ANTIOXIDANT ACTIVITIES AND CHEMICAL COMPOSITION OF HOT WATER-SOLUBLE EXTRACTS AND FRACTIONS OF SELECTED *Schizophyllum commune* Fr. STRAINS

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DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (BIOTECHNOLOGY)

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ANTIOXIDANT ACTIVITIES AND CHEMICAL COMPOSITION OF HOT WATER-SOLUBLE EXTRACTS AND FRACTIONS OF SELECTED *Schizophyllum commune* Fr. STRAINS

ABSTRACT

Oxidative stress is a conjoint pathological mechanism that can be triggered by free radicals leading to cellular senescence or apoptosis. Antioxidants have the potential to inhibit free radicals' generation and balance the irregulated oxidative stress to restore cellular homeostasis. *Schizophyllum commune*, also known as the split-gill mushroom, is well-known for its nutritional values and bioactive compounds. This study aimed to investigate the antioxidant activity and chemical composition of the hot water extracts of selected strains of *S. commune* including natural, gamma-irradiated and hybrid strains. The fruiting bodies of different strains of *S. commune* were subjected to hot water extraction and the extracts were evaluated by radical scavenging, and reducing capacity assays. Results showed that, overall, the antioxidant activity of the various strains was comparable. The natural strain W and the strain obtained when strain W was subjected to gamma-irradiation at a dose of 4000 Gy (W4000) consistently exhibited high antioxidant activities in all assays and they were either comparable or higher than common edible mushrooms, *Pleurotus pulmonarius* and *Agaricus bisporus*. Moreover, the gamma-irradiated strains R2000 and R4000 exhibited higher reducing activities (0.40 and 0.41 mmol Fe^{2+}/g extract) than the parental strain R (0.27 mmol Fe^{2+}/g extract). Furthermore, the hybrid strains WR and RW showed higher scavenging activities towards 2,2 diphenyl-1-picylhydrazyl radicals (1.40 and 1.45 mmol TE/g extract) than the parental strain R (1.17 mmol TE/g extracts), but lower than the parental strain W (1.78 mmol TE/g extract). There is a strong correlation between the antioxidant activity of the extracts and the levels of flavonoid and phenolic compounds ($p < 0.01$). In order to

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determine the nature of antioxidative components in the hot water extracts of *S. commune*, the extracts of strains W and W4000, were fractionated to yield fractions with distinct chemical components. Results indicated that the phenolics- and flavonoids-rich ethyl acetate and ethanol fractions exhibited higher scavenging activities towards DPPH radicals, and ferric reducing activities compared to the fractions containing mostly water-soluble components. Similar trends were observed for both W and W4000 strains. Our findings suggest that the observed antioxidant activities of the hot water extracts of *S. commune* is mainly attributed to the water-soluble, low- molecular-weight compounds rather than the water-soluble components such as polysaccharides and proteins which make up the bulk of the water extracts.

Keywords: *Schizophyllum commune,* hot water extracts, fractionation, antioxidants.

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AKTIVITI ANTIOKSIDAN DAN KOMPOSISI KIMIA DALAM EKSTRAK DAN PECAHAN TERLARUT AIR PANAS STRAIN *Schizophyllum commune* Fr. TERPILIH

ABSTRAK

Tekanan oksidatif adalah mekanisme patologi bergabung yang dapat dicetus oleh radikal bebas yang menyebabkan penuaan sel atau apoptosis. Antioksidan berpotensi menghalang penghasilan radikal bebas dan mengimbangkan tekanan oksidatif yang tidak teratur untuk memulihkan homeostasis selular. *Schizophyllum commune*, juga dikenal sebagai cendawan insang terpisah, terkenal dengan nilai pemakanan dan sebatian bioaktif. Kajian ini bertujuan untuk mengkaji aktiviti antioksidan dan komposisi kimia ekstrak air panas dari strain *S. commune* terpilih termasuk strain semula jadi, strain penyinar gamma dan strain hibrid. Jana buah dari strain *S. commune* yang berlainan dikenakan pengekstrakan air panas dan ekstraknya dikaji melalui ujian mengumpul radikal, dan keupayaan penurunan. Hasil kajian menunjukkan bahawa, secara keseluruhan, aktiviti antioksidan dari pelbagai jenis adalah setanding. Baka semula jadi W dan baka yang diperoleh apabila strain W dikenakan penyinaran gamma pada dos 4000 Gy (W4000) menunjukkan aktiviti antioksidan yang tinggi secara konsisten dalam semua ujian dan mereka setanding lebih tinggi daripada cendawan yang boleh dimakan biasa, *Pleurotus pulmonarius* dan *Agaricus bisporus*. Tambahan baka terdedah kepada sinaran gamma R2000 dan R4000 menunjukkan aktiviti penurunan yang lebih tinggi (0.40 dan 0.41 mmol Fe^{2+}/g ekstrak) daripada baka induk R (0.27 mmol Fe^{2+}/g ekstrak). Selanjutnya, baka hibrid WR dan RW menunjukkan aktiviti mengumpul yang lebih tinggi terhadap radikal 2,2 diphenyl-1-picylhydrazyl (1.40 dan 1.45 mmol TE/g ekstrak) daripada baka induk R (1.17 mmol TE/g ekstrak), tetapi lebih rendah daripada baka induk W (1.78 mmol TE/g ekstrak). Terdapat korelasi yang kuat antara aktiviti antioksidan ekstrak

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dan tahap sebatian flavonoid dan fenolik ($p < 0.01$). Untuk menentukan sifat komponen antioksidan dalam ekstrak air panas *S. commune*, ekstrak strain W dan W4000, diperingkatkan untuk menghasilkan pecahan dengan komponen kimia yang berbeza. Hasil kajian menunjukkan bahawa pecahan etil asetat dan etanol yang kaya dengan fenolik dan flavonoid menunjukkan aktiviti mengumpul yang lebih tinggi terhadap radikal DPPH, dan aktiviti penurunan ferik berbanding dengan pecahan yang kebanyakannya mengandungi komponen terlarut air. Trend yand serupa diperhatikan untuk kedua-dua baka W dan W4000. Penemuan kami menunjukkan bahawa aktiviti antioksidan yang diperhatikan dari ekstrak air panas *S. commune* disebabkan oleh sebatian terlarut air, berat molekul rendah dan bukannya komponen terlarut air seperti polisakarida dan protein yang membentuk sebahagian besar extrak air.

Kata kunci: *Schizophyllum commune*, ekstrak air panas, pecahan, antioksidan.

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β	Beta
°C	degree centigrade
H_2O_2	hydrogen peroxide
µg	microgram
µl	microliter
µM	micromolar
$O_2^{\bullet -}$	superoxide anion
•OH	hydroxyl radical
%	percentage
Ab	*Agaricus bisporus*
ANOVA	analysis of variance
ABTS	2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
Ca	calcium
CAT	catalase
CUPRAC	cupric ion-reducing antioxidant capacity
Cu-Zn	zinc-copper
DNA	deoxyribonucleic acid
DPPH	2,2 diphenyl-1-picrylhydrazyl
dw	dry weight
EA	ethyl acetate

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et al	and others
etc.	Et cetera
Fe	iron
FRAP	ferric reducing antioxidant power
Fe^{2+}	ferrous ion
GAE	gallic acid equivalent
GE	glucose equivalent
GSH-Px	glutathione peroxidase
g	gram
Gy	Gray
h	hour
kGy	kilo gray
K	potassium
mg	miligram
ml	milliliter
mmol	milimol
mM	milimolar
min	minute
M	molar
MDA	malondialdehyde
Mg	magnesium
Mn-dependent	manganese-dependent
Na	sodium

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NADPH	nicotinamide adenine dinucleotide phosphate
nm	nanometers
ORAC	oxygen radical absorbance capacity
Pb	*Pleurotus pulmonarius*
ROS	reactive oxygen species
rpm	revolutions per minute
RE	rutin equivalent
RNS	reactive nitrogen species
SOD	superoxide dismutase
PG	propyl gallate
SPSS	Statistical Package for Social Science
TBHQ	*tert*-butylhydroquinone
TE	trolox equivalent
USA	United States of America
v/v	volume/volume
w/w	weight/weight

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CHAPTER 1: INTRODUCTION

1. Introduction

Oxidative stress is a physiological state triggered by an exposure to excessive levels of free radicals or reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH), all of which can hamper the functionalities of important cell biomolecules, mainly proteins, DNA, and lipids. Rising evidence indicated that the severe impairment caused by the accumulation of ROS contributes to several chronic disorders (Mittal et al., 2014; Wei et al., 2009). According to Phaniendra et al. (2015), there are several sources of free radicals with endogenous free radicals resulting from physiological processes is the most common source. These radicals are generated in the mitochondria (the powerhouse of the cell). However, the energy-generating processes of the mitochondria can also pose contrasting effects to the body due to the generation and accumulation of free radicals in the cell leading to oxidative stress. In addition, Phaniendra et al. (2015) have reported that oxidative stress can cause DNA damage via several modifications of the deoxyribose backbone leading to cancer, senescence and neurodegenerative diseases.

Antioxidants play a significant role in neutralising the oxidative stress by minimising the levels of free radicals and consequently enabling the body cell to carry out useful biological functions without excessive damage. Balance must be reached in the body between free radicals and antioxidants to avoid the risk of oxidative stress (Al-Joudi, 2013; Halliwell, 2006). The antioxidant system can be divided mainly into enzymatic and non-enzymatic. The main components of the enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) (Mirończuk-

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Chodakowska et al., 2018). Enzymes with antioxidant activity are characterized by their ability in stabilizing free radicals and maintain normal cellular redox activities. Moreover, protection against reactive oxygen species is also provided by non-enzymatic dietary antioxidants. Examples of such molecules include phenolics compounds (flavonoids and phenolic acids), carotenoids, vitamin C, polysaccharides, proteins, etc.

There is an increasing demand for natural antioxidants due to safety concerns for synthetic antioxidants. These antioxidants are specifically derived from natural sources, such as spices, plants (fruits, herbs and vegetables) and mushrooms (Lu et al., 2011; Pieszka et al., 2015; Smith et al., 2015). Mushrooms are rich sources of nutraceuticals with several biological properties, such as antioxidant (Khatun et al., 2012), antitumor (Mallick et al., 2010), and antimicrobial activities (Smolskaitė et al., 2015).

Among the bioactive compounds reported to be responsible for the antioxidant activities of mushrooms are polyphenols, tocopherols, vitamins, lycopene, polysaccharides, proteins, and carotenoids (Chowdhury et al., 2015; De Silva et al., 2013; Thongbai et al., 2015). The chemical compositions and antioxidant potentials of mushrooms are influenced by several factors, including mushroom species, habitat composition, stage of harvest, processing techniques, handling conditions, extraction solvent, and others (Pereira et al., 2012; Stajic et al., 2013; Vishwakarma et al., 2016).

Several approaches in mushroom strains improvement may affect the chemical compositions and hence the biological activities of the mushrooms, including their antioxidant abilities. Techniques such as gamma irradiation, molecular breeding and hybridization have been previously applied to generate mushroom strains with modified chemical compositions and enhanced biological activities. The results obtained seem to be

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influenced by the types of mushroom, the dose of gamma irradiation and the hybridization method used (Jaswal et al., 2013; Kumara & Edirimanna, 2009; Sathesh-Prabu & Lee, 2016; Sato et al., 2006).

Among the species of basidiomycetes with notable medicinal significance is *Schizophyllum commune*, a fungus which is typically found on diverse trees and rotting wood across the world (Chowdhary et al., 2013). It is a non-sporulating, wood-decaying and endophytic fungus which is also known as split gills due to the splitting appearance of its underside gills when it is dehydrated (Sim, 2014). Several researches have showed that various extracts of *S. commune* exhibited high scavenging activities toward free radicals and acted as ion reducing agents (Chandrawanshi et al., 2017; Devi et al., 2014; Emsen et al., 2017; Mirfat et al., 2010) but in-depth studies into the chemical constituents are lacking.

It is known that bioactivities of different mushroom strains may be varied. As part of an on-going experiment, several strains of *S. commune* have been generated from two different parental strains by subjecting them to gamma-irradiation and hybridization of the monokaryon; however, it is not known if these strains exhibit antioxidant activity that is higher, lower or comparable to the parental strains. Regarding the nature of antioxidants in *S. commune*, previous studies focused on the organic solvent extracts that are likely to contain mostly low-molecular-weight compounds but the role of the high molecular weight components such as polysaccharides and proteins that are usually present in the hot water extracts are not well studied. Therefore, the aim of this research was to evaluate the antioxidant activities of the crude hot water extracts of selected natural, gamma-irradiated and hybrid strains of *S. commune*.

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The objectives of this study were:

i. To compare the antioxidant activities of the hot water extracts and fractions of the fruiting bodies of selected *S. commune* strains.

ii. To determine the chemical compositions of the *S. commune* crude hot water extracts and fractions of the selected strains.

iii. To correlate the antioxidant activities of *S. commune* extracts and fractions with their chemical compositions.

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CHAPTER 2: LITERATURE REVIEW

2.1 Oxidative Stress

The constant exposure of the body tissues to certain stimulus perturbs the biochemical process at the cellular level and consequently heralds possible physiological changes. This state of imbalance in the body homeostasis is commonly refer to as stress, more specifically an oxidative stress; while the stimulus whether physical or psychological is termed stressors (Rahal et al., 2014). Oxidative stress is further characterized by an imbalance in the amount of oxidant compared to antioxidant, resulting in the outburst of reactive oxygen species (ROS) that leads to an actual damage to the cells (Sies, 1997). Naturally, oxidation in conjunction with reduction (redox) represents a major biochemical reaction involving the removal and addition of electron via electron carrier, e.g., the two forms of nicotinamide adenine dinucleotide, in a regulated process to generate energy from fuel molecules. Although the by-products of oxidation such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are responsible for several important biological process of living organism, their accumulation at higher concentrations can also be damaging (Mittler, 2017).

These reactive species are molecules or atoms having one or more unpaired electron in the external electron shell, capable of stabilizing themselves by abstracting an electron from a nearby target molecule (Di Meo et al., 2016; Phaniendra et al., 2015). The normal cell processes such as oxidative phosphorylation and exposure to toxic environmental contaminants can result in the formation of elevated concentrations of free radicals which may cause serious damage to deoxyribonucleic acid, proteins/peptides and lipids (Rahal et al., 2014). As a result of the damage to the macromolecule, the increase in various diseases and disorders becomes predictable (Sordillo & Aitken, 2009).

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Factors contributing to the formation of free radicals can be categorized into exogenous and endogenous sources. Among the endogenous sources of free radicals includes respiratory oxidases in mitochondria, xanthine oxidase in cytosol, cytochrome in endoplasmic reticulum, oxidases in peroxisomes, cyclooxygenase in lipid bilayer and during Fenton reaction (Di Meo et al., 2016). External or exogenous factors responsible for the production of free radicals are mainly related to unhealthy life style, such as heavy smoking, drug abuse, alcohol, continuous exposure to radiation, and pollutants – pesticides, industrial chemicals and ozone (Wojtunik-Kulesza et al., 2016).

Although there are varying mechanisms by which free radicals react with nearby molecules, the underlying basis involves electron abstraction of free radicals to gain stability and the eventual loss of electron by the target molecule (Dizdaroglu & Jaruga, 2012). The attacked molecules may be biochemical compounds involved in very crucial enzyme reactions, such as protein (Stadtman & Levine, 2000), lipid (Herttuala, 1999) and DNA (Marnett, 2000). Following an attack by free radicals, the chemical structure of these molecules may be altered, consequently impairing their cellular function and thereby contributing to cellular senescence or apoptosis (Ziskoven et al., 2010).

Cell damage caused by free radicals (ROS) are responsible for many severe diseases such as cancer, cardiovascular and neurological diseases, renal and liver disorders as well as age-related disorders like neurodegenerative and diabetes mellitus (Sinha & Kumar, 2015). Therefore, neutralisation of free or peroxide radicals by an antioxidant agent constitutes an important strategy for cell to be protected against oxidative stress (Sen et al., 2010).

