom hot water extract consist a mixture of polar compound that might showed different levels of antioxidant activities in different assays. For example, in this study, UMH001 showed to be a good antioxidant by the DPPH free radical scavenging and inhibition of lipid peroxidation but weak antioxidant by the FRAP and TEAC assay, respectively. Hence, due to the difficulty in comparing the result of antioxidant activities from each assay individually, Antioxidant Index (AI) was constructed in order to rank the mushroom species from low to high antioxidant potential by combining the average result of all four assays which was first introduced by Puttaraju et al. (2006). Antioxidant Index (AI) could be an accurate approach to indicate the antioxidant potential in terms of average relative percentage and comparison was made with BHA that showed excellent antioxidant activities in this study. Therefore, we can conclude that the parent UMP002 had an excellent antioxidant potential compared to other mushroom extracts.

Table 4.6: Grading of *Pleurotus pulmonarius* hybrids and parents for total antioxidant activity according to the method by Puttaraju et al. (2006).

	Relative percentage of each antioxidant capacity assay				
Mushroom species/ positive control	DPPH	LPO	FRAP	TEAC	Average antioxidant activity (Relative percentage= antioxidant index)
BHA	100.00	100.00	100.00	100.00	100.00
UMP002	0.42	0.10	28.71	1.50	7.68
UMH004	0.69	0.12	25.83	0.74	6.85
UMP001	0.48	0.14	23.93	0.95	6.38
UMP003	0.61	0.10	20.29	1.58	5.65
UMH001	0.93	0.30	19.89	1.05	5.54
UMH002	0.69	0.19	19.40	1.19	5.37

The average of DPPH free radical scavenging, inhibition of lipid peroxidation (LPO), ferric reducing antioxidant power (FRAP) and Trolox Equivalent Antioxidant Capacity (TEAC) of BHA is based on 100. Antioxidant potential (average of total antioxidant activity) mushrooms studied was expressed as average relative percentages compared to BHA.

4.5 Screening of fibrinolytic activities

The lytic zone on fibrin plate of hot aqueous extracts of *P. pulmonarius* was illustrated in Figure 4.1. The extracts of *P. pulmonarius* hybrids and parents showed no fibrinolytic activities. There was no lytic zone appeared on the fibrin plate. The diameter of plasmin (positive control) was measured with 1.33 cm. Generally, fibrinolytic activity was measured to screen the ability of some proteolytic enzyme such as urokinase, streptokinase and genetically engineered tissues plasminogen activator to dissolve fibrin in blood clots. Fibrinolytic enzymes are commonly used in managing heart disease and stroke. To date, fibrinolytic agents such as urokinase plasminogen activator and tissues plasminogen activator are available for clinical use.

Study done by Ali et al. (2014) reported that oven dried crude extract (45^{O}C) had lower fibrinolytic activity (5.46 U/mg) as compared to freeze dried crude extract (10.83 U/mg) of *Auricularia polytricha*. It was due to the temperature that may denature the protein. According to Kim et al. (2006) the

enzyme of fibrinolytic activity was active between 20 ^{O}C to 40 ^{O}C with an optimum temperature at 37 ^{O}C. The fibrinolytic activity might decrease dramatically when the temperature is greater than 40 ^{O}C. Cheng et al. (2012) showed thermal stabilities of the protein in mushroom extracts can be varied that categorised into high, slow and middle range thermal stability. Cui et al. (2008) reported that the optimum pH and temperature for the enzyme activity of *Cordyceps militaris* were 6.0 and 25^{O}C, respectively. Meanwhile, study by Zal (2016) reported that the crude extract of freeze-dried *P. pulmonarius* had fibrinolytic activity of 17.29 U/mg with the diameter of lytic zone 1.43 cm. The fibrinolytic enzyme was preserved during the process. Therefore, we can conclude that the negative result of fibrinolytic activity in *P. pulmonarius* hybrids and parents could be due to the high temperature during extract preparation that may denature the protein or enzyme.

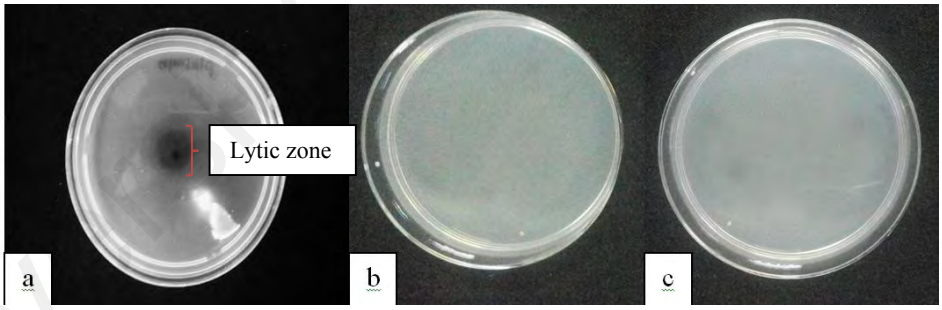
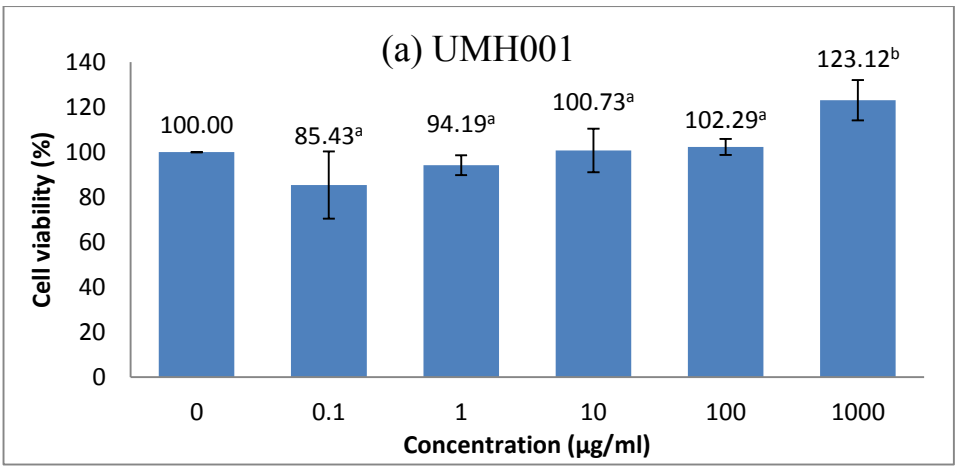