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2.2 Antioxidants

To neutralise the detrimental effects of free radical on the cell, our body has developed scavenging molecules called antioxidants as its primary defense mechanism. These include enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) (Jeeva et al., 2015), and non-enzymatic antioxidants, which include vitamin C, vitamin E, NADPH, glutathione, thioredoxin and certain trace metals (Mirończuk-Chodakowska et al., 2018; Rahal et al., 2014). Similarly, antioxidant molecules such as flavonoids, ascorbic acid, uric acid and lipoic acid can be obtained exogenously from food substances (Rahal et al., 2014).

A natural antioxidant is identified as any substance derived from natural sources (herbs, vegetables and fruit), accumulates in concentration, which is high enough to inhibit the oxidation process of the reactive substrate (Krishnaiah et al., 2007). An optimal antioxidant substance should be readily absorbed by the cells and must possess the ability to prevent or suppress the chain reaction of free radicals as well as chelates redox metal at physiologically related levels. Similarly, the ideal antioxidant should perform very well in both aqueous and non-aqueous medium and have a positive impact on the cell (Ratnam et al., 2006).

The homeostasis of cellular oxidoreduction is regulated by a complicated internal antioxidant defense pathway that composed of enzymatic molecules such as superoxide dismutase which occurs as a Cu-Zn enzyme that resides inside the cell plasma, or a Mn-dependent catalyst that is located in the cell powerhouse (Kwon et al., 2012). Similarly, catalase is a high molecular weight, Fe-dependent enzyme that found in all cells types and localized in the cytosol (Nicholls, 2012). Moreover, low molecular weight glutathione

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peroxidases play a significant role in reducing several organic and inorganic hydroperoxides to their equivalent hydroxyl derivatives, using glutathione as a reductant (Brigelius-Flohé & Maiorino, 2013).

As for exogenous antioxidants, they are classified into two groups. The first group includes vitamins, flavonoids, anthocyanins and certain mineral compounds, which are derived from natural sources, mainly plants. On the other hand, the second group includes synthetic compounds (butylhydroxyanisole, butylhydroxytoluene, gallates, etc.) with antioxidant properties (Sen & Chakraborty, 2011). Antioxidants defense mechanisms work precisely by acting against oxidative stress via scavenging of radicals, donation of an electron, decomposition of peroxides, singlet oxygen quenching, enzymes inhibition and metal-chelating actions. Both enzymatic and non-enzymatic antioxidants are present in both internal and external environments to neutralize ROS (He et al., 2017).

There are two major techniques of antioxidant to overcome oxidative stress. The first one is a chain-breaking reaction involving the donation of electron by the primary/natural antioxidant to the highly reactive unpaired electron of free radicals located in the systems. In this case, the antioxidant acts directly on the unstable radicals and deactivate them, while they become less reactive free radicals or stabilized by other antioxidants and eventually terminating the chain reaction (Sen et al., 2010). This ability of antioxidant to donate electrons without itself becoming highly unstable is mostly due to the presence of aromatic ring in their structure, which allows for delocalization of unpaired electron. For example, vitamin C works by reacting with hydroxyl or alkoxyl radicals to generate water and alcohol respectively. The resulting vitamin C radical is stabilized via delocalization of its structure,

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and with the aid of NADH or NADPH-dependent reductases, vitamin C is restored (Lü et al., 2010).

The second mechanism is the removal of several oxygen species via inhibition of their activities by mostly enzymatic antioxidants. These enzymes function by blocking the development of free radicals from the onset. For example, enzyme such as superoxide dismutase is responsible for the dismutation of superoxide anion free radical ($O_2^{\bullet -}$) into oxygen molecule and hydrogen peroxide, subsequent to its conversion into water and oxygen molecule by either catalase (CAT) or glutathione peroxidase (GPx) (Santos-Sánchez et al., 2019). The concomitant elimination of H_2O_2 by CAT or GPx prevent the formation of hydroxyl radical from hydrogen peroxide via Fenton Reaction (Lü et al., 2010). Also, other processes of antioxidant defense system include metal ion chelation, up-regulation of antioxidants gene expression, and inhibition of gene expression of radical-producing enzymes (Kostuyk & Potapoyich, 2009).

2.2.1 Natural Antioxidants

Natural antioxidants can be found in food sources, such as fruits, vegetables and mushrooms. Therefore, several natural antioxidants are consumed from our daily diet. In recent years, the need for natural antioxidants has intensified due to the growing concern of consumers regarding the possible harmful consequences of synthetic antioxidants (Juntachote et al., 2006; Naveena et al., 2008). Moreover, the natural antioxidant is well-known for its high aqueous solubility, easy availability, purity, and high activity. Thus, it represents a better choice for most consumers as a functional ingredient to foods and food products (Jiang & Xiong, 2016).

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Antioxidant properties of many edible plants' species such as fruits, vegetables, herbs, and spices have been reported (Curimbaba et al., 2020; Gunathilake et al., 2016; Qasim et al., 2017). Most plant-based natural antioxidants contain polyphenols and carotenoids, which are known for various biological activities including antiviral, antibacterial and anti-inflammatory (Rathore et al., 2017). However, to complement the green nature of the antioxidants coupled with overcoming the degradation associated with the conventional extraction techniques (maceration and Soxhlet) and increase and preserve its activity, different improved eco-friendly extraction and accurate evaluation methods have been developed (Azmir et al., 2013; Barba et al., 2016; Xu et al., 2016). Some of these extraction methods include enzyme-assisted extraction, ultrasound-assisted extraction, supercritical fluid extraction, pulsed electric field extraction, and others (Xu et al., 2017). Similarly, the antioxidant content can be evaluated via cellular antioxidant activity assay, trolox equivalent antioxidant capacity (TEAC), oxygen radical absorbance capacity (ORAC), ferric ion reducing antioxidant assay, etc. (Xu et al., 2017).

The mechanisms of natural antioxidants depend on their classification, for example, they can deactivate free radicals via stabilizing them, thereby minimising the cellular damage and risk of oxidative related diseases. Also, dietary antioxidants such as carotenoids prevent chain reaction formation by scavenging and deactivating the free radicals (Krinsky, 2001). Moreover, mineral antioxidants such as selenium, copper and iron; they function as co-factors for enzymatic antioxidants (Allen & Cornforth, 2007). In the case of metal-binding proteins such as ferritin, lactoferrin and albumin; they are responsible for binding metals, thereby preventing their availability for Fenton reaction (Igielska-Kalwat et al., 2015; Perez-Jimenez et al., 2008; Wang et al., 2011). Therefore, increasing the levels of antioxidants in

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the body through the dietary intake of supplements rich in vitamins and minerals will protect the human cells against oxidative stress and prevent health disorders (Landete, 2013).

2.2.2 Synthetic Antioxidants

Synthetic antioxidants are antioxidant compounds synthesized and purified chemically. The most common features of these antioxidants include cost-effective, shorter assay duration and efficient and better pharmacological activity against different diseases. Some synthetic antioxidants are fat-soluble substances and are therefore able to effectively prevent lipid peroxidation in many processed food, pharmaceutical and supplements. The ideal synthetic compounds are mostly added or applied to different food item or other products to extend the shelf life of foods and food product (Berdahl et al., 2010; Karovičová & Šimko, 2000; Saad et al., 2007).

Synthetic antioxidants work mainly by capturing free radicals to stop the formation of chain reaction. Among the most utilized synthetic antioxidants are butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), *tert*-butylhydroquinone (TBHQ), propyl gallate (PG), and hexylresorcinol. These substances are mostly utilized in food as an antioxidant to preserve food and prevent rancidity (Chen et al., 2004; Freitas et al., 2010; Pop et al., 2013; Rahmanpour & Khalilzadeh., 2016; Yehye et al., 2015). All these antioxidants vary in their usage due to their properties. However, they all function by scavenging radical via donation of hydrogen atom from their hydroxyl substituted phenols structure (Berdahl et al., 2010).

The public concern towards synthetic antioxidants has grown due to several reasons. First, artificial antioxidants have been implicated in promoting tumor growth and forming

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reactive oxygen species, which may alter the genetic material of the cell (Haas, 2006; Goodman et al., 1990). Second, these antioxidants exhibit low aqueous solubility and a higher degree of toxicity (Sarkar & Ghosh, 2016). Third, they may produce unwanted effect on products color, taste and odor. As a result of the growing concern, countries such as Australia and Japan have either restrict or limit the usage of some of the synthetic antioxidant (Chan et al., 2011).

2.3 Mushrooms as a Source of Antioxidants

The nutritional value of mushrooms is well known (Rathee et al., 2012). Mushrooms provide a remarkable group of nutritional substances, such as vitamins B1, B2, B12, C, D and E (Feeney et al., 2014). Besides, due to its high protein contents, mushrooms can be used as a substrate to avoid protein malnutrition (Miles & Chang, 2004). Similarly, their low cholesterol level as well as a carbohydrate contents make it a suitable substitute food for diabetic and cardiac patients (Lee et al., 2019). Besides, mineral compositions of mushroom includes K, P, Ca, Mg, Na, Fe and some other trace elements (Wang et al., 2014).

Mushrooms are rather economical and technologically quite simple crop to cultivate as it requires small area and resources, and it can be grown anywhere in all seasons with low cost starting martials. Thus, the availability of mushroom made it a good candidate in food and nutraceutical industries (Ferraro et al., 2020). Regarding food industry, fruiting body of mushrooms have been consumed directly in fresh, processed or converted into powder and used as additives to certain food products to increase the nutritional quality of these products (Li et al., 2014). On the other hand, the chemical composition of mushrooms includes several bioactive compounds which give them the ability to exert beneficial effects at different levels (Elkhateeb et al., 2019). Thus, nutraceuticals in the form of food extracts or a single natural

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compound in a pharmaceutical form (pills, tablets, etc.) consumed as a part of dietary supplements to prevent the consequences of chronic diseases have been developed from mushrooms (Ustun et al., 2018). Some commercially available nutraceuticals include Organis ReiShi-Gen a synergistic blend of 50% *Lentinula edodes* and 50% *Ganoderma lucidum* which is a daily health supplement providing immune strength (Taofiq et al., 2017).

Mushrooms have gained significant attention for their biological activities, such as antitumor, antivirals, anticomplements, anticoagulants, antidiabetics, lipid-lowering drugs, hepatoprotectors, and immunostimulators, all of which made it suitable for use in food industry, cosmetics, biomedicine, agriculture, wastewater management and environmental protection (Moon & Lo, 2014). Moreover, a highly rich nutritional diet filled with natural antioxidants has been reported to lower the side effects of several chronic disorders (Oboh & Shodehinde, 2009).

Many mushrooms have been reported to possess antioxidant properties, which enable them to neutralize free radicals. Mushroom's antioxidant components are found in the fruiting bodies, mycelium and culture broth. For example, Gasecka et al. (2018) have estimated the content of macroelements (Ca, K, Mg, Na) as well as ascorbic acid and phenolic compounds in several mushrooms, and correlated the presence of these components to the ability of the extracts to act as free radical scavengers. Likewise, certain types of medicinal mushrooms and their extracts are alternative sources of antioxidant components, and their defense abilities against oxidative effects have been demonstrated by De Silva et al. (2012). While, Bozdogan et al. (2018) have worked on two edible mushrooms *Lactarius deliciosus* and *Pleurotus ostreatus* and found that the ion reducing capability of the isolated extracts is correlated to the presence of phenolic compounds and lycopene content. Therefore,

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mushroom is one of the main sources of various types of natural antioxidants (Abugri & McElhenney, 2013).

2.3.1 Strains Improvement of Mushrooms

To meet the growing demands of the increasing population, scientists are now focusing on improving both the phenotypic and genotypic properties of mushrooms by manipulating the chemical compositions to obtain higher yields, better quality, texture, color and taste (Chakravarty, 2011; Dan, 2010; Gogoi et al., 2019). Hybridization, molecular breeding, chemical mutagenesis, and irradiation are the most common ways to improve mushroom strains (Barh et al., 2019; Chattopadhyay et al., 2014; Fernandes et al., 2016).

In general, a hybrid is "the offspring produced by crossing two individuals of unlike genetic make-up", and the hybridization technique has contributed significantly to the improvement of crops and specifically mushroom production (Chakraborty & Sikdar, 2008; Sikdar et al., 2008). The most common type of mushrooms hybridization is practical protoplast fusion approach, which is able to generate different new species for research (Chakraborty & Sikdar, 2010; Mani et al., 2016). The technique includes the isolation of a single spore of fungi species via a spore print or serial dilution. This is followed by the assessment of the monokaryotic mycelium subsequent to its crossing (dikaryotization) with other strains (Baral et al., 2018).

Some studies have shown that hybrids exhibited better bioactivities compared to the parental stains such as Maity et al. (2011) who worked on hybrid of two different mushrooms, namely *Calocybe indica* var. APK2 and *Pleurotus florida* that was obtained by protoplast fusion. The study showed that polysaccharides extracted from the hybrid demonstrated better

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scavenging activities at higher concentrations. Likewise, a study was conducted on multiple hybrids of two *Pleurotus* spp. namely *P. ostreatus* and *P. djamor* and it was shown that the two hybrid strains exhibited antioxidant activities and have higher total phenolic contents than the parental strains (Oropeza-Guerrero et al., 2018).

Subsequent to the advancement of DNA technology, physical (UV and gamma irradiation) and chemical mutagens (N'-methyl-N'-nitro-N'-nitrosoguanidine) were employed to produce a point mutation in mushroom strain. Different doses of gamma-irradiation can be used on mushroom mycelium to observe the changes in the chemical components of mushrooms, and to measure the antioxidant activities towards free radicals. For instance, the use of gamma-irradiation on the mycelia of *Pleurotus florida* yielded several mutants. Among the obtained mutants, PO-4 mutant had higher antioxidant activities than the natural strain (Djajanegara, 2008). Likewise, multiple doses of gamma-irradiation were used on the mycelia of *Antrodia camphorata*, the study showed that extracts from irradiated mycelia exhibited antioxidant activities significantly higher than that of the extracts of non-irradiated mycelia (Huang & Mau, 2007).

2.3.2 Bioactive Compounds in Mushrooms

Mushrooms represent a boundless store of biologically active compounds, whose structural and molecular properties are of great importance to the pharmaceutical industry. Mushrooms produce different types of bioactive compounds - primary and secondary metabolites that possess different restorative or curative properties. The primary metabolites such as protein, carbohydrates, etc., are biomolecules that are involved in function of the major organism activities (reproduction, growth, and development). On the other hand, secondary metabolites such as alkaloids, terpenes, steroids, fatty acid, etc., are biochemical

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compound that less involved in major physiological functions of organism, although it is necessary for competitive survival (Wang et al., 2014). Also, based on sizes, the biologically active compounds in mushrooms are categorized into high and low molecular weight. Polysaccharides (β-glucans) and proteins represent the high molecular weight compound, while ascorbic acid, carotenoids, steroids, organic germanium and selenium constitute some of the low molecular weight functional compounds (Cohen et al., 2014; Feeney et al., 2014; Ferreira et al., 2010).

Most of these biomolecules can be extracted and purified from normal strain fruiting bodies or cultured mycelia. Examples of bioactive molecules with known properties are proteoglycan as immunostimulatory; lectins as antidiabetic; polysaccharides as antiviral; lectinans as antitumor; acidic polysaccharides as anti-inflammatory; terpenes as hepatoprotective and carotenoids as antioxidative agents (Dulay et al., 2017; Friedman, 2016; Jiang, 2014; Muszyńska et al., 2018).

Bioactive proteins in mushrooms that are of potential medicinal interest include lectins and hydrophobins. Besides, many reports have showed the high digestibility of the proteins found in mushroom (Walser et al., 2004; Wani et al., 2010). A study on *Pholiota nameko* demonstrates the antioxidant property of a novel protein in addition to its antitumor properties (Zhang et al., 2014). Similarly, the ability of water-soluble proteins isolated from *Ganoderma lucidum* to chelate prooxidant and donate hydrogen to free radicals was attributed to the high contents of selenium and amino acid residues as reported by Du et al. (2007).

Secondary metabolites (alkaloids, terpenoids, phenolic compounds, steroids, and polyketides) are small molecules produced during morphological and chemical

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differentiation against external stimulus. They are usually located and accumulated in the cell wall. Also, the biologically active metabolites of mushrooms possess antioxidant properties and other useful biological potentials (Asatiani et al., 2010; Cheung, 2010; Keles et al., 2011). Likewise, Kumari et al. (2011) have estimated the total contents of phenolic, flavonoid and ascorbic acid of three species of *Cantharellus* spp. and correlated the antioxidant activities of the tested mushroom to the presence of these bioactive compounds.

2.3.3 Proteins

The functional values of mushroom are mostly dependent on its protein contents, which varies depending on the external factors and on the species of the mushroom. The biologically active proteins and peptides produced by mushrooms are varied in structure. Some of the proteins found in the mushroom are lectins, immunomodulatory proteins, ribosome-inactivating proteins, antimicrobial proteins, ribonucleases and laccases, all of which are the crucial and functional components with great values for pharmaceutical products (Sheu et al., 2009; Wong et al., 2008).

Recently, the antioxidative role of proteins and peptides derived from mushrooms have gained significant interest. Also, molecules such as cysteine and tyrosine are normally amino acids with side chains that can donate electrons (Reczek & Chandel, 2015; Schieber & Chandel, 2014; Van Lancker et al., 2011). Also, various studies have proved that proteins and peptides are good inhibitors of lipid peroxidation, scavengers of free radicals and metal-ions chelators. For example, a novel protein was isolated from *Pholiota nameko* exhibited significant antioxidant activities by effectively scavenging hydroxyl and 1,1-diphenyl-2-picrylhydrazyl radicals (Zhang et al., 2014). Moreover, both Farzaneh et al. (2018) and Kimatu et al. (2017) have worked on different mushrooms, they used ammonium sulphate

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precipitation and chromatography techniques to isolate and purify water-soluble protein. The isolated protein extracts were hydrolyzed by sequential enzymatic processes to obtain several peptide fractions, the peptides and their related initial proteins showed metal chelating abilities and radical scavenging activities.

The enzymatic hydrolysis of proteins is one effective approach that can be used to release antioxidant peptides without affecting the nutritive value. The antioxidant activities of peptides have been reported to depend on enzyme specificity, molecular weight, the degree of hydrolysis, amino acid composition, and hydrophobicity (He et al., 2013; Sarmadi et al., 2010). Moreover, bioactive peptides derived from proteins contain amino acids with aromatic residues that can donate electron/hydrogen to free radicals (Yuwa-amornpitak et al., 2020).

2.3.4 Polysaccharides

Polysaccharides extracted from mushrooms have been studied extensively and found to contribute to fungal antitumor activities (Tao et al., 2006). Moreover, different polysaccharides with extra nutritional components have been extracted and purified from the sporocarps of various types of mushrooms (Chen et al., 2010; Yan et al., 2019). Polysaccharides, glycoproteins and heteropolysaccharides are water-soluble compounds that consist of monosaccharides such as glucose, mannose, galactose, fucose, xylose and arabinose in different ratios coupled with varying glycosidic and peptide linkages (Chen et al., 2008; Nie et al., 2013). Since polysaccharides molecules are considered very complex, elucidation of their specific characteristics is essential to ascertain the effect of structure on the bioactivity of the polysaccharides (Villares et al., 2012). Due to biological properties of mushroom polysaccharides, there has been a massive increase in the industrial use of polysaccharides extracted from various types of edible mushrooms (Rathore et al., 2017).

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Tseng et al. (2008) have found that the antioxidant activity of mushroom extracts was found to be correlated with their polysaccharide contents. Moreover, a previous study illustrated the antioxidative potentials of polysaccharides by scavenging free radicals, thereby minimising the damage caused by oxidative stress (Thetsrimuang et al., 2011).

Practically, the antioxidant activities of polysaccharides may vary between one assay to another and also depends on the structural characteristics that involve molecular weight, branching degree, glycosidic bond and chemical composition (Gong et al., 2020; Li et al., 2013). For example, a study on *Flammulina velutipes* showed varying antioxidant properties of four different polysaccharides extracted via different techniques, suggesting the possible influence of the extraction method on the polysaccharides structure and in turn on its antioxidant activity (Zhang et al., 2013). Furthermore, supporting the aforementioned supposition is the study on *S. commune*, Chen et al. (2019) reported that polysaccharides extracted from *S. commune* by the ultrasonic-assisted method demonstrated higher hydroxyl radicals scavenging activity and reducing power. The *in vivo* antioxidative role of polysaccharides have been demonstrated. For instances, Liu et al. (2016) found that polysaccharides from *Cordceps militaris* inhibited the formation of MDA in multiple organs (liver, kidney and heart). This particular study has consolidated the previous findings that polysaccharides have high radicals scavenging activities and protecting abilities against oxidative stress *in vivo*.

Molecular weight is one of the most important structural features of polysaccharides as it is related to the number of reductive hydroxyl group terminals that are responsible for accepting and eliminating free radicals as well as increasing the solubility of polysaccharides in water and other polar solvents (Cor et al., 2018). Related to the previously mentioned facts,

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Hu et al. (2019) found that polysaccharides with low molecular weight exhibit better antioxidant activities due to increased solubility and surface as indicated in study of polysaccharides isolated from *F. velutipes*. Kao et al. (2012) have demonstrated that low molecular weight polysaccharides isolated from *Ganoderma lucidum* were able to significantly reduce the reactive oxygen species formation by increasing the viability of a mouse leukaemic monocyte macrophage cell line. Moreover, previous reports indicated that polysaccharides with antioxidant activity were characterised by average molecular weights mainly distributed between 10 and 1000 kDa, composed of glucose, mannose and galactose residues (Chen et al., 2012; Tahmouzi, 2014; Wang et al., 2014).

2.3.5 Secondary Metabolites

Much significance has been attached to mushrooms, owing to its rich nutritional and medicinal values which stem from its plentiful bioactive secondary metabolites (Wasser, 2014), such as phenolic compounds, alkaloids, terpenes, steroids and polyketides. The secretion and the activities of these low molecular weight metabolites are regulated by various cellular and physiological mechanisms (Chanda et al., 2009; Mukherjee et al., 2012). Besides, these compounds are produced by fungi in response to unfavorable environment condition and a threat to their survival (Zhong & Xiao, 2009).

Phenolic compounds are the most known secondary metabolites and represent a large class of phytochemicals with favorable biological properties. Anthocyanins, catechins, lignans, capsaicinoids, stilbenes, curcumin and ellagic acid are some of the phenolic compounds (Palacios et al., 2011). Previous studies have discussed the role of phenolic compounds in stopping the free radical chain reactions due to their hydroxyl group which neutralizes the reactive oxygen/nitrogen species (Asatiani et al., 2010; Yadav et al., 2014).

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Alkaloids are also very important organic compounds, which contain basic nitrogen atoms and are commonly used in the pharmaceutical field for drugs development. However, the antioxidant properties of a mushroom may differ depending on the type of alkaloids (Wieczorek et al., 2015). A scientific research study was carried *in vitro* on the methanolic extracts of *Cyclocybe cylindracea*, and β-carboline alkaloid was identified and purified. This particular type of alkaloid was soluble in water and methanol, also exhibited explicit radical scavenging activity (Krüzselyi et al., 2019).

Terpenes are aromatic compound composed of unsaturated hydrocarbon (isoprene) units. Different types of terpenes demonstrating therapeutic efficiency have been identified from mushroom species to date (Jeong et al., 2008; Yue et al., 2010). Terpenes in macrofungi are present in modified forms, such as terpenoids or isoterpenoids and are the most investigated forms of terpenes. The antioxidative activity of terpenoids have been evaluated and measured in several research studies. Their ability to scavenge free radicals and power to reduce ferric ions to ferrous were also demonstrated (Zhong & Xiao, 2009). Modified terpenes (neogrifolin) isolated from *Albatrellus ovinus* showed high scavenging activities towards DPPH radicals (Nukata et al., 2002).

2.4 *Schizophyllum commune*

Schizophyllum commune (Figure 2.1) is an edible fungus species in the phylum Basidiomycota. This species usually grows abundantly during the rainy season and frequently appears on dead wood (Nieuwenhuis et al., 2011; Ohm et al., 2010; Teoh et al., 2012). It is well-known for its split gills, which function to produce basidiospores on their surface. The individual fruiting bodies are flexible, stiff and well-adapted to a climate with sporadic rains. The color of this species of mushroom is very unique and wavers between

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white-grey and brown. Moreover, the shape of the fruiting body is like a fan and has a very short strip with the frizzy upper surface (Kuo, 2003).

Figure 2.1: The fruiting bodies of *Schizophyllum commune* cultivated in Mycology Laboratory, Institute of Biological Sciences, Faculty of Science, Universiti Malaya.

With the increasing rate of disease associated with diet, foods containing bioactive compounds are crucial for health improvement and received great significance, particularly in the food industry. In this regard, *S. commune* is of a great interest due to its rich nutritional value and therefore serves as an important source of protein, polysaccharides, vitamins, lipids, mineral and variety of secondary metabolites (Chandrawanshi et al., 2017). These functioning bioactive components contribute to the nutraceutical values of *S. commune* and more specifically its antioxidant activity against (ROS) resulting from biochemical reactions in the body.

The medicinal use of *S. commune* has been widely reported in recent years. Moreover, the genetic make-up of *S. commune* has been researched thoroughly (Acharya et al., 2016).

Among the potential application of *Schizophyllum* species that includes biological response modification, non-specific stimulator of the immune system, enhancement of vaccines, and others (Hao et al., 2010).

As shown in Table 2.1, the antioxidant activity of *S. commune* has been studied previously but most work are rather preliminary with regard to the role of different antioxidative components. For instance, Abd Razak et al. (2019) have reported that temperature and extraction time influenced the scavenging activity of *S. commune* hot water extracts in which temperature (4°C) and longer extraction time (30 min) enhanced the radical scavenging activity of the extracts. Moreover, Devi et al. (2014) have worked on the ethanolic extracts of *S. commune* and correlated the presence of phenolic and flavonoids content to the radical scavenging activity of the extracts. Thus, these antioxidant properties make *S. commune* as a source of natural antioxidants. Although the antioxidant activities of polysaccharides of several mushroom species have been reported, the role of polysaccharides and other water-soluble components in the hot water extracts of *S. commune* has yet to be elucidated. While the antioxidant activity of the hot water extract of *S. commune* has been reported, little is known regarding the active components that might be responsible for the observed activity.

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Table 2.1: Previous studies conducted on various *Schizophyllum commune* extracts to determine their antioxidant activities.

Mushroom part	Extraction solvent	Major findings	References
Mycelia	Acid-alkaline extraction	Melanin-like pigments extracts showed scavenging activity towards DPPH radicals. The scavenging activity increased to 96% at higher concentration (10 µg/µl).	Arun et al. (2015)
Fruiting body	Hot water	Extraction at (4°C) and longer extraction time (30 min) produced extracts with a remarkable scavenging activities towards DPPH radicals ($p < 0.05$).	Abd Razak et al. (2019)
Fruiting body	Hot water Methanol Ethanol	The water extracts exhibited higher DPPH radical scavenging activity (IC_{50}: 19.26 µg/µl) than methanol extracts (IC_{50}: 24.82 µg/µl) but slightly lower than ethanol extracts (IC_{50}: 18.56 µg/µl). However, the reducing power ability of hot water (IC_{50}: 20 µg/µl) and methanol extracts (IC_{50}: 21.68 µg/µl) were comparable.	Chandrawanshi et al. (2017)

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Table 2.1, continued

Mushroom part	Extraction solvent	Major findings	References
Fruiting body	Hot water Different extraction techniques.	Polysaccharides extracted through ultrasound-assisted technique had the highest antioxidant activities. Conversely, hot water extraction had the highest polysaccharides yield and content but exhibited the lowest antioxidant activities.	Chen et al. (2019)
Fruiting body	Ethanol	The antioxidant activities were correlated to the presence of phenolic and flavonoid compounds.	Devi et al. (2014)
Fruiting body	Hot water Methanol Chloroform *n*-hexane	Chloroform extracts exhibited the highest scavenging activity (IC_{50}:7.652 mg/ml) and metal chelating abilities (IC_{50}: 6.590 mg/ml).	Emsen et al. (2017)
Fruiting body	Ethanol	Ethanol extracts exhibited ferric ion reducing ability equivalent to vitamin C (13.5 VCE g/L).	Gevorgyan et al. (2017)
Fruiting body	Water Acetone Ethanol	All extracts showed hydroxyl and DPPH radicals scavenging activity.	Kumar et al. (2018)

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Table 2.1, continued.

Mushroom part	Extraction solvent	Major findings	References
Fruiting body	Hot water Methanol Ethyl acetate Dichloromethane	The methanol extracts exhibited the highest radical scavenging activity (IC_{50}: 0.145 ± 0.01 mg/ml) and was correlated to the total phenolic content of the extracts.	Mirfat et al. (2010)
Fruiting body	Absolute methanol Methanol 70% Absolute ethanol Ethanol 70%	The aqueous-methanol extracts exhibited the highest radical scavenging activity up to 70% and ion reducing ability (76.61 ± 1.79 mM Fe^{2+}/g extract).	Razak et al. (2018)
Mycelium Culture filtrate	Ethyl acetate	The extracts exhibited scavenging activities towards DPPH (IC_{50}: 6.88 ± 0.41 µg/ml). In addition, the extracts showed ion reducing abilities (17,328.23 ± 310.72 mM Fe^{2+}/g extract).	Tangjitjaroenkun et al. (2020)

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CHAPTER 3: MATERIALS AND METHODS

3.1 Chemicals

The following are of analytical grade: ethanol, methanol (Kollin Chemicals, Korea); ethyl acetate, sulphuric acid and neocuproine (Friendemann Schmidt Chemical, Australia); Folin-Ciocalteu's phenol reagent, D-glucose (Merck Millipore, Germany). Sodium carbonate, iron(II) sulphate, acetic acid, 2,4,6-tripyridyl-s-triazine (TPTZ), trolox (6-hydeoxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and butylated hydroxytoluene (BHT) were obtained from R&M Chemicals, Malaysia. 2,2-azinobis-3-ethylbenzothiazonline-6-sulfonic acid (ABTS), potassium persulphate, 2,2 diphenyl-1-picylhydrazyl (DPPH), aluminium chloride were purchased from Sigma-Aldrich, Germany. Standards (purity > 95%) such as gallic acid, rutin hydrate and ascorbic acid were obtained from Sigma-Aldrich, Germany. Iron(III) chloride, sodium hydroxide, sodium nitrite and sodium acetate were obtained from Friendemann Schmidt Chemical, Australia. Ammonium sulphate was obtained from QReC Chemicals, Malaysia. The Micro BCA™ Protein Assay Kit and SnakeSkin™ dialysis Tubing 5 kDa MWCO were obtained from Thermo Scientific Rockford, IL, USA.

3.2 Mushrooms samples

The mycelium cultures of *S. commune* were obtained from the Mycology Laboratory, Institute of Biological Sciences, Faculty of Science, Universiti Malaya. The mycelium cultures on agar plate were irradiated with gamma-ray at two doses, 2000 and 4000 Gy (representing the IC_{25} and IC_{50} of the treatment) for 10 minutes. The cultures were subsequently used in the production of fruiting bodies via the solid-substrate fermentation

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method. Sawdust supplemented with rice bran (20%, w/w) were used as substrate. The entire growing process from the inoculation to the harvesting took approximately 2 months. The strains used in this study have minor differences with respect to the size and colour of the fruiting bodies. The fruiting bodies of *P. pulmonarius* (grey oyster mushroom) and *A. bisporus* (white button mushroom) were purchased from a local supermarket in Petaling Jaya, Selangor, Malaysia. All mushroom samples were cleaned and dried using a food dehydrator (WAKimart, Malaysia) between 5 to 7 hours. The dried mushroom samples were powdered using a Waring blender. Table 3.1 shows the list of all mushroom samples used in this study.