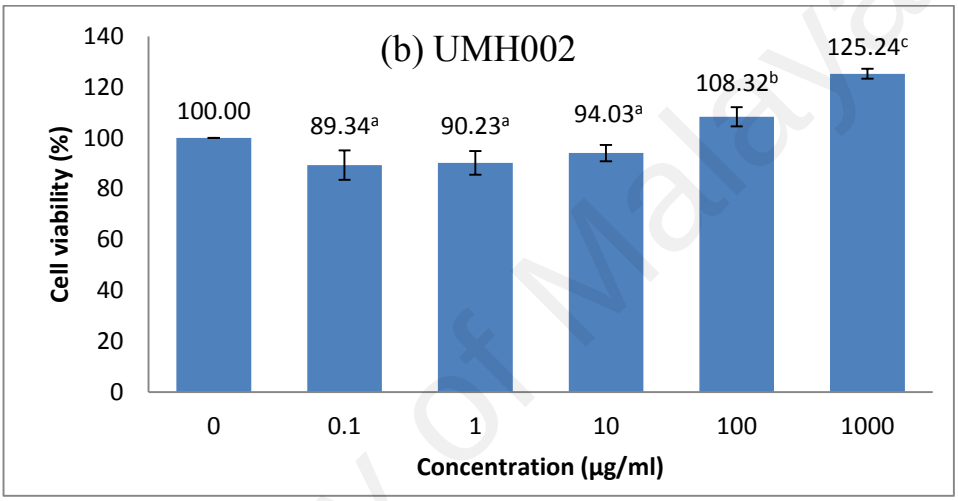
Figure 4.1 The lytic zones on fibrin plate of hot aqueous extracts of *P. pulmonarius*. (a) 2.28 mg/ml of plasmin (positive control). (b) distilled water (negative control). (c) hot aqueous extract of *P. pulmonarius*.

4.6 Measurement of cell viability (MTT assay)

MTT assay was used in this study to evaluate the cytotoxic effects of *P. pulmonarius* hybrids and parents on RAW264.7 murine macrophage cells. The hybrids were all from the crosses between three parents namely UMP001, UMP002 and UMP003. The cross-breeding between the parents may in result genetic variations in the crosses. Avin et al. (2016) reported that genetic variations for the most traits were associated with the genes that were dominant. The results are illustrated in Figure 4.2 and Figure 4.3. It was found that all the *P. pulmonarius* hybrids and parents had no cytotoxic effects on RAW264.7 cells at the concentration 1000 µg/ml. UMP002 showed 30% cell proliferate followed by UMH004 and UMH002 with 28% and 25%, respectively. At 0.1 µg/ml, all mushroom extracts showed cytotoxic on RAW264.7 cells. The cell viability of the positive control (cells without any treatment) was denoted as 100%. The concentration of mushroom extracts that used in this study was varies from 0.1 µg/ml to 1000 µg/ml. Originally, MTT assay was described by Monsmann (1983) for measuring cell survival or proliferation. The pale yellow of MTT reagent is reduced to purple formazan when incubated with live cells. The amount of purple formazan produced by cell treated with the MTT reagent was compared with the amount of purple formazan that produced by untreated control cells.

Apart for being recognised as a nutritious food, certain mushroom could be categorised as a toxic. Most of the fungal toxin may cause mild or moderate even severe poisoning. The result of mix-up between edible and toxic fungi might be accidently produce mushroom poisoning (Persson, 2012).





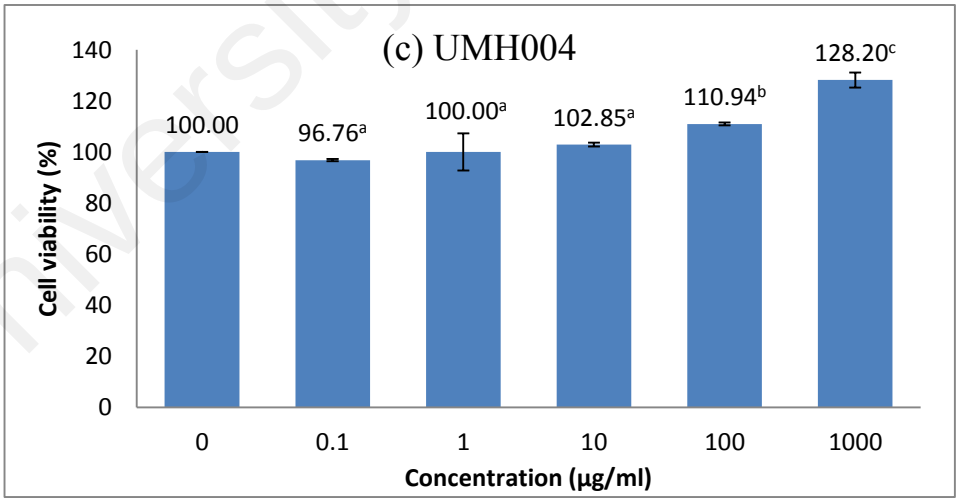
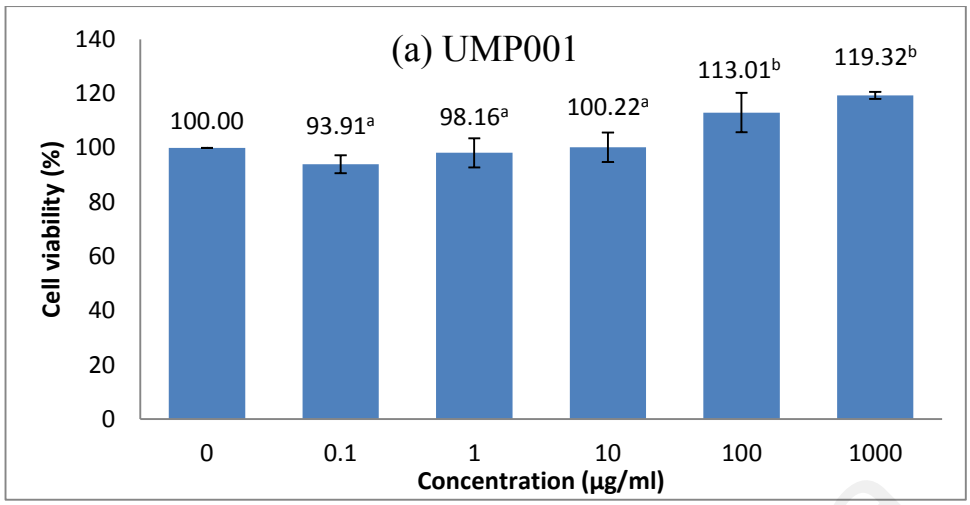
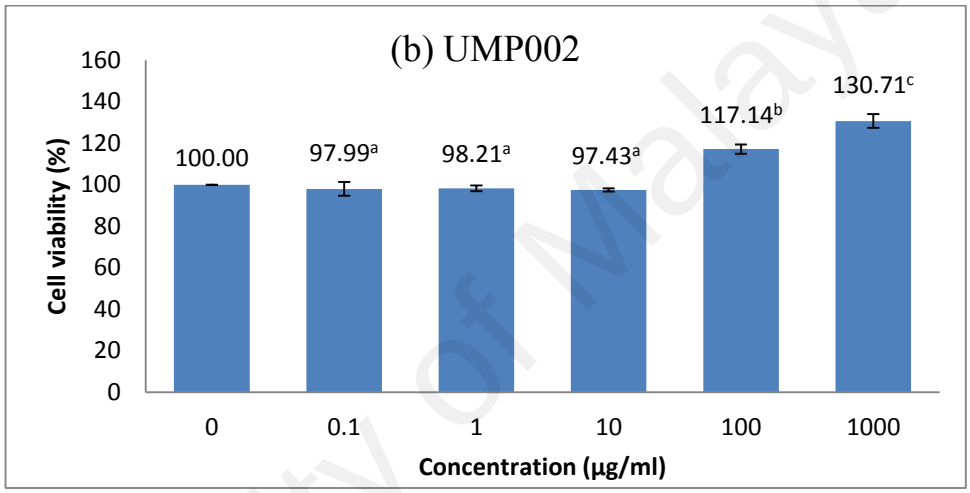


Figure 4.2: Effect of hot aqueous extracts of *Pleurotus pulmonarius* hybrids on RAW264.7 cells after 2 hours incubation.

*values were presented as mean ± standard deviation of three replicates

mean values in a column with different lowercase letters (a-c) indicates significant difference ($p<0.05$).





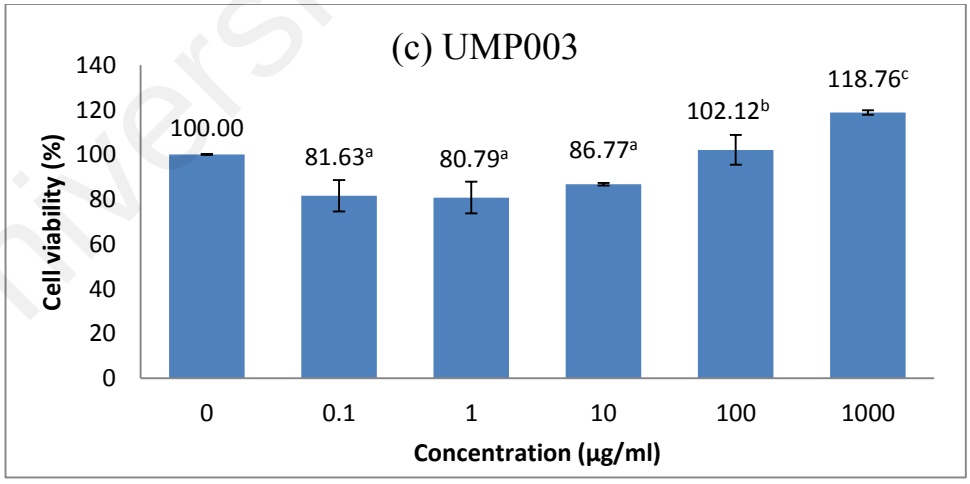


Figure 4.3: Effect of hot aqueous extract of *Pleurotus pulmonarius* parents on RAW264.7 cells after 2 hour incubation.

*values were presented as mean ± standard deviation of three replicates

mean values in a column with different lowercase letters (a-c) indicates significant difference ($p<0.05$).

In a work done by Finimundy et al. (2013) reported that *P. sajor-caju* extracts were more effective against tumour cells and might induced cytotoxicity in cells compared to extracts of *Lentinula edodes*. Studies done by Chan (2013) reported that all extracts of *Amauroderma rugosum* had no cytotoxic effects on RAW264.7 cell except for aqueous fraction. Moreover, *Ganoderma lucidum* extracts was supressed the proliferation of RAW264.7 cells may contribute to its anti-inflammatory properties (Dudhgaonkar et al., 2009). In the study by Makropoulou et al. (2012) revealed that the cytotoxic effects of dichloromethane (DCM) extract of *Gomphus clavatus* against human breast cell and prostate cancer cell could be due to some ergosterol derivatives. On the other hand, living cell require active mitochondrion to generate a strong signal and has possibility that the amount of formazan generated per cell depend on the level of energy metabolism in the cells (Monsmann, 1983). Hence, we can conclude that the extracts of *P. pulmonarius* hybrids and parents may promote cell proliferation of RAW264.7 cells and had no cytotoxic effects.

4.7 Nutritional value of *P. pulmonarius* hybrid and parents

In Table 4.7, the nutritional value (protein, total carbohydrate, total fat and total dietary fibre) of *P. pulmonarius* hybrid and parents is presented. The values of protein ranged from 21.6% to 28.6%, the parent UMP005 had highest protein content compared to others. The protein of *P. pulmonarius* hybrid and parents were in line and within the range measured by Chang and Buswell (1996), who stated that the cultivated mushrooms normally contain 19-35% of protein. Our results showed high protein content (21.6 to 28.6%) except *P. pulmonarius* (15 to 18%) compared to previuos reported study by Khantun et al. (2014). Reis et al. (2012) revealed that the white, brown and king oyster mushrooms had the highest level of protein compared to others edible mushroom. Supported by Longvah and Deosthale (1998) reported that the composition of amino acids in mushrooms is comparable to animal protein. Sanmee et al. (2003) reported that cultivated mushrooms have higher protein content as compared to most vegetables.

Obviously, there were high carbohydrates content in *P. pulmonarius* hybrid and parents ranging from 50.2-60.2%. The parent of UMP006 had highest carbohydrates content with 60.2%, followed by the parent UMP005 (50.2%) and hybrid UMH004 (58.4%). The carbohydrates content in the present study were within the range measured by Chang and Buswell (1996), who concluded that the fresh mushroom contain relatively a large amount of carbohydrates ranging from 51-88%. Reis et al. (2012) suggested that higher carbohydrates content in mushroom is probably due to high level of non-fiber carbohydrates. Besides that, study by Kalać (2009) revealed that the fibre such as structural polysaccharides ß-glucans, chitin, hemicellulose and pectin substances are the other carbohydrates content in mushrooms. Our finding

showed that there was high fibre content in parent of UMP005 (38.4%) and hybrid of UMH004 (36.8%). In support to our study, Diez and Alvarez (2001) reported that high level of fibre in *Tricholoma portentosum* (30.1%) and *Tricholoma terreum* (30.1%) seems to be nutritionally desirable.

As shown in Table 4.7, there were low content of fat in *P. pulmonarius* hybrid and parent. The parent UMP005 (3.4%) had low content of fat compared to the hybrid UMH004 (4.0%). Study done by Crisan and Sands (1978) concluded that mushrooms contained 2-8% of fat content that possess several classes of lipid compound, free fatty acids, mono, di, and triglycerides, sterols, sterol esters and phospholipids. Ouzouni et al. (2009) revealed that there was low fat content in ten analysed mushrooms from 2.10% (*Armillaria mellea*) to 6.00% (*Hygrophorus russula*). The low content of fat in mushrooms provides low calories in foods (León-Guzmán et al., 1997).

Table 4.7: Nutritional values of *P. pulmonarius* hybrid and parents.

Sample	Nutritional value (%)			
	Protein	Total Carbohydrates	Total Fat	Total Dietary Fibre
UMH004	21.6	58.4	4.0	36.8
UMP005	28.6	50.2	3.4	38.4
UMP006	23.4	60.2	-	-

CHAPTER 5

RECOMMENDATIONS FOR FURTHER STUDIES AND CONCLUSIONS

5.1 Recommendations for further studies

a) The potential bioactive compound should be identified in this mushroom which responsible for the observed antioxidant.
b) Optimization of the volume, pH and temperature is recommended in order to evaluate the activity of the fibrinolytic enzyme.
c) Identify the potential anti-inflammatory properties of this mushroom is recommended that beneficial on inflammatory disorders.