Table 3.1: Mushroom samples used in this study

Sample	**Description**
Natural strains of *Schizophyllum commune*	
W	Natural strain isolated from Malaysia
R	Natural strain isolated from Thailand
Gamma-irradiated strains of *S. commune*	
W2000	Strain W subjected to gamma irradiation at a dose of 2000 Gy
W4000	Strain W subjected to gamma irradiation at a dose of 4000 Gy
R2000	Strain R subjected to gamma irradiation at a dose of 2000 Gy
R4000	Strain R subjected to gamma irradiation at a dose of 4000 Gy
Hybrid strains of *S. commune*	
WR	Hybrid of strain W and R with dominant characteristics of W
RW	Hybrid of strain W and R with dominant characteristics of R
Common edible mushrooms	
Pleurotus pulmonarius	Obtained from commercial sources
Agaricus bisporus	Obtained from commercial sources

*The fruiting bodies of all *S. commune* strains were cultivated using sawdust as the main substrates.

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3.3 Preparation of crude hot water extracts

The powdered fruiting bodies were soaked in 80% (v/v) aqueous ethanol at a ratio of 1:10 (w/v) for two days at room temperature under shaking condition. The extracts were then filtered and the residues were air-dried before subjected to hot water extraction at a ratio of 1:20 (w/v) at 121°C for 30 min under high pressure in an autoclave. The mixtures were filtered through Fioroni filter paper 185 mm, and the residues were re-extracted twice. Supernatants from each hot water extraction were combined and freeze-dried. The crude hot water extracts of selected mushrooms were kept at -20°C prior to analysis.

3.4 Determination of chemical compositions of the crude hot water extracts

3.4.1 Total sugar content

Total sugar content of the extracts was assayed by the phenol-sulphuric acid method (Masuko et al., 2005). A 50 µl of sample was added in a well followed by 150 µl of concentrated sulphuric acid and 30 µl of 5% (w/v) phenol in water. The mixture was shaken then heated for 5 min at 90°C in a static water bath. After cooling to room temperature, the absorbance was measured at 490 nm. A calibration curve of glucose (0-1000 µg/ml) was plotted and results were expressed as mg carbohydrate/g extract.

3.4.2 Total protein content

Total protein content of the extracts was estimated using the Micro BCA^{TM} Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). A 200 µl of the working reagent, prepared by mixing 50 part of reagent A and 1 part of reagent B, was added to 25 µl of sample. The mixture was incubated at 37°C for 30 min. After cooling to room temperature,

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the absorbance was measured at 562 nm. A calibration curve of bovine serum albumin (0-2000 µg/ml) was prepared and results were expressed as mg protein/g extract.

3.4.3 Total phenolic content

Total phenolic content of the extracts was measured using the Folin-Ciocalteu method by Singleton and Rossi (1965) with modifications. A 25 µl of 1 N Folin-Ciocalteu reagent was added into 50 µl of sample and the mixture was incubated for 5 min. Then, 100 µl of sodium carbonate solution (0.57 M) and 75 µl of milliQ water were added respectively. The final volume was 250 µl. The mixture was incubated for 2 h and the absorbance was measured at 760 nm. A calibration curve of gallic acid (0-0.2 mg/ml) was prepared and results were expressed as mg gallic acid equivalents (GAE)/g extract.

3.4.4 Total flavonoid content

Total flavonoid content (TFC) of the extracts was determined using the aluminium chloride colorimetric method by Liu et al. (2008) with slight modifications. A 100 µl of extracts was mixed with 10 µl of 5% (w/v) sodium nitrite. After incubation for 5 min, 10 µl of 10% (w/v) aluminium chloride was added. Following 6 min incubation, 100 µl of 1 M sodium hydroxide and 30 µl of milliQ water were added respectively. The final volume was 250 µl. The absorbance was read at 510 nm. A calibration curve of rutin (0-0.1 mg/ml) was prepared and results were expressed as mg rutin equivalents (RE)/g extract.

3.5 Assessment of antioxidant activity of crude hot water extracts

Different *in vitro* antioxidant assays, such as 2,2-diphenyl--picrylhydrazyl (DPPH) scavenging activity assay, trolox equivalent antioxidant capacity (ABTS), ferric reducing antioxidant power (FRAP), and cupric ion-reducing antioxidant capacity (CUPRAC) were

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used to evaluate the antioxidant activity of the extracts. The crude hot water extracts of mushrooms were initially dissolved in water to produce stock solutions (20 mg/ml) and diluted further to the desired concentrations for the assays.

3.5.1 Diphenyl-2-pricryl-hydrazyl (DPPH) radical scavenging assay

The DPPH free radical scavenging activity of the extracts was measured according to the method of Brand-Williams et al. (1995) with slight modifications. Briefly, 50 µl of the extract was mixed with 195 µl of DPPH methanolic solution, and the mixture was incubated for 30 min in the dark. The absorbance values were measured at 515 nm. The percentage of DPPH radical scavenging activity was calculated according to the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [\ (A_{control} - A_{sample}) / A_{control}] \times 100$$

Where; $A_{control}$ = absorbance of DPPH radicals without sample or standard and A_{sample} is the absorbance of DPPH radicals with sample.

Trolox was used as a standard (0-2 mM) and the results were expressed as trolox equivalents (mmol TE/g extract). Gallic acid, rutin, ascorbic acid and butylated hydroxytoluene (1 mg/ml) were used as positive controls.

3.5.2 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) or $ABTS^+$ scavenging assay

ABTS is a stable radical cation used to measure the trolox equivalent antioxidant capacity. The assay was done according to Re et al. (1999). The reagent was prepared by mixing 7 mM of ABTS solution with 2.45 mM potassium persulphate. The mixture was incubated for 16 h at room temperature in the dark. The stock solution of ABTS was adjusted to obtain the desired absorbance of 0.70 ± 0.05 at 734 nm. A 300 µl of the ABTS reagent was

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added to 3 µl of extract. After 6 min the absorbance values were measured at 734 nm. The percentage of ABTS scavenging activity was calculated according to the following equation:

$$\text{ABTS radical scavenging activity (\%)} = [\ (A_{control} - A_{sample}) / A_{control}] \times 100$$

Where; $A_{control}$ is the absorbance of ABTS radical cations without sample or standard, and A_{sample} is the absorbance of ABTS radical cations with sample or standard.

Trolox was used as a standard (0-4 mM) and the results were expressed as trolox equivalents (mmol TE/g extract). Gallic acid, rutin, ascorbic acid and butylated hydroxytoluene (1 mg/ml) were used as positive controls.

3.5.3 Ferric reducing antioxidant power (FRAP) assay

The assay was done according to the method of Benzie and Strain (1996). The FRAP reagent was prepared by adding 300 mM acetate buffer, 10 mM of TPTZ in 40 mM HCl and 20 mM of ferric chloride solution at a ratio of 10:1:1 (v/v/v). Briefly, 300 µl of FRAP reagent was added to 5 µl of extract, and the mixture was incubated for 30 min at 37°C. The absorbance was measured at 595 nm. A calibration curve of iron(II) sulphate (0-4 mM) was prepared and the results were expressed as mmol Fe^{2+}/g extract. Gallic acid, rutin, ascorbic acid and butylated hydroxytoluene (BHT) (1 mg/ml) were used as positive controls.

3.5.4 Cupric ion-reducing antioxidant capacity (CUPRAC) assay

The CUPRAC assay was done according to Apak et al. (2005) with slight modifications. The CUPRAC test solution was prepared by adding 50 µl of copper(II) chloride (10 mM), neocuproine (7.5 mM) and acetate buffer (1000 mM, pH 7). A 150 µl of the CUPRAC reagent was added to 50 µl of extract. The mixture was incubated in the dark at room temperature for 30 min. The absorbance was measured at 450 nm. A calibration

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curve of trolox (0-1 mM) was prepared and the results were expressed as trolox equivalents (mmol TE/g extract). Gallic acid, rutin, ascorbic acid (0.1 mg/ml) and butylated hydroxytoluene (1 mg/ml) were used as positive controls.

3.6 Fractionation of selected crude hot water extracts of *S. commune*

The crude hot water extracts of the *S. commune* strains W and W4000 were selected for further study. The powdered fruiting bodies (20 g) of the selected strains were again subjected to aqueous ethanol and hot water extraction as described in Section 3.3. After that, the crude hot water extracts were collected and subjected to liquid-liquid extraction and precipitation procedures. The processes of hot water extraction and fractionation of the selected *S. commune* strains are shown in Figure 3.1.

3.6.1 Liquid-liquid extraction

The resulting hot water extracts were partitioned with ethyl acetate (1:1, v/v) to give the ethyl acetate-soluble and water fractions. The ethyl acetate fractions of strains W and W4000 were termed as W-EA and W4000-EA, respectively. The water fractions of strains W and W4000 were termed as W-H_2O and W4000-H_2O, respectively. Excess solvents in the organic and water fractions were removed using a rotary evaporator. The water-soluble components in the water fractions were then precipitated using two different methods namely ammonium sulphate precipitation and ethanol precipitation.

3.6.2 Ammonium sulphate precipitation

Powdered ammonium sulphate (60.3 g) was gradually added into the aqueous fraction until 90% (w/v) saturation was achieved and the mixture was left overnight with continuous stirring at 4°C. Then, the mixture was centrifuged and the pellet was re-dissolved in distilled

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water. The fraction was extensively dialyzed (SnakeSkin™ dialysis Tubing 5 kDa MWCO, Thermo Scientific, USA) against distilled water at 4°C for 3 days with the water being changed every 12 h before being freeze-dried. These fractions were termed as WS1. The fractions obtained from strains W and W4000 were termed as W-WS1 and W4000-WS1, respectively. The samples were kept at -20°C prior to analysis.

3.6.3 Ethanol precipitation

Ethanol was added to the water fractions until the final concentration of ethanol was 80% (v/v). The mixture was left overnight at 4°C without stirring. Following that, the mixture was centrifuged at 10,000 ×g for 20 min and the pellet was washed with acetone and ethanol, successively, before re-dissolved in water. The fraction was also subjected to dialysis against distilled water at 4°C for 3 days before being freeze-dried. Theses fractions were termed as WS2. The fractions obtained from strains W and W4000 were termed as W-WS2 and W4000-WS2, respectively. The ethanol supernatant was also collected and dried to yield the EtOH fractions. The fractions W-EtOH and W4000-EtOH were obtained from strains W and W4000, respectively. The samples were kept at -20°C prior to analysis.

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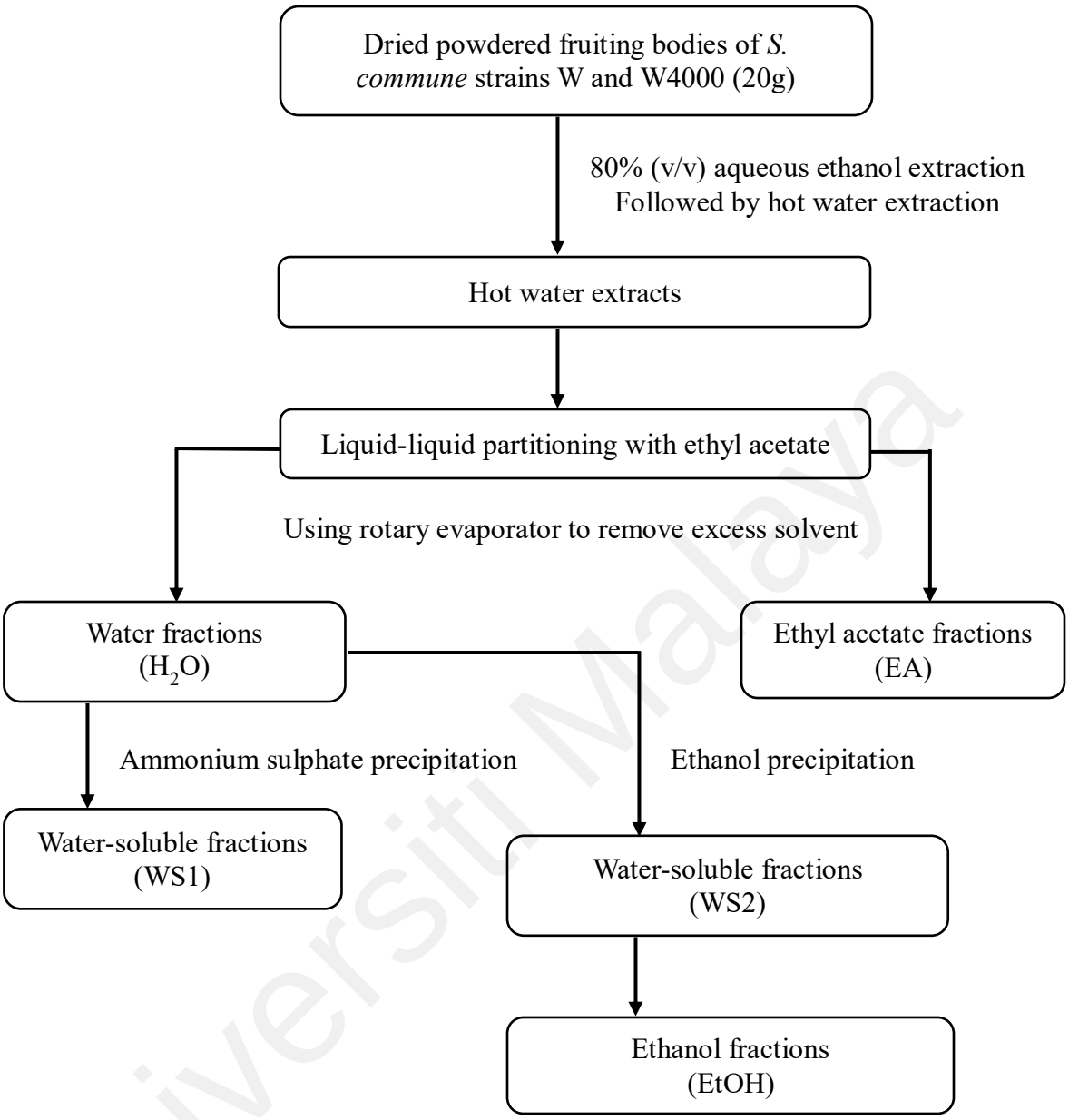

Figure 3.1: Extraction and fractionation of selected strains of *S. commune*.
The process of extraction and fractionation of the two selected strains of *S. commune* W and W4000 to obtain water-soluble fractions (H_2O, WS1 and WS2), and organic fractions (EA and EtOH).

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3.7 Assessment of chemical compositions and antioxidant activities of *S. commune* fractions

The water-soluble and organic solvent fractions of *S. commune* were dissolved in water and dimethyl sulphoxide, respectively, to produce stock solutions (20 mg/ml). The chemical compositions of the fractions were determined as described in Section 3.4. The antioxidant activities of the fractions were assessed as described in Section 3.5.

3.8 Statistical analysis

All analyses were performed in triplicates. Results were expressed as mean ± standard deviation (n=3). All data were statistically analyzed using Statistical Package for the Social Sciences software, version 25.0 (SPSS Inc., Chicago, Illinois, USA). One-way ANOVA was used to compare means among the groups. Pearson's correlation was used to analyse the relationship between chemical components and antioxidant activities of the extracts. A p value < 0.05 will be regarded as significant.

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CHAPTER 4: RESULTS

4.1 Yield of crude hot water extracts

The yields of the crude hot water extracts of *S. commune* strains, *P. pulmonarius* and *A. bisporus* are shown in Table 4.1. The overall yield of the extracts (%, w/w) of *S. commune* (9.0 – 13.4%) was comparatively higher than those of *P. pulmonarius* (11.2%) and *A. bisporus* (white button) (8.6%). Amongst the *S. commune* strains, the yield of both natural strains W and R were lower than that of the strains obtained from gamma irradiation and the hybrids of both strains.

Table 4.1: The yields of crude hot water extracts of selected mushroom samples

Samples	Yield (%, w/w)
Schizophyllum commune strains	
W	9.02
W2000	13.07
W4000	12.76
R	9.32
R2000	11.88
R4000	13.38
WR	10.92
RW	12.76
Common edible mushrooms	
P. pulmonarius	11.24
A. bisporus	8.57

*W; natural strain from Malaysia; W2000 and W4000, two gamma-irradiated strains; WR and RW, two hybrid strains; R, natural strain isolated from Thailand; R2000 and R4000, two gamma-irradiated strains.

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4.2 Chemical compositions of crude hot water mushroom extracts

The chemical compositions of the crude hot water extracts of *S. commune* and common edible mushrooms are shown in Table 4.2.

4.2.1 Total sugar content

The total sugar contents of the crude extracts of *S. commune* strains, ranging from 229.95 to 596.22 mg carbohydrate/g extract, were found to be higher than both common edible mushrooms (Table 4.2). Among the *S. commune* strains, the gamma-irradiated strain R2000 have the highest sugar content (596.22 mg carbohydrate/g extract) followed by the hybrid strains WR (449.65 mg carbohydrate/g extract) and RW (468.97 mg carbohydrate/g extract).

4.2.2 Total protein content

The total protein contents of the crude extracts of *S. commune* strains ranged from 395.75 to 539.69 mg protein/g extract and these were lower than the protein contents in the extracts of both edible commercial mushrooms (Table 4.2). Regarding *S. commune* strains, the highest protein content was observed in the two natural strains W and R with protein contents (mg protein/g extract) of 539.69 and 526.66, respectively.

4.2.3 Total phenolic content

The crude extracts of *S. commune* strains have higher phenolic contents (15.63 – 25.78 mg GAE/g extract) than *P. pulmonarius* (13.87 mg GAE/g extract) but lower than that of *A. bisporus* (29.39 mg GAE/g extract) as shown in Table 4.2. Overall, the natural strain W and its gamma-irradiated strains (W2000 and W4000) have higher phenolic content than those of natural strain R and its gamma-irradiated strains (R2000 and R4000).

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4.2.4 Total flavonoid content

The crude extracts of *S. commune* strains have significantly higher flavonoid contents (4.04 – 13.45 mg RE/g extract) compared to *P. pulmonarius* (1.40 mg RE/g extract) but lower than that of *A. bisporus* (24.18 mg RE/g extract) as shown in Table 4.2. The natural strain W has the highest level of flavonoids while the other natural strain R has the lowest flavonoid content.

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Table 4.2: The chemical compositions of crude hot water extracts of selected mushroom samples

Mushrooms	Chemical Composition			
	Total sugars (mg carbohydrate/g extract)	Total proteins (mg protein/g extract)	Total phenolic content (mg GAE/g extract)	Total flavonoid content (mg RE/g extract)
S. commune				
W	327.99 ± 4.36^{e}	539.69 ± 6.05^{c}	25.78 ± 0.57^{a}	13.45 ± 0.31^{b}
W2000	400.73 ± 1.55^{d}	442.42 ± 12.20^{e}	20.14 ± 0.79^{bc}	7.78 ± 0.95^{de}
W4000	414.85 ± 17.11^{c}	430.60 ± 39.31^{d}	22.07 ± 0.87^{b}	7.01 ± 0.73^{de}
R	229.95 ± 1.61^{f}	526.66 ± 40.98^{cd}	15.95 ± 0.89^{def}	4.04 ± 0.62^{f}
R2000	596.22 ± 1.72^{a}	480.00 ± 19.68^{de}	19.21 ± 0.22^{c}	8.76 ± 0.93^{cde}
R4000	395.14 ± 13.27^{de}	438.18 ± 26.78^{e}	18.27 ± 0.49^{cde}	9.51 ± 0.46^{bcd}
WR	449.65 ± 9.07^{bc}	440.90 ± 8.33^{e}	18.59 ± 0.36^{d}	9.92 ± 0.65^{bc}
RW	468.97 ± 12.61^{b}	395.75 ± 6.82^{e}	15.63 ± 0.21^{ef}	6.41 ± 0.37^{ef}
P. pulmonarius	219.06 ± 14.98^{fg}	666.96 ± 12.60^{b}	13.87 ± 1.95^{f}	1.40 ± 0.64^{g}
A. bisporus	204.85 ± 8.25^{g}	684.24 ± 63.58^{a}	29.39 ± 0.16^{a}	24.18 ± 0.89^{a}

$^{a, b, c....}$ means with different superscripts on the same column differ significant ($p < 0.05$).

*Data are expressed as mean ± standard deviation (n = 3).

*GAE, gallic acid equivalent; RE, rutin equivalent.

*W; natural strain from Malaysia; W2000 and W4000, two gamma-irradiated strains; WR and RW, two hybrid strains; R, natural strain isolated from Thailand; R2000 and R4000, two gamma-irradiated strains.

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4.3 Antioxidant activities of crude mushroom extracts

The radical scavenging activities and reducing capacities of the extracts of *S. commune* were evaluated and compared to the selected common edible mushrooms and positive controls.

4.3.1 DPPH radical scavenging activities

The abilities of the mushrooms' hot water extracts to scavenge the DPPH free radicals, expressed as trolox equivalents, is depicted in Figure 4.1. The extracts of *S. commune* strains (1.17 – 1.78 mmol TE/g extract) showed either higher or comparable antioxidant capacity compared to *P. pulmonarius* (1.35 mmol TE/g extract) but were weaker than that of *A. bisporus* (2.01 mmol TE/g extract). Amongst the strains of *S. commune*, the natural strain W showed the highest antioxidant activity while the other natural strain R showed the lowest antioxidant activity. The positive controls including ascorbic acid (2.66 mmol TE/g), gallic acid (2.62 mmol TE/g), rutin (2.52 mmol TE/g) and BHT (2.50 mmol TE/g) showed relatively high antioxidant capacity compared to the mushroom extracts.

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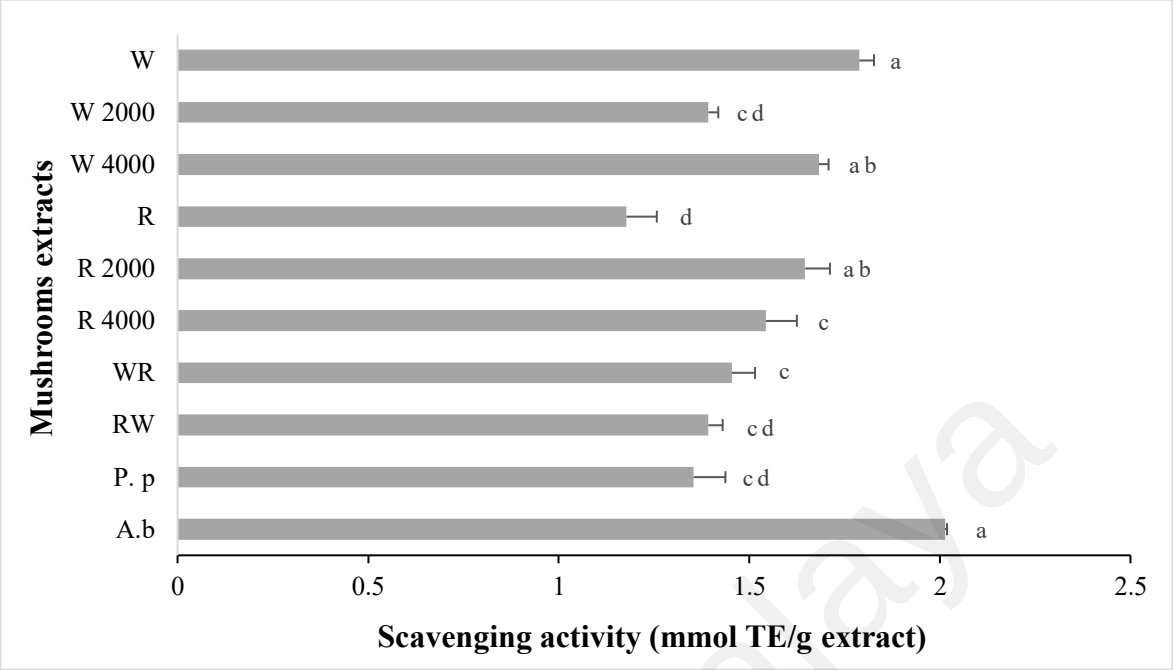

Figure 4.1: DPPH free radical scavenging activities of the crude hot water extracts of selected mushrooms.

[a, b, c....] means with different superscripts on the bars ($p < 0.05$). W; natural strain from Malaysia; W2000 and W4000, two gamma-irradiated strains; WR and RW, two hybrid strains; R, natural strain isolated from Thailand; R2000 and R4000, two gamma-irradiated strains; P. p, *Pleurotus pulmonarius;* A. b, *Agaricus bisporus.*

4.3.2 $ABTS^{+}$ scavenging activities

The abilities of the mushrooms' hot water extracts to scavenge the ABTS radicals, expressed as trolox equivalents, is depicted in Figure 4.2. The antioxidant capacities of the crude extracts of *S. commune* strains (0.11 – 0.15 mmol TE/g extract) were higher compared to *P. pulmonarius* (0.08 mmol TE/g extract) but lower than that of *A. bisporus* (0.19 mmol TE/g extract). The natural strain W has the most potent ABTS radical scavenging ability amongst the strains of *S. commune*. The positive controls including ascorbic acid (4.12 mmol TE/g), gallic acid (4.07 mmol TE/g), rutin (1.97 mmol TE/g) and BHT (0.08 mmol TE/g) showed relatively high antioxidant capacity compared to the mushroom extracts.

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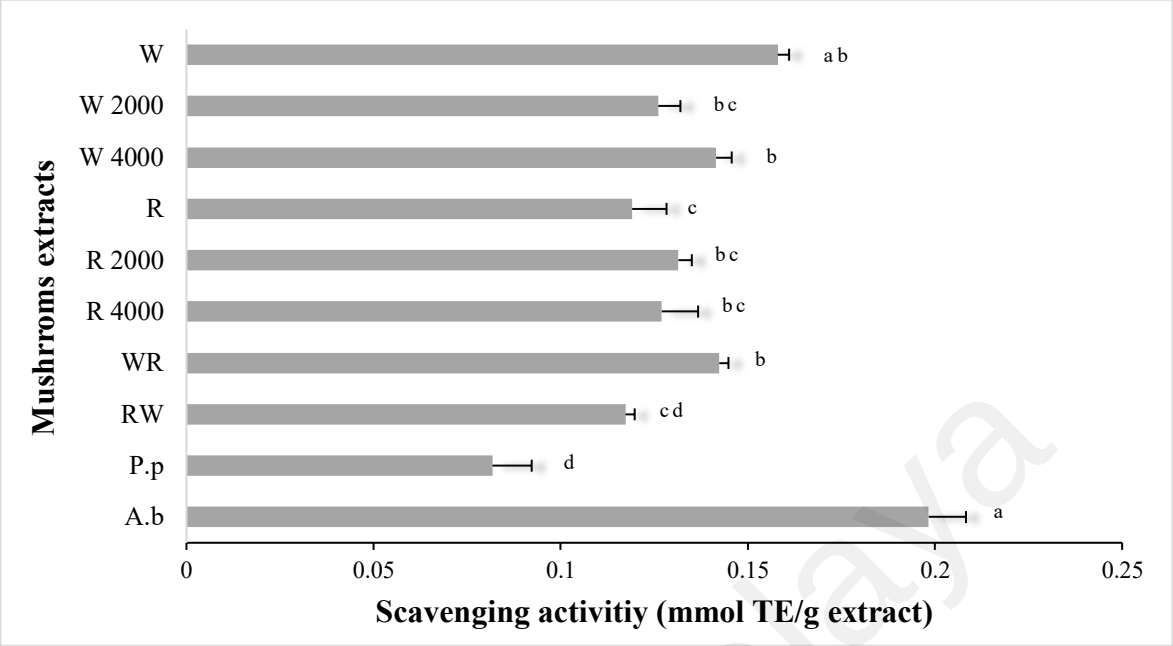

Figure 4.2: ABTS^{+} scavenging activities of the crude hot water extracts of selected mushrooms.

[a, b, c....] means with different superscripts on the bars ($p < 0.05$). W; natural strain from Malaysia; W2000 and W4000, two gamma-irradiated strains; WR and RW, two hybrid strains; R, natural strain isolated from Thailand; R2000 and R4000, two gamma-irradiated strains; P. p, *Pleurotus pulmonarius;* A. b, *Agaricus bisporus.*

4.3.3 Ferric reducing antioxidant power (FRAP)

The ferric ion-reducing capacity of the mushroom extracts is shown in Figure 4.3. With the exception of natural strain R, the crude extracts of all *S. commune* strains (0.35 – 0.50 mmol Fe^{2+}/g extract) showed higher FRAP value than *P. pulmonarius* (0.27 mmol Fe^{2+}/g extract); however, *A. bisporus* (0.72 mmol Fe^{2+}/g extract) have showed the highest ferric ion reducing capacity. Amongst the strains, the highest FRAP value was observed for the natural strain W while strain R gave the lowest value. The ferric ion reducing capacity (mmol Fe^{2+}/g) of the positive controls including ascorbic acid, gallic acid, rutin and BHT are 13.15, 6.70, 20.91 and 4.56, respectively.

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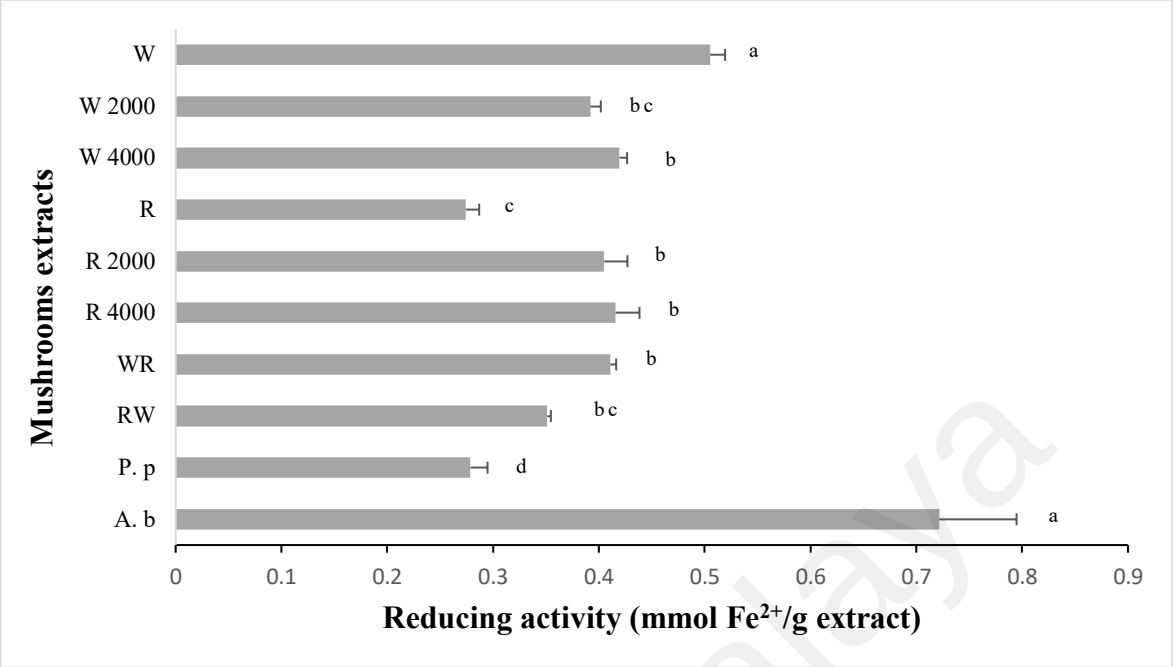

Figure 4.3: Ferric ion-reducing antioxidant power of the crude hot water extracts of selected mushrooms.

[a, b, c....] means with different superscripts on the bars ($p < 0.05$). W; natural strain from Malaysia; W2000 and W4000, two gamma-irradiated strains; WR and RW, two hybrid strains; R, natural strain isolated from Thailand; R2000 and R4000, two gamma-irradiated strains; P. p, *Pleurotus pulmonarius;* A. b, *Agaricus bisporus.*

4.3.4 Cupric ion-reducing antioxidant capacity (CUPRAC)

The reducing capacity of the crude extracts of selected mushrooms evaluated using the CUPRAC assay is presented in Figure 4.4. The reducing capacity of the extracts of *S. commune* strains (0.08 – 0.15 mmol TE/g extract) was higher than that of *P. pulmonarius* (0.06 mmol TE/g extract) but lower than that of *A. bisporus* (0.16 mmol TE/g extract). The natural strain W also showed the highest reducing capacity while the lowest reducing capacity was observed in the natural strain R. The gamma-irradiated strains of W showed lower activity than the natural strain W; however, an opposite trend was observed for the gamma-irradiated strains of R which showed higher activity than the natural strain R. The cupric ion reducing capacity (mmol TE/g) of the positive controls including ascorbic acid, gallic acid, rutin and BHT are 6.97, 11.95, 5.16 and 0.83, respectively.