5.2 Conclusions

In this study, the antioxidant activities, fibrinolytic activities, cell proliferation and nutritional component of *P. pulmonarius* hybrids and parents were determined. Hence, we can conclude that:

a) Based on the four antioxidant assays that were carried out, UMH001 exhibited higher free radical scavenging activity and inhibition of lipid peroxidation. Meanwhile, UMP002 had highest ferric reducing power at 1.0 mg/mL and UMP003 showed higher TEAC value compared to all mushroom extracts.
b) UMP002 (7.68%), UMH004 (6.85%) and UMP001 (6.38%) showed relatively high antioxidant index (AI). Therefore, it can be conclude that UMP002 exhibited significantly ($P<0.05$) higher antioxidant activity among all mushroom extracts.
c) The extracts of *P. pulmonarius* hybrids and parents were showed no fibrinolytic activities.

d) The extracts of *P. pulmonarius* hybrids and parents showed no cytotoxic effect the RAW264.7 cell and may promote cell proliferation.

e) The nutritional analysis revealed that the hybrid UMH004 and parent UMH005 exhibited high protein, carbohydrates content and low fat level which makes *P. pulmonarius* a source for dietary fibre rich low calorie food.

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APPENDICES

Appendix A: Sample preparation (hot aqueous extracts) ratio 1:10

Pleurotus pulmonarius hybrids and parents were prepared using hot aqueous extracts at the ratio 1:10 (w/v) at 100^{o}C for 30 minutes.

Hybrid No.	Wet weight(g)	Dry weight(g)	Crude extract (g)
UMH001	748.130	84.281	14.931
UMH002	894.813	98.841	19.011
UMH004	1030.947	104.477	10.290
UMP001	710.965	76.858	13.911
UMP002	978.243	128.260	23.199
UMP003	1491.988	192.115	27.086

Appendix B: Experimental and statistical data for DPPH radical scavenging activity of BHA (positive control) and samples

(B1) DPPH radical scavenging activity of BHA and samples

(a) Percentage of inhibition in triplicates. Concentration of stock extract was 50 mg/ml.

Sample	IC_{50} (mg/mL)			Average IC_{50} (mg/ml)	Std
	R1	R2	R3		
BHA	0.12	0.12	0.08	0.11	0.02
UMH001	11.444	11.868	12.276	11.863	0.416
UMH002	11.858	19.305	16.972	16.045	3.809
UMH004	16.075	16.070	15.368	15.837	0.407
UMP001	23.645	23.101	22.130	22.959	0.767
UMP002	28.125	27.169	23.750	26.348	2.300
UMP003	18.847	17.693	17.262	17.934	0.819

Calculation of IC_{50} (mg/mL):

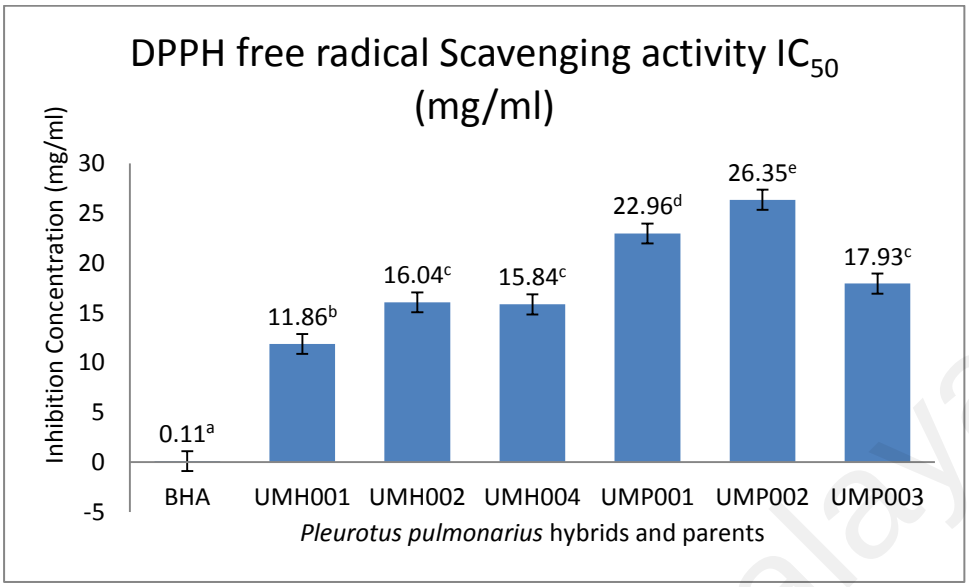
For example IC_{50} of UMH001 (R1):

From the graph $y = 2.4385x + 22.093$

$x = (50-22.093)/2.4385$

= **11.444 mg/mL**

(b) Graph for the inhibition concentration (IC_{50}) of BHA and samples.



(B2) Statistical analysis of DPPH scavenging activity of BHA and samples

(a) Analysis of variance for mean of inhibition concentration (IC_{50}) for BHA and samples.

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		1286.487	6	214.414	70.132	.000
	Linear Term	Contrast	22.343	1	22.343	7.308	.017
		Deviation	1264.144	5	252.829	82.697	.000
Within Groups			42.802	14	3.057		
Total			1329.289	20			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one level of concentrations to another at the 95% confidence level.

(b) Multiple range tests

Method: 95.0 percent LSD			
Sample	Number of replicates	Mean	Homogenous gp
BHA	3	0.1083	X
UMH001	3	11.8613	X
UMH002	3	15.8377	X
UMH004	3	16.0450	X
UMP003	3	17.9340	X
UMP001	3	22.9587	X
UMP002	3	26.3480	X

*The mean differences were significant (P<0.05), ANOVA test)

Appendix C: Experimental and statistical data for inhibition of lipid peroxidation of buffered egg yolk

(C1) Inhibition of lipid peroxidation of buffered egg yolk of BHA and samples

(a) Percentage of inhibition in triplicates. Concentration of stock extract was 50 mg/ml.