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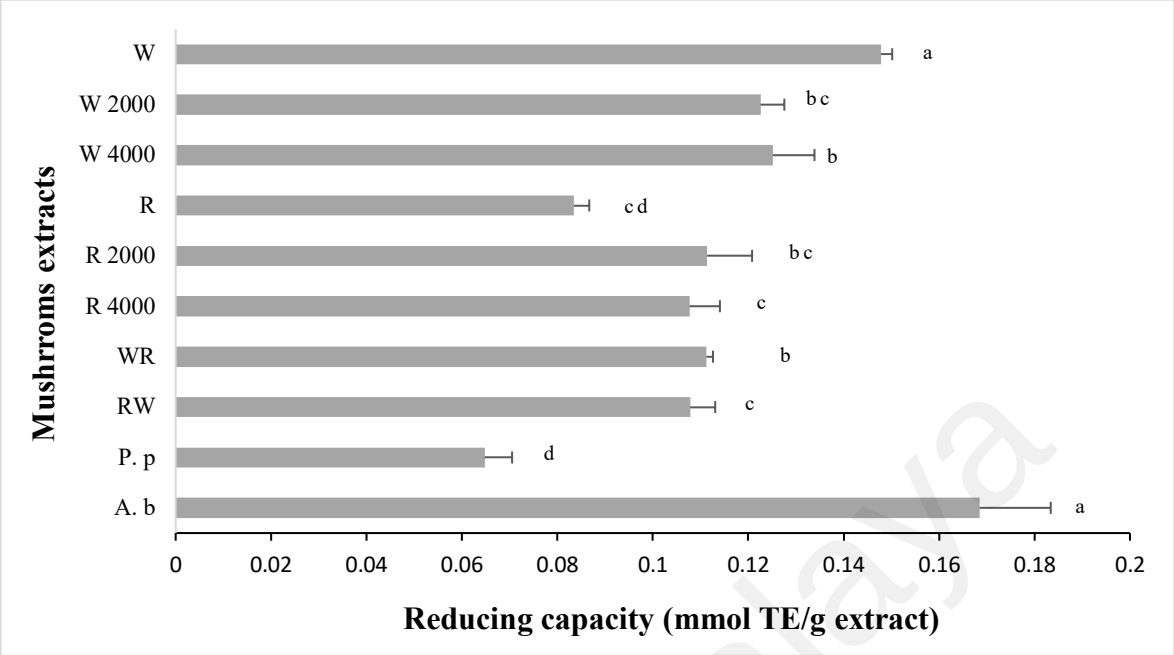

Figure 4.4: Cupric ion-reducing antioxidant capacities of the crude hot water extracts of selected mushrooms.

[a, b, c....] means with different superscripts on the bars ($p < 0.05$). W; natural strain from Malaysia; W2000 and W4000, two gamma-irradiated strains; WR and RW, two hybrid strains; R, natural strain isolated from Thailand; R2000 and R4000, two gamma-irradiated strains; P. p, *Pleurotus pulmonarius;* A. b, *Agaricus bisporus.*

4.3.5 Pearson's correlation analysis of antioxidant components and antioxidant activities of extract from *S. commune* samples

Pearson's correlation analysis of the chemical composition and antioxidant activities of the crude extracts was conducted and results were presented in Table 4.3. Significant and strong positive relationships ($p < 0.01$) were obtained between total flavonoid content and the antioxidant activities of the extracts assessed by the two reducing power assays FRAP ($r = 894$) and CUPRAC ($r = 0.728$). Likewise, the correlation coefficients between the reducing activities of the extracts and total phenolic content showed highly significant and strong positive relationships FRAP ($r = 0.827$) and CUPRAC ($r = 0.826$). Significant and strong positive relationships between the scavenging activities of the extracts and total phenolic content DPPH ($r = 0.782$) and ABTS ($r = 0.796$). The correlation between ABTS radical

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scavenging activity of the extracts and total protein content was significant but moderate positive relationship ($r = 0.518$, $p < 0.01$). The correlations between antioxidant activities and the total sugar content of the extracts were very weak ($r = 0.139 – r = 0.374$).

Table 4.3: Pearson's correlation coefficients between various chemical compositions parameters and antioxidant activities of the crude hot water extracts.

Parameters	DPPH (mmol TE/g extract)	ABTS (mmol TE/g extract)	FRAP (mmol Fe^{2+}/g extract)	CUPRAC (mmol TE/g extract)
Total sugar content	0.374	-0.130	0.233	0.139
Total protein content	0.285	0.518**	0.305	0.333
Total phenolic content	0.782**	0.796**	0.827**	0.826**
Total flavonoid content	0.723**	0.672**	0.894**	0.728**

** Correlation is significant at the 0.01 level (2-tailed)

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4.4 Yield of *S. commune* fractions

The crude hot water extracts of *S. commune* strains W and W4000 consistently showed high antioxidant activity in all assays; therefore, both strains were selected for further fractionation. In the first step, liquid-liquid extraction yielded the ethyl acetate (EA) and water (H_2O) fractions. A portion of the water fraction was then subjected to two different procedures i.e., ammonium sulphate precipitation and ethanol precipitation to recover fractions that were enriched with water-soluble components, including WS1 and WS2. The ethanol supernatant in the ethanol precipitation method was dried to yield the EtOH fraction. The yields of all fractions (expressed on a dry weight basis) are shown in Table 4.4.

Table 4.4: The yields of fractions of *S. commune* strains W and W4000

Fraction	Yield (%, w/w)	
	***S. commune* strain W**	***S. commune* strain W4000**
Ethyl acetate (EA)	0.07	0.05
Water (H_2O)	9.41	6.01
Water-soluble component (WS1)	18	22.71
Water-soluble component (WS2)	53.36	71.28
Ethanol (EtOH)	3.0	2.3

*The fractions were derived from the crude hot water extracts of *S. commune* strains W and W4000.

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4.5 Chemical compositions of *S. commune* fractions

The total sugars, proteins, phenolic and flavonoid of the fractions derived from the crude hot water extracts of *S. commune* strains W and W4000 were determined. Table 4.5 shows the chemical compositions of the fractions.

4.5.1 Total sugar content

The water-soluble fractions of both *S. commune* strains contained higher sugar content (273.57 – 628.38 mg carbohydrate/g extract) than the crude extracts (224.16 – 263.57 mg carbohydrate/g extract) (Table 4.5). Amongst the water-soluble fractions, the sugar content decreased in the order of WS1 and H_2O. The EtOH fractions have the lowest sugar content.

4.5.2 Total protein content

The water-soluble fractions, in general, contained higher protein content than the organic solvent fractions of *S. commune* strains (Table 4.5). It can be observed that the water-soluble fraction obtained by ammonium sulphate precipitation (WS1) of both strains (729.39 and 490.30 mg protein/g extract) have the highest protein content than the fraction obtained from ethanol precipitation (133.93 and 312.12 mg protein/g extract). Moreover, the protein content of the crude extracts was comparable to that of the water fractions W-H_2O and W4000-H_2O.

4.5.3 Total phenolic content

Similar trend was observed for both strains in which the ethyl acetate fractions showed the highest level of phenolics among the fractions (Table 4.5). The concentration of phenolics in W-EA (34.59 mg GAE/g extract) and W4000-EA (37.87 mg GAE/g extract)

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was approximately two-fold higher than those of the respective crude extracts. The water-soluble fractions also contained phenolics that were either higher or comparable to the crude extracts.

4.5.4 Total flavonoid content

Amongst the fractions of *S. commune* strains, high concentrations of flavonoids were observed in the ethyl acetate fractions that were almost ten-fold higher than the concentration of flavonoids in the crude extracts (Table 4.5). For strain W, water-soluble fractions contained flavonoid content that was comparable to the crude except for WS1. A different trend was observed for strain W4000 in which the level of flavonoids in all fractions were higher than that of the crude except for W4000-H_2O.

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Table 4.5: The chemical compositions of the fractions derived from the selected strains of *S. commune*.

Fractions	Chemical composition			
	Total sugars (mg carbohydrate/g extract)	**Total proteins (mg protein/g extract)**	**Total phenolic (mg GAE/g extract)**	**Total flavonoid (mg RE/g extract)**
S. commune strain W				
W crude	224.16 ± 11.06^{c}	457.87 ± 5.01^{b}	22.28 ± 0.28^{b}	8.69 ± 0.42^{d}
W-EA	ND	ND	34.59 ± 2.46^{a}	84.55 ± 4.09^{a}
W-H_2O	400.53 ± 6.81^{b}	458.18 ± 7.10^{b}	19.45 ± 0.24^{cd}	12.52 ± 1.18^{c}
W-WS1	549.46 ± 12.65^{a}	729.39 ± 25.26^{a}	29.89 ± 1.22^{a}	42.69 ± 1.74^{b}
W-WS2	597.30 ± 10.30^{a}	267.87 ± 7.35^{c}	12.11 ± 1.44^{d}	12.60 ± 0.66^{c}
W-EtOH	16.81 ± 0.45^{d}	133.93 ± 4.67^{cd}	6.43 ± 0.26^{e}	12.25 ± 0.87^{c}
S. commune strain W4000				
W4000 crude	263.57 ± 13.34^{c}	395.45 ± 8.08^{b}	18.46 ± 0.62^{b}	8.77 ± 0.80^{cd}
W4000-EA	ND	ND	37.87 ± 9.70^{a}	71.44 ± 2.94^{a}
W4000-H_2O	273.57 ± 24.92^{c}	415.45 ± 13.39^{a}	17.80 ± 0.09^{bc}	5.77 ± 0.40^{d}
W4000-WS1	409.38 ± 6.97^{b}	490.30 ± 9.64^{ab}	19.94 ± 1.14^{b}	18.21 ± 2.68^{bc}
W4000-WS2	628.38 ± 38.93^{a}	296.06 ± 10.45^{c}	11.70 ± 0.44^{d}	12.70 ± 0.61^{c}
W4000-EtOH	51.12 ± 2.50^{d}	312.12 ± 8.15^{bc}	18.17 ± 1.75^{bc}	29.81 ± 2.38^{b}

$^{a, b, c....}$ means with different superscripts on the same column differ significantly ($p < 0.05$).

*EA, ethyl acetate; H_2O, water-soluble fraction; WS1, water-soluble protein; WS2, water-soluble polysaccharides; EtOH, ethanol fractions.

*ND = not determined. *The total sugars and total proteins of the ethyl acetate fractions were not determined due to the low yield of the fractions.

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4.6 The antioxidant activities of *S. commune* fractions

The radical scavenging activities and reducing capacities of the fractions derived from the crude hot water extracts of *S. commune* strains W and W4000 were assessed.

4.6.1 DPPH radical scavenging activities

Figure 4.5 shows the DPPH free radical scavenging activities of the fractions of *S. commune* strains expressed as trolox equivalents. The organic solvent fractions exhibited significantly higher antioxidant capacity compared to water-soluble fractions. Similar trend was observed for both strains. It is important to note that the activities of the ethyl acetate and ethanol fractions (0.42 – 0.48 mmol TE/g extract) were about four times higher than those of the crude extracts (0.09 – 0.12 mmol TE/g extract). Both ethyl acetate and ethanolic fractions also showed higher antioxidant capacity than some of the positive controls namely ascorbic acid (0.27 mmol TE/g) and gallic acid (0.26 mmol TE/g).

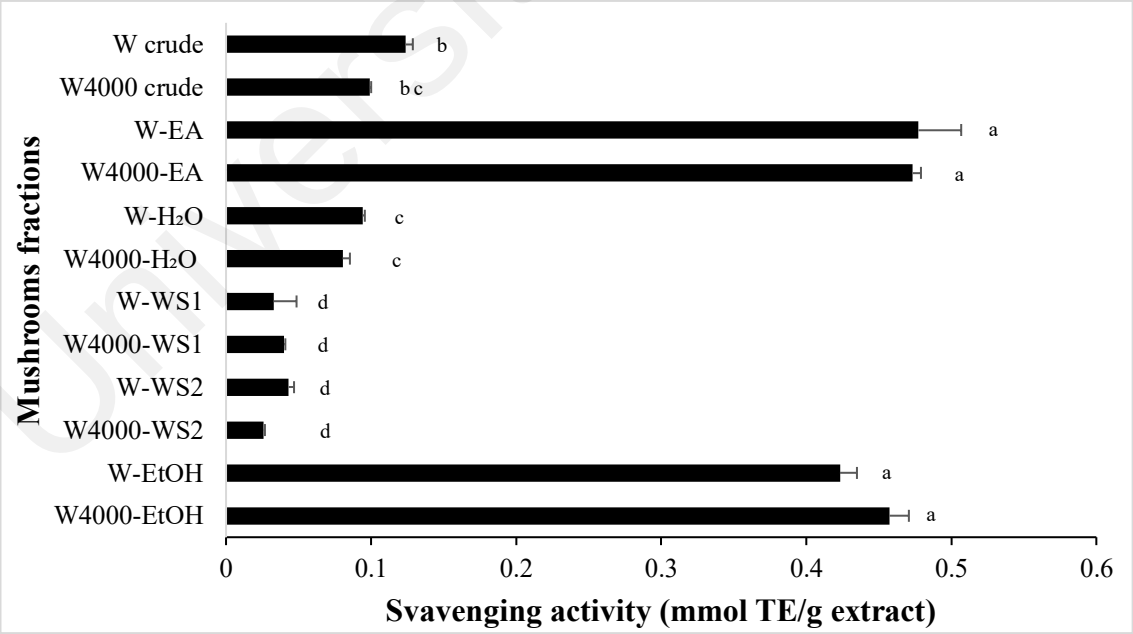

Figure 4.5: DPPH free radical scavenging activities of the fractions derived from the crude hot water extracts of *S. commune* strains W and W4000.

[a, b, c....] means with different superscripts on the bars ($p < 0.05$). EA, ethyl acetate; H_2O, water-soluble fraction; WS1, water-soluble protein; WS2, water-soluble polysaccharides; EtOH, ethanol fractions.

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4.6.2 ABTS$^+$ scavenging activities

Figure 4.6 shows the ABTS$^+$ scavenging activities of the fractions of *S. commune* strains expressed as trolox equivalents. The crude extracts of both strains exhibited higher antioxidant activity than the most of the fractions. It is noted that the fractions derived from the H_2O fraction, namely WS1, WS2 and EtOH fractions (0.03 – 0.08 mmol TE/g extract), showed significantly lower antioxidant capacity compared to EA (0.15 – 0.16 mmol TE/g extract) and H_2O (0.18 – 0.19 mmol TE/g extract). Some of the fractions showed higher activity than BHT (0.17 mmol TE/g) that was used as a positive control. The antioxidant activities of other controls expressed as mmol TE/g, including ascorbic acid, gallic acid and rutin were 4.31, 4.27 and 1.34, respectively.