Sample	IC_{50} (mg/mL)			Average IC_{50} (mg/ml)	Std
	R1	R2	R3		
BHA	0.090	0.107	0.070	0.089	0.02
UMH001	30.149	30.002	28.548	29.566	0.89
UMH002	45.492	47.706	49.848	47.682	2.18
UMH004	81.747	77.246	75.435	78.143	3.25
UMP001	62.575	71.269	65.051	66.298	4.48
UMP002	88.914	80.596	98.886	89.465	9.16
UMP003	90.489	90.920	95.102	92.170	2.55

Calculation of IC_{50} (mg/mL):

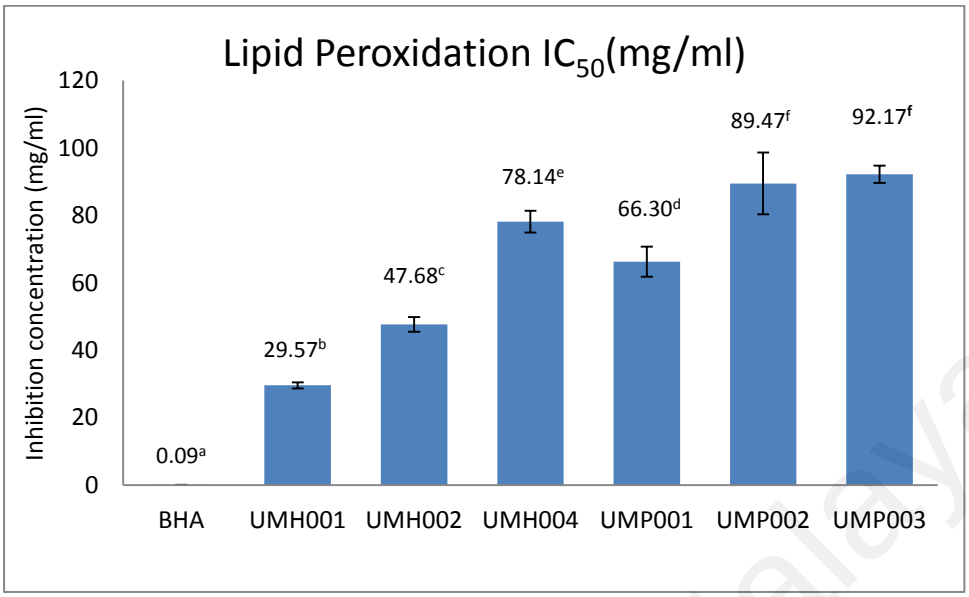
For example IC_{50} of UMH001 (R1):

From the graph $y = 1.5002x + 4.7699$

$x = (50-4.7699)/1.5002$

= **30.149 mg/mL**

(b) Graph for the inhibition concentration (IC_{50}) of BHA and samples.



(C2) Statistical analysis of inhibition of lipid peroxidation of BHA and samples

(a) Analysis of variance for mean of inhibition concentration (IC_{50}) for BHA and samples.

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		20699.794	6	3449.966	190.899	.000
	Linear Term	Contrast	189.307	1	189.307	10.475	.006
		Deviation	20510.487	5	4102.097	226.983	.000
Within Groups			253.011	14	18.072		
Total			20952.805	20			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one level of concentrations to another at the 95% confidence level.

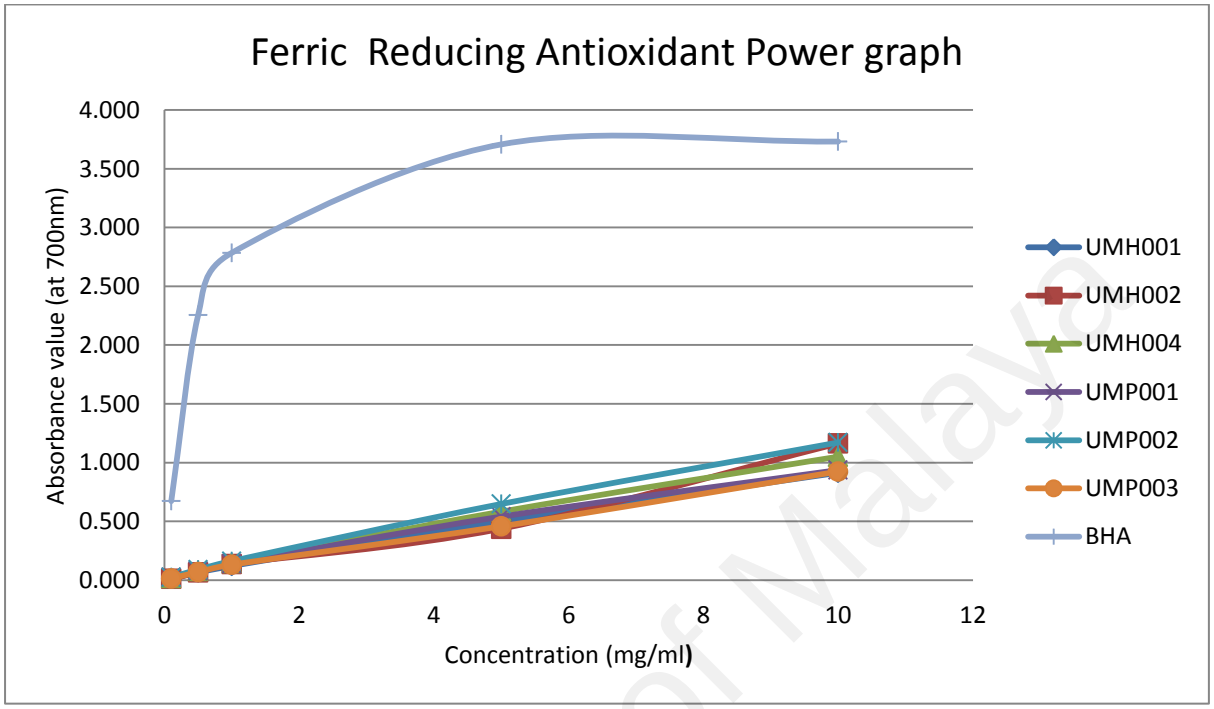
(b) Multiple range tests

Method: 95.0 percent LSD			
Sample	Number of replicates	Mean	Homogenous gp
BHA	3	0.09	X
UMH001	3	29.57	X
UMH002	3	47.68	X
UMP001	3	66.30	X
UMH004	3	78.14	X
UMP002	3	89.47	X
UMP003	3	92.17	X

*The mean differences were significant (P<0.05), ANOVA test)

Appendix D: Experimental and statistical data for Ferric Reducing Antioxidant Power (FRAP)

(D1) Graph ferric reducing antioxidant power



(D2) Statistical analysis of ferric reducing antioxidant power of BHA and samples

Concentration at 0.1 mg/mL

(a) Analysis of variance for mean of FRAP for BHA and samples.

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		1.363	6	.227	10148.133	.000
	Linear Term	Contrast	.526	1	.526	23480.179	.000
		Deviation	.837	5	.167	7481.723	.000
Within Groups			.000	14	.000		
Total			1.363	20			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one level of concentrations to another at the 95% confidence level.

(b) Multiple range tests

Method: 95.0 percent LSD			
Sample	Number of replicates	Mean	Homogenous gp
UMH002	3	0.011	X
UMH001	3	0.012	X
UMP003	3	0.015	X
UMH004	3	0.016	X X
UMP001	3	0.025	X
UMP002	3	0.025	X
BHA	3	0.672	X

*The mean differences were significant (P<0.05), ANOVA test)

Concentration at 0.5 mg/ml

(a) Analysis of variance for mean of FRAP for BHA and samples.

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		13.086	6	2.181	1468.573	.000
	Linear Term	Contrast	4.975	1	4.975	3349.976	.000
		Deviation	8.111	5	1.622	1092.292	.000
Within Groups			.021	14	.001		
Total			13.107	20			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one level of concentrations to another at the 95% confidence level.