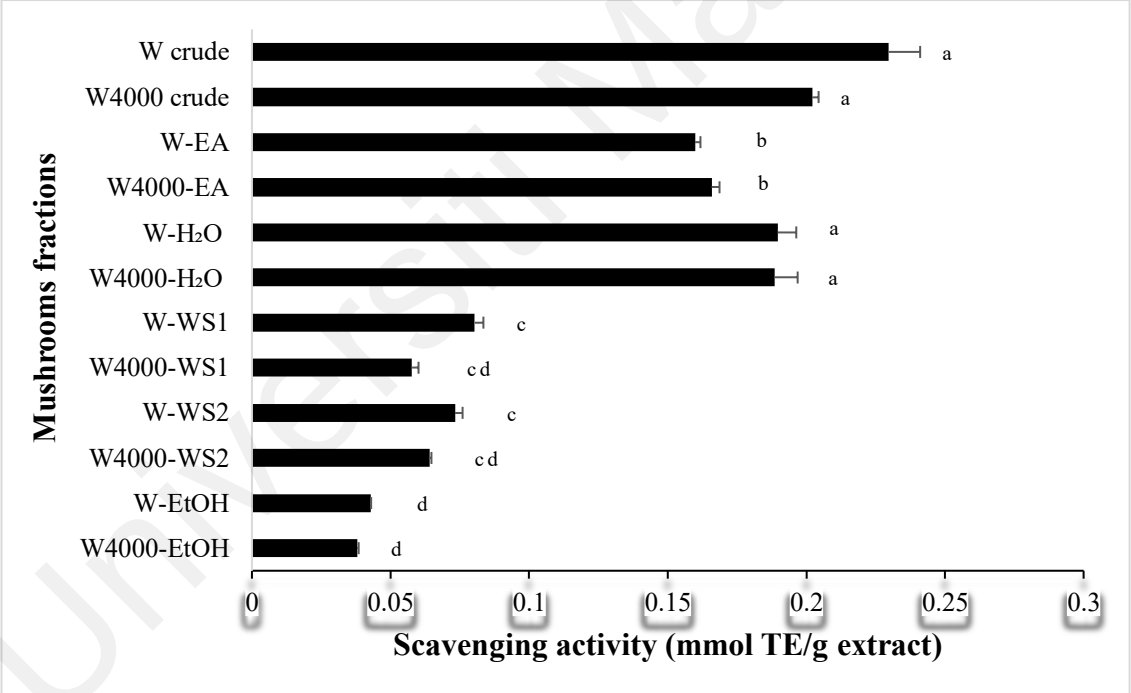

Figure 4.6: ABTS$^+$ scavenging activities of the fractions derived from the crude hot water extracts of *S. commune* strains W and W4000.

[a, b, c....] means with different superscripts on the bars ($p < 0.05$). EA, ethyl acetate; H_2O, water-soluble fraction; WS1, water-soluble protein; WS2, water-soluble polysaccharides; EtOH, ethanol fractions.

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4.6.3 Ferric reducing antioxidant power (FRAP)

The reducing capacities of the fractions of *S. commune* strains, as determined by the FRAP assay, are shown in Figure 4.7. The organic solvent fractions showed better reducing capacities than the water-soluble fractions and crude extracts. In particular, both ethyl acetate fractions (1.09 – 1.17 mmol Fe^{2+}/g extract) displayed the highest reducing capacity followed by the ethanol fractions (0.25 – 0.38 mmol Fe^{2+}/g extract). The reducing capacities of the water-soluble fractions of both strains ranged from 0.08 to 0.18 mmol Fe^{2+}/g extract. The positive controls showed relatively higher reducing capacity in the range of 4.56 to 13.15 mmol Fe^{2+}/g.

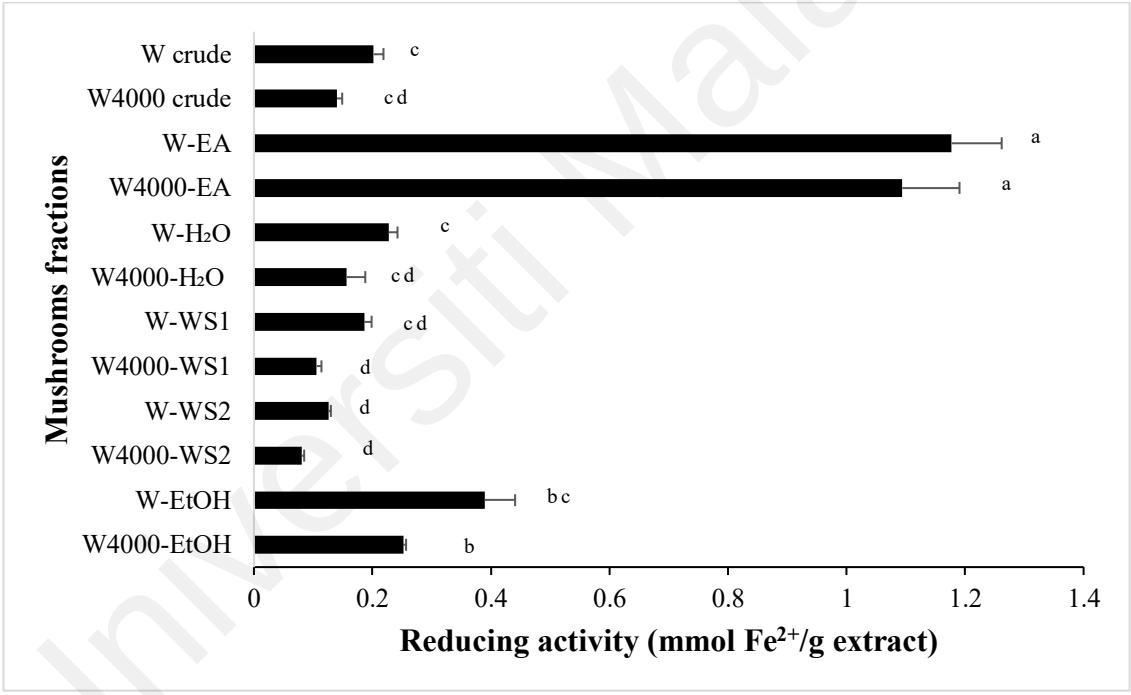

Figure 4.7: Ferric ion-reducing antioxidant power of the fractions derived from the crude hot water extracts of *S. commune* strains W and W4000.

[a, b, c....] means with different superscripts on the bars ($p < 0.05$). EA, ethyl acetate; H_2O, water-soluble fraction; WS1, water-soluble protein; WS2, water-soluble polysaccharides; EtOH, ethanol fractions.

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4.6.4 Cupric ion-reducing antioxidant capacity (CUPRAC)

The reducing capacities of the fractions of *S. commune* strains, as determined by the CUPRAC assay, are shown in Figure 4.8. The reducing capacities of the fractions ranged from 0.07 to 0.12 mmol TE/g extract with the exception of W-EtOH that showed lower activity (0.04 mmol TE/g extract). It is also noted that the reducing capacities of crude extract and fractions of *S. commune* strain W were consistently higher than those of strain W4000 except for the EtOH fractions in which an opposite trend was observed. The positive controls showed relatively higher reducing capacity in the range of 0.83 to 11.95 mmol TE/g.

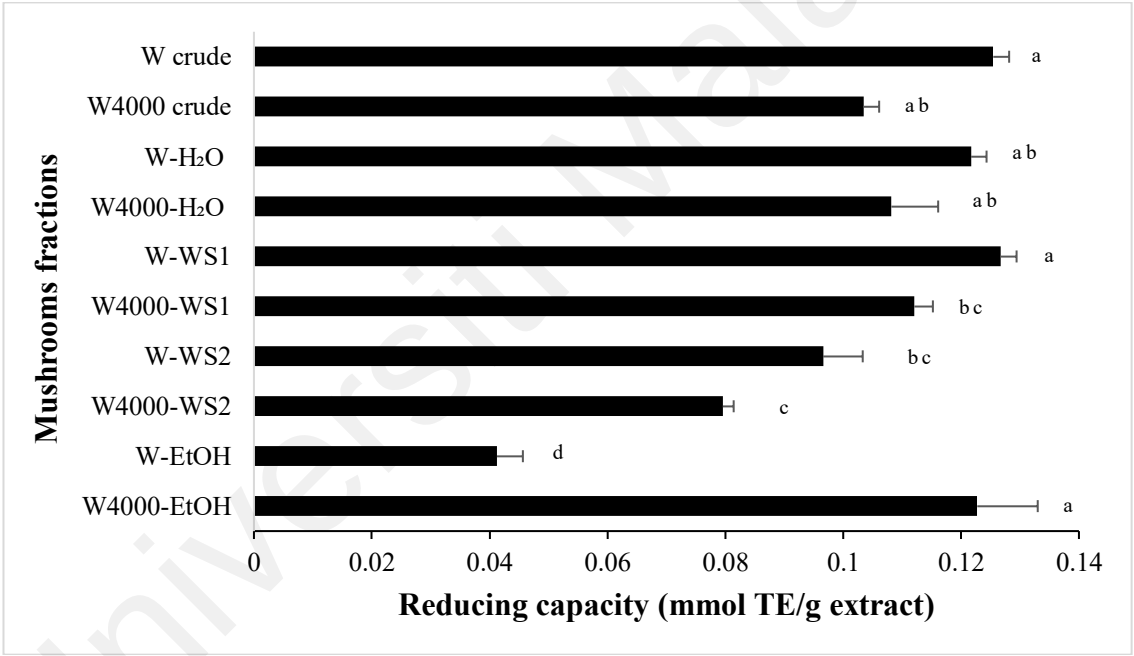

Figure 4.8: Cupric ion-reducing antioxidant capacities of the fractions derived from the crude hot water extracts of *S. commune* strains W and W4000.

[a, b, c....] means with different superscripts on the bars ($p < 0.05$). EA, ethyl acetate; H_2O, water-soluble fraction; WS1, water-soluble protein; WS2, water-soluble polysaccharides; EtOH, ethanol fractions.

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4.6.5 Pearson's correlation analysis between chemical composition and antioxidant activities of the fractions derived from the crude hot water extracts of *S. commune* strains W and W4000

Table 4.6 shows the Pearson's correlation coefficient for analysis between antioxidant activities and chemical composition of the extracts. A significant ($p < 0.01$) and moderate to strong relationships between the reducing activities of the fractions and total phenolic content FRAP ($r = 0.686$) and CUPRAC ($r = 0.823$). Same trend was observed between total flavonoid content and reducing activities of the fraction. Significant correlation but moderate positive relationship was observed between DPPH radical scavenging activity of the fractions and total flavonoid content ($r = 0.626$, $p < 0.01$). Furthermore, significant and strong negative relationships ($p < 0.01$) were obtained between total sugar content and the scavenging activity of the fractions towards DPPH radicals ($r = -0.778$). Same trend but positive relationship was observed between reducing activity of the fraction and total protein content ($r = 0.753$, $p < 0.01$).

Table 4.6: Pearson's correlation coefficients between various chemical compositions parameters and antioxidant activities of the fractions.

Parameters	DPPH (mmol TE/g extract)	ABTS (mmol TE/g extract)	FRAP (mmol Fe^{2+}/g extract)	CUPRAC (mmol TE/g extract)
Total sugar content	-0.778**	-0.005	-0.532**	-0.128
Total protein content	-0.549**	0.176	-0.361	0.753**
Total phenolic content	0.332	0.012	0.686**	0.823**
Total flavonoid content	0.626**	-0.181	0.900**	0.479*

** Correlation is significant at the 0.01 level (2-tailed)
*Correlation is significant at the 0.05 level (2-tailed)

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CHAPTER 5: DISCUSSION

5.1 The yields of *S. commune* hot water extracts and their selected fractions

Efficient extraction of antioxidants and other biologically active molecules requires the use of solvents with different polarities. Certain antioxidant molecules are more soluble in polar solvents such as methanol and water while non-polar solvents like hexane or dichloromethane are preferable for isolating lipophilic compounds. In this current study, sequential extraction methods were utilized to obtain hot water extracts that were mainly composed of water-soluble compounds and eliminate the presence of other low molecular weight molecules. Ethanol extraction was initially conducted to remove apolar compounds, such as lipids and other secondary metabolite molecules, followed by hot water extraction to isolate the high molecular weight components such as polysaccharides and proteins.

The yields (w/w) of crude hot water extracts of *S. commune* ranged between 9.02 and 13.38%, and these values were lower than in previous studies that used direct hot water extraction method. For example, the yield of hot water extract obtained by Chandrawanshi et al. (2019) was 19.60% whereas the yields obtained by Abd Razak et al. (2019) ranged between 12.94 and 17.79% using two different temperatures; 4°C and 30°C, and at different extraction times. Furthermore, the yields of *S. commune* crude extracts were slightly higher than that of *Agaricus bisporus* (8.57%) but comparable to the yield of *Pleurotus pulmonarius* (11.24%). It is noticeable that *S. commune* strains subjected to gamma irradiation showed higher yield than the natural or hybrid strains. Similarly, Rashid et al. (2015) have found that the yields of *Pleurotus sajor-caju* irradiated mycelium increased gradually as the dose of γ-irradiation increased from 23.10% to 39.82%. Likewise, the extraction yields of *Antrodia camphorate* mycelia

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increased with higher doses of gamma irradiation up to 15 kGy from 47.6% to 53.1% (Huang & Mau, 2007).

The conventional liquid-liquid partitioning was used for the extraction of low-molecular weight compounds from water extracts. The extraction yields of the fractions derived from crude hot water extracts of *S. commune* strains W and W4000 varied from one strain to another. The highest yields were obtained from the water-soluble fractions W-WS2 (53.36%) and W4000-WS2 (71.28%). The yields from semi-polar solvents such as ethyl acetate were found at very low levels that ranged between 0.05 to 0.07% compared to polar solvents (ethanol and water). This suggests that the crude extracts consist of mainly water-soluble components with a small proportion of secondary metabolites that are likely to be retained in the ethyl acetate fractions. The findings here are comparable to a previous study conducted by Kim (2012) who reported that the yield of the ethyl acetate fraction was (1.05%) which was lower than the yield of water-soluble fraction (35.68%).

5.2 Chemical compositions and antioxidant activities of crude hot water extracts of *S. commune*

The chemical composition of mushroom species may be affected by several variables such as genetic structure, strains, maturation stage, environmental conditions as well as irradiation and hybridisation (Appels et al., 2018; Bellettini et al., 2019; Cardoso et al., 2019; Mleczek et al., 2020; Oropeza-Guerrero et al., 2018; Sakamoto, 2018;). The results obtained in the current work have shown that crude hot water extracts of different strains contain carbohydrates, protein, phenolic and flavonoid compounds. However, the total content of these components varied from one strain to another.

It was reported that, in mushroom hot water extracts, the predominant components are carbohydrates and proteins (Singh et al., 2020) and a similar trend was observed in

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the present study. Different doses of gamma irradiation have influenced the total sugar contents of the *S. commune* strains W and R. The total sugar content increased with the increased doses of gamma irradiation up to 2 and 4 kGy. Gamma irradiation might cause genetic mutation leading to an overexpression of certain genes encoding polysaccharides synthesis (Mahmood et al., 2019). Similarly, the carbohydrate content of *Pleurotus osteratus* ethanol extracts increased gradually as the dose of gamma irradiation increased (San et al., 2019). The two hybrid strains with different dominant characteristics (WR and RW) also yielded higher sugar content than the two natural strains of *S. commune* (W and R). These results are consistent with those of other studies and suggest that the increase of total sugar contents of the hybrid strains is most likely related to the changes in the genetic structure leading to an overexpression of genes encoding polysaccharides synthesis (Liu et al., 2017; Raman et al., 2021).

Protein is the major component next to carbohydrates in mushroom. According to the results, the total soluble protein contents of *S. commune* strains (W and R) has decreased gradually with gamma irradiation increments up to the dose 4 kGy. These results differed from some published studies. For instance, Weng et al. (2004) reported that the gamma irradiated strains of *Agaricus bazei* Murrill contain higher protein content than the non-irradiated strains. According to Sathesh-Prabu and Lee (2016), mutation altering the non-coding region of a gene will cause different protein production, the estimated protein concentration between the natural and gamma irradiated strains will change according to the dose of mutagenesis.

Several studies have investigated the total phenolic content of *S. commune* hot water extracts. The total phenolic contents recorded in this study are comparable to those reported by Emsen et al. (2017) who recorded 12.32 mg GAE/g of phenolic content in the water extract of *S. commune.* Generally, gamma irradiation either decreased or increased

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the total phenolic content. For the natural strain W, different doses of gamma irradiation have slightly decreased the total phenolic content. Surprisingly, for the natural strain R, gamma irradiation doses 2 and 4 kGy significantly influenced the phenolic content in comparison with non-irradiated strain. The reason for such changes is still unclear but based on previous studies, the effect of gamma irradiation on the chemical composition of mushroom may be influenced by factors such as genetic mutation that may cause certain changes in the synthesis of bioactive components, species type, geographical and environmental conditions, and dose of gamma irradiation (Tsai et al., 2014).

Furthermore, the total flavonoid contents of the *S. commune* crude hot water extracts were lower than their total phenolic contents. The findings of the current study are consistent with those of Emsen et al. (2017) who reported that the amount of total flavonoid content of *S. commune* water extract was significantly lower than total phenols. Another important finding was that gamma irradiated strains (R2000 and R4000) have higher flavonoid contents than the parental strain (R). Both hybridised strains (WR and RW) have higher flavonoid contents compared to the natural strain R. Our results are in agreement with a previous study conducted by Oropeza-Guerrero et al. (2018) who reported that the hybrid strains of *Pleurotus djamor* exhibited higher flavonoid content (6.89 mg QE/100 g extract) than the parental strains (2.22 mg QE/100 g extract).

An initial objective of this work was to determine the antioxidant activities of *S. commune* crude hot water extracts used, and highlight the potential links between the chemical composition and antioxidant activities. Thus, various *in vitro* chemical assays have been developed to measure the antioxidant capacities of mushrooms products. A number of these assays depend on the generation of free radicals as their initial step and later they would be scavenged by the particular sample. Based on the reaction mechanisms involved, antioxidant assays can be divided into two major groups; those

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based on hydrogen transfer (HAT) reactions and others involving single electron transfer (SET) reactions (Shahidi & Zhong, 2015). In this study, four antioxidant assays were used to evaluate the antioxidant activity of the extracts. The crude hot water extracts of *S. commune, P. pulmonarius* and *A. bisporus* were evaluated.

The $ABTS^+$ and DPPH assays were utilized to measure the radical scavenging activities of crude hot water extracts of selected mushrooms. Our results showed that the natural strain W possessed the highest phenolic and flavonoid contents among the different *S. commune* strains and showed higher scavenging activities than *P. pulmonarius* water extract. Comparable results were reported by a study conducted on five different mushrooms including *S. commune* reported that hot water extracts of *S. commune* exhibited higher DPPH radical scavenging activity than *P. pulmonarius* extracts (Tepsongkroh et al., 2019). It has been reported that the IC_{50} of *S. commune* crude hot water extract (19.36 µg/µl) was higher than the methanol (24.82 µg/µl) and ethanol (18.56 µg/µl) extracts (Chandrawanshi et al., 2017). Furthermore, several studies showed that crude hot water extracts of *S. commune* had the ability to scavenge DPPH radicals (Emsen et al., 2017; Kumar et al., 2018). Nonetheless, the $ABTS^+$ scavenging activities of *S. commune* crude hot water extracts were lower than that of Tepsongkroh et al. (2019). This result may be explained by the fact that bioactive compounds of the water extracts may act differently towards different free radicals. Also, these assays use different chromogenic redox reagents with different standard potentials (Apak et al., 2007).

The ion reducing activity of the crude water extracts was tested by two assays, FRAP and CUPRAC. The present study tested the crude hot water extracts of *S. commune* W and gamma-irradiated strains and were found to have ion reducing activities higher than that of *P. pulmonarius* water extract. Similarly, in a previous study, the crude hot water extract of *S. commune* exhibited better reducing activity than several *Pleurotus* spp.

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(Abdullah et al., 2012). Water extracts of *S. commune* possessed electron-transfer capacities indicating the significant reducing power of the extracts (Sudha et al., 2012). In the CUPRAC assay, the crude hot water extracts of *S. commune* showed stronger reducing capacities than those of *P. pulmonarius* and this is in agreement with the previous findings by Abdullah et al. (2012).

The natural strains of *S. commune* showed different degrees of antioxidant activities in all assays. The strain obtained from Malaysia demonstrated higher radical scavenging abilities than the strain obtained from Thailand. The geographical origin and corresponding climatic conditions have been reported by several researchers to impose a significant impact on the genetic structure and the chemical composition of mushroom's mycelium. Hence, the changes in the antioxidant activities of the cultivated fruiting bodies under the same growth conditions, are correlated to the genetic diversity of the strains (Al-Laith, 2010; Rašeta et al., 2017; Wu et al., 2020).

In the current study, the strains were exposed to irradiation and were grown under the same conditions. Thus, the exposure of the mycelium to gamma rays at different doses might cause genetic mutation and generate new strains (Huang & Mau, 2007; Djajanegara, 2008). Our data showed that gamma irradiated strains R2000 and R4000 exhibited better scavenging activity and reducing power in all assays than the natural strain R. Similarly, San et al. (2019) found that the antioxidant activity of *Pleurotus osteratus* extract increased with higher doses of gamma irradiation. The observed increase in the antioxidant activities of the extracts could be attributed to genetic mutation because gamma rays are high-energy electromagnetic waves that have the ability to penetrate cell walls of mushroom's mycelia causing a breakdown in DNA structure and changing the purine and pyrimidine bases (Wang et al., 2007; Zhao et al., 2015). Thus, gamma irradiation may induce mutations in terms of chromosome number, morphological

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structure, and growth behavior (Mohajer et al., 2014). Recently, Liu et al. (2019) demonstrated the genetic differences between the gamma irradiated and parental strains using random amplified polymorphic DNA (RAPD) markers suggesting a change in the DNA of the gamma irradiated strain. According to Djajanegara (2008), mutation might cause changes in the gene expression encoding biosynthetic enzymes resulting in an increase in the production and accumulation of bioactive components with antioxidative activity. Furthermore, Pelcaru et al. (2021) have found that the mutation caused by gamma irradiation may stimulate the synthesis of bioactive compounds with antioxidant activities in *Fomes fomentarius* mycelium. Moreover, Jyothi and Thara (2021) reported the DNA variation of the mutants from single base pair change to repeated sequences. The polymorphism analysis by RAPD markers revealed that *P. djamor*, *P. florida* and *P. ostreatus* had 16.7%, 25% and 22% polymorphism with their respective improved strains.

Hybridisation is a method used to exchange the genetic information between two compatible nuclei and generate a recombinant genome with a probable expression for a desirable trait (Kaur et a., 2008). Furthermore, the exchange of genes between two nuclei during hybridisation may produce hybrids with diverse genetic materials and this may also result in differential expression of genes between the hybrid strains compared to the parental strains. According to Raman et al. (2021), the genetic profile of ten hybrids was detected using inter-simple sequence repeats (ISSR) and RAPD markers, the results depicted those hybrids contain polymorphic bands indicating the rearrangement and deletion in genetic structure. Moreover, Liu et al. (2017) reported that certain hybrids have higher concentrations of polysaccharides and triterpenes than that of parental strains. Similarly, in the current study, higher antioxidant activity of the hybrid strains (WR and RW) than the natural strain R may be due to the differences in the chemical composition between the strains. The present findings seem to be consistent with other research which found that hybrid strains of *Pleurotus* spp. showed higher antioxidant activity than the

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parental strains (Oropeza-Guerrero et al., 2018). It can thus be suggested that the potency of hybrid strains to scavenge free radicals is mainly contributed to the presence of higher phenolic compounds as compared with the parental strains (Oropeza-Guerrero et al., 2018).

In general, the chemical compositions and antioxidant activities of *S. commune* hot water extracts were relatively higher than those of *P. pulmonarius* and comparable to *A. bisporus*. This study confirms that *S. commune* water extracts contain an abundant amount of bioactive compounds that act as antioxidants. Moreover, this finding has important implications on future studies to take into consideration that *S. commune* can be a source of natural antioxidants.

Based on the results of the correlation analysis, it is pertinent to suggest that total phenolic contents and total flavonoid contents present in the *S. commune* crude hot water extracts were the main contributors to the observed antioxidant effect. This is in agreement with previous reports by Cheung et al. (2003) and Shah et al. (2018). Both reported that the water extracts showed a significant correlation between the scavenging activity and the total phenolic and flavonoid contents.

5.3 Chemical compositions and antioxidant activities of the fractions derived from the crude hot water extracts of *S. commune* strains W and W4000

The second objective of this study was to determine the bioactive compounds responsible for the antioxidant activities of *S. co*mmune strains. Thus, liquid-liquid partitioning by ethyl acetate of the crude water extracts was used to isolate low-molecular weight compounds from high-molecular weight compounds such as proteins and polysaccharides.

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Overall, according to the results obtained, the water-soluble fractions of *S. commune* are mainly enriched with carbohydrates and proteins. The WS2 of the gamma irradiated strain W4000 have higher total sugar content compared to the WS2 of the natural strain W. It is encouraging to compare this result with those of Wang et al. (2005) who reported that gamma-irradiated strains have higher polysaccharides than non-irradiated strain. These findings further support the idea that mutagenesis such as gamma rays might cause changes in the genetic structure, hence increases the synthesis of bioactive compounds. Moreover, the water-soluble protein fraction of *S. commune* strain W possesses the highest phenolic and flavonoid contents among all water-soluble fractions. Similarly, the total phenolic contents of the water-soluble protein fractions obtained from *P. pulmonarius* was higher than the total phenolic contents of water-soluble polysaccharides and the crude extracts (Abidin et al., 2016).

Two organic fractions were produced in the fractionation stage. Based on our results, the ethanolic and ethyl acetate fractions were enriched with low-molecular weight compounds such as phenolic and flavonoid compounds. It is essential to determine the total contents of phenolic compounds in the organic fractions derived from water extracts because these fractions are mostly active due to the presence of these secondary metabolites (Buruleanu et al., 2018; Siu et al., 2014). This study confirms that ethyl acetate fractions W-EA and W4000-EA contain the highest phenolic content among all fractions. The findings of the current study are consistent with those of Rajput et al. (2020) who worked on different fractions derived from *Ophiocordyceps sinensis* water extracts, and found that the ethyl acetate fractions have high content of phenolic compounds than the crude extracts. Furthermore, the ethyl acetate fractions showed the highest level of flavonoid contents among all water-soluble fractions and crude extracts.

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High contents of organic compounds in semi-polar fractions derived from polar extracts may be explained by the fact that the polarity and solubility of phenolic molecules may increase due to the presence of sugars or unsubstituted –OH group (Moreno & Peinado, 2012). Furthermore, hot water extraction might cause disruption to the cell wall structure resulting in easier release of bound polyphenolic and flavonoid compounds (Tepsongkroh et al., 2019). Moreover, phenolic compounds have a polar and semi-polar nature. Thus, through fractionation process, ethyl acetate fractions derived from water extracts may contain phenolic compounds presence in the cell wall and attached to sugar molecules through glycosidic bond (Cheung, 2008; Ferreira et al., 2009).

The antioxidant activities of the water-soluble and organic fractions were determined and compared to the activities of the crude extracts that were prepared in the fractionation stage. The activities of the tested fractions towards free radicals were based on the soluble components and the principle of the assay. However, it should be noted that, in some cases, a mixture of compounds may display better bioactivity than a single isolated active fraction because of the synergistic effects (Williamson, 2001; Caesar & Cech, 2019).

Based on the current results, the ethyl acetate fractions exhibited strong scavenging activity towards free radicals amongst water-soluble fractions and crude extracts, similar to the obtained findings by Kim (2011). In addition, the total reduction activities served as a significant indicator of the potential antioxidant activity. In the current study, the ethyl acetate fractions showed the highest reducing activities amongst all tested fractions. Similar observations on the potency of the ethyl acetate fractions, representing the compounds with intermediate polarity, have been reported earlier by several researchers (Mishra et al., 2018; Rajput et al., 2020).

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Overall, separation of bioactive compounds based on molecular weight using a semi-polar solvent such as ethyl acetate, was successful. Due to high temperatures used in preparing water extracts, Jeong et al. (2004) suggested that soluble polyphenols can be liberated by heat treatment. Furthermore, Liu et al. (2014) reported that during hot water extraction, the viscosity and surface tension of water decrease, while diffusivity and solubility of the polyphenols increase. According to Minatel et al. (2017), polyphenols can be found either combined with mono- and polysaccharides in the cell wall or linked to one or more phenolic group. Thus, thermal processing method may liberate polyphenols compounds from insoluble portion of mushroom, that increases the accumulation of bioaccessible antioxidant compounds (Choi et al., 2006). In addition, the unique molecular structure, particularly the number and position of hydroxyl groups give polyphenols the ability to inactivate free radicals (Choi et al., 2006; Minatel et al., 2017). Moreover, these bioactive compounds are highly soluble in semi-polar solvents such as ethyl acetate. Therefore, ethyl acetate fractions displayed the highest antioxidant activities in DPPH and FRAP assays among water-soluble fractions and the crude extracts. To identify the exact type of compounds, further work is required which involve complete purification of the components and further analysis using chromatographic and spectroscopic techniques.

However, the crude polysaccharides and other water-soluble components in the fractions showed only moderate to weak antioxidant activities. Our results are in contrast with previous reports which indicated that polysaccharides showed better antioxidant activity than the crude extracts (Vas et al., 2011; Wang et al., 2018). As mentioned in the literature review, the antioxidant activities of polysaccharides may vary between one assay to another, and depends on the structural characteristics that involve glycosidic bond and chemical compositions (Gong et al., 2020). According to Thetsrimuang et al. (2011), crude polysaccharides might contain soluble compounds responsible for the

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antioxidant activities of the extracts such as reducing sugars. These soluble compounds might be active towards certain types of free radicals. For example, Chen et al. (2015) found that the crude polysaccharides obtained from several mushrooms possessed remarkable hydroxyl and DPPH radicals scavenging activities.

On the other hand, Cai et al. (2015) have reported that crude polysaccharides obtained from *Auricularia auricular-judae* showed high scavenging activity towards $ABTS^{+}$, and this indicates that the soluble compounds in the crude polysaccharides have hydrogen donating ability. While the antioxidant activities of the high-molecular weight components of *S. commune* might be low, the fractions may exhibit other bioactivities like antitumor and immunomodulating activities (Yelithao et al., 2019; Zhong et al., 2015).

In general, according to our results, the functional groups of organic compounds from the analysed water extracts of *S. commune* are mainly phenolic compounds. Therefore, organic fractions were more active as antioxidants than water-soluble fractions and the crude extracts. In accordance with the present results, Choi et al. (2006) suggested that hot water extraction might cause disruption of the cell walls resulting in easier release of bound polyphenolic compounds which, in turn, increases the antioxidant activities of the extracts. Data were published indicating that the presence of phenolic compounds in the water extracts/fractions was highly involved in the antioxidant activities of the extracts (Cheung et al., 2003; Ramírez-Anguiano et al., 2007). These results are supported by Pearson's correlation analysis as shown in the Table 4.6, where the total phenolic and flavonoid contents of the fractions showed significant correlations with their ion reducing activities/capacities. The presence of low molecular weight compounds, possibly phenolic compounds, in the fractions/crude extracts are the main contributors to the antioxidant activity of *S. commune* crude hot water extracts.

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CHAPTER 6: CONCLUSION

In the present study, the antioxidant activities of the various strains of *S. commune* were investigated and compared with two commonly eaten mushrooms, *P. pulmonarius* and *A. bisporus*. Chemical composition of the extracts and fractions of selected strains were determined and correlated to their radical scavenging and reducing capacities. Our results indicated that, in general, the antioxidant activities of the crude hot water extracts of *S. commune* strains were higher than those of *P. pulmonarius.* The crude hot water extracts of *S. commune* strains W and the gamma-irradiated strain W4000 displayed the highest antioxidant activities. Further fractionation of strains W and W4000 yielded several water-soluble and organic fractions. It was observed that the organic fractions, in particular the ethyl acetate fractions exhibited the highest scavenging activities towards DPPH radicals and ion reducing activities in FRAP assay among all tested fractions. Correlation analysis showed that phenolic and flavonoid compounds present in both crude hot water extracts and their fractions are the main contributors to the antioxidant activities of the extracts and fractions of *S. commune* strains, suggesting that these low-molecular-weight compounds played a bigger role in the overall antioxidant activities of the hot water extracts of *S. commune*.

There are several recommendations for further study. First of all, strains improvement in this study via irradiation and hybridisation needs to be further investigated. An increase in the antioxidant activities of certain gamma-irradiated strains was observed in this study. Thus, further research regarding the effect of gamma-irradiation on the levels of bioactive components in mushrooms would be worthwhile. Also, identification and characterization of the polar low-molecular weight compounds would help us to understand better the antioxidant activities of the hot water extracts of *S. commune*.

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In conclusion, the current comparative study showed that the crude hot water extracts of *S. commune* contain a variety of metabolites with diverse antioxidant activities. Our results contributed additional evidence that the low molecular-weight compounds, in addition to the high-molecular-components like polysaccharides and proteins, are also responsible for the overall antioxidant activities exhibited by the water extracts of *S. commune*. With all certainty, *S. commune* contains an abundant amount of natural antioxidants and thus, is recommended as a valuable constituent of the daily diet.

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