(b) Multiple range tests

Method: 95.0 percent LSD			
Sample	Number of replicates	Mean	Homogenous gp
UMH001	3	0.065	X
UMH002	3	0.066	X
UMP003	3	0.068	X
UMH004	3	0.077	X
UMP001	3	0.080	X
UMP002	3	0.090	X
BHA	3	2.257	X

*The mean differences were significant (P<0.05), ANOVA test)

Concentration at 1 mg/ml

(a) Analysis of variance for mean of FRAP for BHA and samples.

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		19.012	6	3.169	4141.494	.000
	Linear Term	Contrast	7.241	1	7.241	9464.486	.000
		Deviation	11.771	5	2.354	3076.896	.000
Within Groups			.011	14	.001		
Total			19.023	20			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one level of concentrations to another at the 95% confidence level.

(b) Multiple range tests

Method: 95.0 percent LSD			
Sample	Number of replicates	Mean	Homogenous gp
UMH001	3	0.120	X
UMP003	3	0.132	X
UMH002	3	0.138	X
UMP001	3	0.140	X
UMH004	3	0.144	X
UMP002	3	0.162	X
BHA	3	2.785	X

*The mean differences were significant (P<0.05), ANOVA test)

Concentration at 5mg/ml

(a) Analysis of variance for mean of FRAP for BHA and samples.

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		27.305	6	4.551	6012.079	.000
	Linear Term	Contrast	10.607	1	10.607	14013.313	.000
		Deviation	16.698	5	3.340	4411.832	.000
Within Groups			.011	14	.001		
Total			27.316	20			

0.05, there is statistically significant difference between the mean absorbance from one level of concentrations to another at the 95% confidence level.

(b) Multiple range tests

Method: 95.0 percent LSD			
Sample	Number of replicates	Mean	Homogenous gp
UMH002	3	0.438	X
UMP003	3	0.458	X X
UMH001	3	0.499	X X
UMP001	3	0.540	X X
UMH004	3	0.583	X
UMP002	3	0.648	X
BHA	3	3.708	X

*The mean differences were significant (P<0.05), ANOVA test)

Concentration at 10mg/ml

(a) Analysis of variance for mean of FRAP for BHA and samples.

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		20.066	6	3.344	586.754	.000
	Linear Term	Contrast	7.413	1	7.413	1300.487	.000
		Deviation	12.654	5	2.531	444.007	.000
Within Groups			.080	14	.006		
Total			20.146	20			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one level of concentrations to another at the 95% confidence level.

(b) Multiple range tests

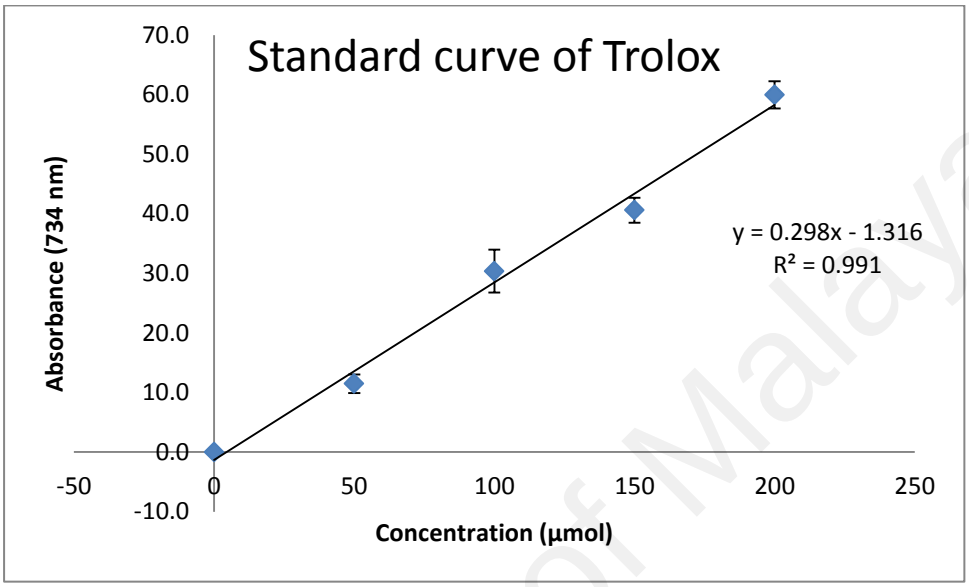
Method: 95.0 percent LSD			
Sample	Number of replicates	Mean	Homogenous gp
UMH001	3	0.913	X
UMP003	3	0.924	X
UMP001	3	0.936	X
UMH004	3	1.050	X X
UMH002	3	1.163	X
UMP002	3	1.171	X
BHA	3	3.732	X

*The mean differences were significant (P<0.05), ANOVA test)

Appendix E: Experimental and statistical data for Trolox Equivalent Antioxidant Capacity (TEAC)

(E1) Standard curve of Trolox (an analog of vitamin C)

(a) Absorbance graph at 734 nm in triplicate of Trolox. Concentration of stock extract from 0-200 µmol.



- Each value is expressed as mean ± standard deviation (n=3)

(b) Analysis of variance of 5 concentrations

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		6719.662	4	1679.915	334.889	.000
	Linear Term	Contrast	6658.870	1	6658.870	1327.439	.000
		Deviation	60.792	3	20.264	4.040	.040
Within Groups			50.163	10	5.016		
Total			6769.825	14			

The ANOVA table shows the F-ratio and p-value. Since the P- value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one level of concentrations to another at the 95% confidence level.

(E2) Trolox equivalent antioxidant activity (TEAC) of BHA and samples

(a) TEAC value of BHA

BHA concentration: 5 mg/ml

BHA	Absorbance			Average	µmol TE/g of BHA	Std
	R1	R2	R3			
	0.006	0.007	0.011	0.008	4442.953	8.878

Calculation:

5 mg/mL of BHA= 0.005g of BHA in 1 mL of distilled water

From standard curve, y = 0.298x – 1.316

µmol TE = (0.008 + 1.316)/ 0.298

= 4.442953 µmol TE

So, µmol TE/g of BHA= (4.442953 x 5)/0.005g BHA

= **4.44 X 10^3 µmol TE/g of BHA**

(b) TEAC value of samples

Sample	Absorbance			µmol TE			µmol TE/100g dry weight			Average	std
	R1	R2	R3	R1	R2	R3	R1	R2	R3		
UMH001	0.825	0.818	0.841	7.185480	7.161210	7.238421	46.494	46.337	46.837	46.56	0.26
UMH002	0.535	0.560	0.565	6.211750	6.294885	6.311459	52.561	53.264	53.405	53.08	0.45
UMH004	0.814	0.812	0.823	7.148834	7.140334	7.176278	32.765	32.727	32.891	32.79	0.09
UMP001	0.715	0.742	0.802	6.813787	6.905487	7.108957	41.640	42.200	43.444	42.43	0.92
UMP002	0.624	0.708	0.691	6.510151	6.790673	6.736101	65.102	67.907	67.361	66.79	1.49
UMP003	0.398	0.406	0.398	5.751060	5.778221	5.752175	70.291	70.623	70.304	70.41	0.19

Calculation:

In 1g processed fruiting bodies:

For example sample UMH001

Dry weight: 84.281g

Crude extract: 14.931g = 14931 mg

So, 1g of extract = 2.5 mg extract/ 14931 mg

= **0.000167g**

Based on standard curve of Trolox:

Y = 0.298x – 1.316

So, mg µmol TE = (absorbance (R1) + 1.316)/ 0.298

= (0.825+1.316)/0.298

= **7.18 µmol TE**

Hence, based on working concentration:

Sample load = 100 µL

Total volume of assay mixture = 1100 µL

(7.18 µmol TE/1000) x 1100 µL = 7.898 µmol TE

7.898 µmol TE/1000 = 0.007898 µmol TE

So, 0.007898 µmol TE/ 100 g dry weight = 0.007898 µmol TE/ 0.00017 g dry weight

= **46.5 µmol TE/100 g dry weight**

(E3) Statistical analysis of TEAC value of BHA and samples

(a) Analysis of variance for mean of total phenolic content for BHA and samples.

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		49581323.925	6	8263553.988	703745.332	.000
	Linear Term	Contrast	16034377.015	1	16034377.015	1365528.438	.000
		Deviation	33546946.911	5	6709389.382	571388.710	.000
Within Groups			164.392	14	11.742		
Total			49581488.317	20			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one level of concentrations to another at the 95% confidence level.

(b) Multiple range tests

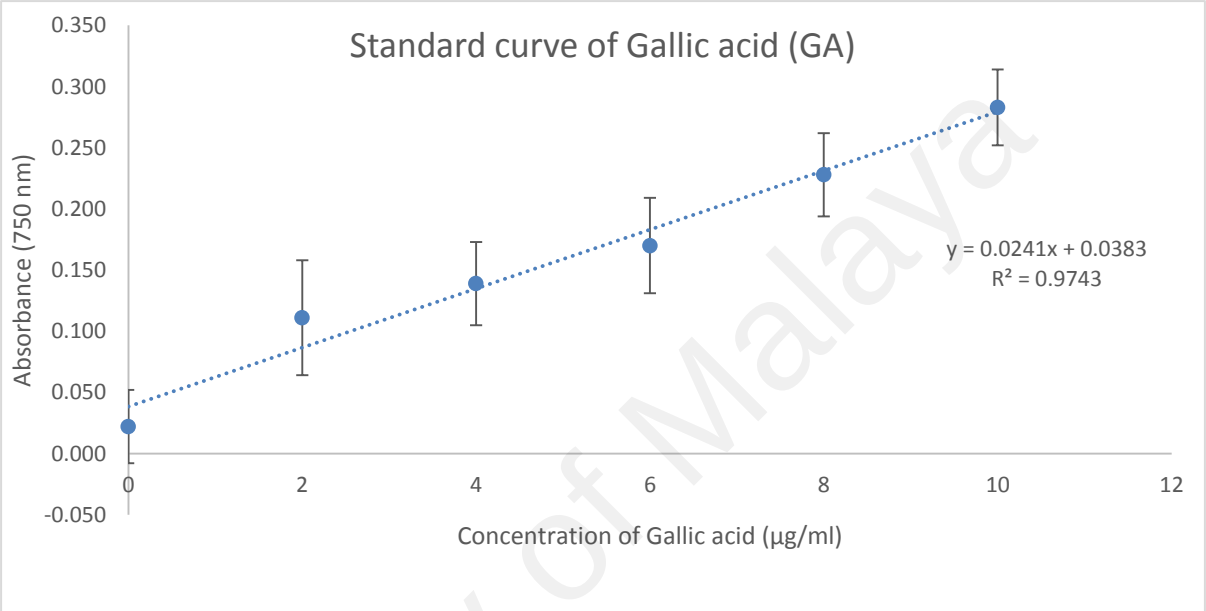
Method: 95.0 percent LSD			
Sample	Number of replicates	Mean	Homogenous gp
UMH004	3	32.79	X
UMP001	3	42.43	X
UMH001	3	46.56	X
UMH002	3	53.08	X
UMP002	3	66.79	X
UMP003	3	70.41	X
BHA	3	4.44×10^3	X

*The mean differences were significant ($P<0.05$), ANOVA test

Appendix F: Experimental and statistical data for Total Phenolic Content (TPC)

(F1) Standard curve of Gallic Acid (GA)

(a) Absorbance graph at 750 nm in triplicates of Gallic Acid. Concentration of stock extract was 50 mg/ml.



- Each value is expressed as mean ± standard deviation (n=3)

(b) Analysis of variance at 5 concentrations

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		.125	5	.025	21.857	.000
	Linear Term	Contrast	.122	1	.122	106.455	.000
		Deviation	.003	4	.001	.708	.602
Within Groups			.014	12	.001		
Total			.139	17			

The ANOVA table shows the F-ratio and p-value. Since the P- value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one level of concentrations to another at the 95% confidence level.

(F2) Total Phenolic Content of BHA (positive control) and samples

(a) Total phenolic content of BHA

BHA concentration: 1 mg/mL

BHA	Absorbance			Average	mg GAE in 1g BHA	Std
	R1	R2	R3			
	3.561	3.524	3.440	3.508	1.44×10^4	257.27

Calculation:
1mg/mL BHA = 0.01g BHA in 1 mL of distilled water
mg GAE = (3.508-0.0383)/0.0241
= 143.97
So, mg GAE/g BHA = 143.97/ 0.01g BHA
= **1.44×10^4 mg GAE/g BHA**

(b) Total phenolic content of samples

Sample	Crude extracts (mg)	Absorbance			mg GAE in 2.5 mg extract			mg GAE/100g of dry weight			Average	Standard deviation
		R1	R2	R3	R1	R2	R3	R1	R2	R3		
UMH001	14931.000	0.968	0.971	1.039	0.019	0.019	0.021	115.198	115.570	123.995	118.254	0.040
UMH002	19011.000	0.758	0.792	0.764	0.015	0.016	0.015	113.545	118.909	114.492	115.649	0.018
UMH004	10290.000	1.182	1.042	1.003	0.024	0.021	0.020	97.67	85.71	82.38	88.585	0.094
UMP001	13911.000	0.912	0.904	0.906	0.018	0.018	0.018	100.863	99.940	100.171	100.325	0.004
UMP002	23199.000	0.584	0.568	0.590	0.011	0.011	0.011	105.060	101.979	106.215	104.418	0.011
UMP003	27086.000	0.493	0.441	0.481	0.009	0.008	0.009	102.208	90.519	99.510	97.412	0.027

Calculation:

Sample concentration: 5 mg/mL

Sample load: 500 µL/ cuvette

Total volume for assay mixture: 2000 µL (2 mL)

5 mg/mL = 5 mg extract in 1 mL of distilled water

500 µL = 0.5 mL

So, extract (mg) = 0.5 x 5 mg = **2.5 mg extract**

In 1g processed fruiting bodies:

For example sample UMH001

Dry weight: 84.281g

Crude extract: 14.931g = 14931 mg

So, 1g of extract = 2.5 mg extract/ 14931 mg

= **0.000167g**

Based on standard curve of Gallic acid (GA):

Y = 0.0241x + 0.0383

So, mg GAE = (absorbance (R1) – 0.0383)/ 0.0241

= (0.968-0.0383)/0.0241

= **38.5768 µg GAE**

Hence, based on working concentration:

5 mg/mL = 38.5768 µg GAE

2.5g extract in 5mL = 38.5768 µg GAE

2.5g extract = 38.5768µg GAE x 0.5mL

= 19.2884 µg GAE/mL

= 0.0192884 mg GAE/ml

So, mg GAE/100g of dry weight = 0.0192884 mg GAE/ 0.000167g

= **115.499 mg GAE/100g of dry weight**

(F3) Statistical analysis of total phenolic content of BHA and samples

(a) Analysis of variance for mean of total phenolic content for BHA and samples.

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		5.25E+8	6	8.76E+7	9237.209	.000
	Linear Term	Contrast	1.67E+8	1	1.67E+8	17642.49	.000
		Deviation	3.58E+8	5	7.16E+7	7556.152	.000
Within Groups			132714.9	14	9479.633		
Total			5.26E+8	20			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one level of concentrations to another at the 95% confidence level.

(a) Multiple range tests

Method: 95.0 percent LSD			
Sample	Number of replicates	Mean	Homogenous gp
UMH004	3	88.585	X
UMP003	3	97.412	X
UMP001	3	100.325	X
UMP002	3	104.418	X
UMH002	3	115.649	X
UMH001	3	118.254	X
BHA	3	1.44×10^4	X

*The mean differences were significant (P<0.05), ANOVA test)

Appendix G: Experimental and statistical data for cell viability (MTT assay)

(G1) Calculation of cell count

$$N = n \times 10^4 \times 2$$

So, $N = 1100 \times 10^4 \times 2$

$= 1100{,}0000$ cells

$(1100{,}0000)(V_1) = 50{,}000$ (30 ml of media)

$V_1 = 1500000/11000000$

$V_1 = 0.136$ ml of cells needed + 29.86ml of media

Percentage of cell viability:

$$\% = \frac{\text{absorbance of sample}}{\text{Absorbance of control}} \times 100$$

For example: percentage of cell viability of sample UMH001, at 0.1 µg/ml

$\%\ (R1) = (0.407/0.597) \times 100$

$= 68.17\%$

(G2) Statistical data for cell viability

(a) UMH001

(i) Analysis of variance for mean of cell viability of samples.

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		2338.700	4	584.675	6.789	.007
	Linear Term	Contrast	1914.040	1	1914.040	22.223	.001
		Deviation	424.660	3	141.553	1.644	.241
Within Groups			861.272	10	86.127		
Total			3199.972	14			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one level of concentrations to another at the 95% confidence level.

(ii) Multiple range tests

Method: 95.0 percent LSD			
Concentration	Number of replicates	Mean	Homogenous gp
0.1	3	85.43	X
1	3	94.19	X
10	3	100.73	X
100	3	102.29	X
1000	3	123.12	X

*The mean differences were significant (P<0.05), ANOVA test)

(b) UMH002

(i) Analysis of variance for mean of cell viability of samples.

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		2818.016	4	704.504	41.821	.000
	Linear Term	Contrast	2338.215	1	2338.215	138.802	.000
		Deviation	479.801	3	159.934	9.494	.003
Within Groups			168.457	10	16.846		
Total			2986.473	14			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one level of concentrations to another at the 95% confidence level.

(ii) Multiple range tests

Method: 95.0 percent LSD			
Concentration	Number of replicates	Mean	Homogenous gp
0.1	3	89.34	X
1	3	90.23	X
10	3	94.03	X
100	3	108.32	X
1000	3	125.24	X

*The mean differences were significant (P<0.05), ANOVA test)

(c) UMH004

(i) Analysis of variance for mean of cell viability of samples.

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		1899.336	4	474.834	38.097	.000
	Linear Term	Contrast	1685.685	1	1685.685	135.245	.000
		Deviation	213.650	3	71.217	5.714	.015
Within Groups			124.640	10	12.464		
Total			2023.975	14			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one level of concentrations to another at the 95% confidence level.

(ii) Multiple range tests

Method: 95.0 percent LSD			
Concentration	Number of replicates	Mean	Homogenous gp
0.1	3	96.76	X
1	3	100.00	X
10	3	102.85	X
100	3	110.94	X
1000	3	128.20	X

*The mean differences were significant (P<0.05), ANOVA test)

(d) UMP001

(i) Analysis of variance for mean of cell viability of samples.

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		1385.081	4	346.270	14.118	.000
	Linear Term	Contrast	901.967	1	901.967	36.775	.000
		Deviation	483.114	3	161.038	6.566	.010
Within Groups			245.264	10	24.526		
Total			1630.345	14			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one level of concentrations to another at the 95% confidence level.

(ii) Multiple range tests

Method: 95.0 percent LSD			
Concentration	Number of replicates	Mean	Homogenous gp
0.1	3	93.91	X
1	3	98.16	X
10	3	100.22	X
100	3	113.01	X
1000	3	119.32	X

*The mean differences were significant (P<0.05), ANOVA test)

(e) UMP002

(i) Analysis of variance for mean of cell viability of samples.

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		2719.539	4	679.885	114.588	.000
	Linear Term	Contrast	2111.558	1	2111.558	355.882	.000
		Deviation	607.981	3	202.660	34.156	.000
Within Groups			59.333	10	5.933		
Total			2778.872	14			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one level of concentrations to another at the 95% confidence level.

(ii) Multiple range tests

Method: 95.0 percent LSD			
Concentration	Number of replicates	Mean	Homogenous gp
10	3	97.43	X
0.1	3	97.99	X
1	3	98.21	X
100	3	117.14	X
1000	3	119.32	X

*The mean differences were significant (P<0.05), ANOVA test)

(f) UMP003

(i) Analysis of variance for mean of cell viability of samples.

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		3176.353	4	794.088	27.530	.000
	Linear Term	Contrast	2549.959	1	2549.959	88.404	.000
		Deviation	626.394	3	208.798	7.239	.007
Within Groups			288.445	10	28.845		
Total			3464.798	14			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one level of concentrations to another at the 95% confidence level.

(ii) Multiple range tests

Method: 95.0 percent LSD			
Concentration	Number of replicates	Mean	Homogenous gp
1	3	80.79	X
0.1	3	81.63	X
10	3	86.77	X
100	3	102.12	X
1000	3	118.76	X

*The mean differences were significant (P<0.05), ANOVA test)

Appendix H: Sample preration for nutritional analysis

Sample	Fresh fruiting bodies (g)	Dry weight (g)
UMH004	1849.551	222.615
UMP001	1905.681	178.846
UMP002	597.923	88.546