COMPARATIVE EVALUATION OF ANTIOXIDANT ACTIVITIES AND CHEMICAL COMPOSITION OF SELECTED *Schizophyllum commune* Fr. NATURAL, GAMMA-IRRADIATED AND HYBRID STRAINS

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COMPARATIVE EVALUATION OF ANTIOXIDANT ACTIVITIES AND CHEMICAL COMPOSITION OF SELECTED *Schizophyllum commune* Fr. NATURAL, GAMMA-IRRADIATED AND HYBRID STRAINS

ABSTRACT

Due to the uncontrolled production of reactive oxygen species, excessive level of free radicals leads to oxidative stress which is linked to many diseases such as cancers, diabetes, neurological disorder and hypertension. The major countermeasure to balance the excessive presence of free radicals in the body is to obtain substantial amounts of dietary antioxidants. Mushrooms contain many bioactive compounds with health-promoting effects including antioxidants. Therefore, this study was designed to investigate the antioxidants activities and chemical compositions of different strains of *Schizophyllum commune* Fr. The crude extracts of the natural (isolated from Malaysia and Thailand), gamma-irradiated (strains obtained when natural strains subjected to different doses of gamma radiation) and hybrid (strains obtained from hybridisation between the natural strains) strains of *S. commune* were prepared using 80% (v/v) aqueous ethanol. The antioxidant activities of the extracts were then measured using 2,2 diphynel-1-picrylhydrazyl radical scavenging activity (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), ferric reducing antioxidant power (FRAP) and cupric ion-reducing antioxidant capacity (CUPRAC) assays, and correlated to their chemical compositions. *S. commune* strains showed moderate total phenolic content (11.7-22.8 mg GAE/g extract) and high protein content (293.5-486.8 mg protein/g extract). The crude extracts of *S. commune* strains showed comparable scavenging activities in both DPPH radicals scavenging activities ranged from 2.30 to 2.48 (mmol TE/g extract) and ABTS radicals scavenging activities ranged from 0.07 to 0.15 (mmol TE/g extract). In FRAP (0.22 to 0.30 mmol Fe^{2+}/g extract) and CUPRAC (0.10 to 0.13 mmol TE/g extract) assays, the crude extracts showed comparable reducing activities.

The antioxidant activities of the crude extracts showed moderate correlation with the total phenolic (r = 0.625) and flavonoid (r = 0.535) content. Further investigation was on the liquid-liquid fractionation of the crude extracts of the strains W, W2000 and W4000 to yield the hexane, ethyl acetate and water fractions. For all selected strains, the water fractions contained the highest sugar, protein and phenolic content while the hexane fractions gave the highest flavonoid content. The ethyl acetate fractions of all selected strains also showed stronger antioxidant activities approximately one time higher than water fractions and two times higher than hexane fractions in all antioxidant assays. These findings suggest that the antioxidant compounds in the crude aqueous ethanol extracts of *S. commune* were successfully concentrated in the ethyl acetate fractions and the strong radical scavenging and reducing activities of the fractions might be attributed to the abundance both phenolics and flavonoids based on the correlation studies. Our findings have shown that *S. commune* strains and ethyl acetate fractions of the selected strains (W, W2000 and W4000) are a good source of natural antioxidants and hence, they will be potentially useful for medicinal and food applications.

Keyword: *Schizophyllum commune*, mushrooms, antioxidant activities, chemical compositions, hybrids, gamma irradiation and liquid-liquid fractionation.

PENILAIAN PERBANDINGAN AKTIVITI ANTIOKDSIDAN DAN KOMPOSISI KIMIA *Schizophyllum commune* Fr. BAKA SEMULA JADI, TERDEDAH KEPADA SINARAN GAMMA DAN HYBRID TERPILIH

ABSTRAK

Oleh kerana pengeluaran spesies oksigen reaktif yang tidak terkawal, tahap radikal bebas yang berlebihan menyebabkan tekanan oksidatif yang dikaitkan dengan banyak penyakit seperti kanser, diabetes, gangguan neurologi dan hipertensi. Tindakan pencegahan utama untuk mengimbangi kehadiran radikal bebas yang berlebihan dalam tubuh adalah mendapatkan sejumlah besar antioksidan daripada makanan. Cendawan mengandungi banyak sebatian bioaktif dengan kesan yang menggalakkan kesihatan termasuk antioksidan. Oleh itu, kajian ini dirancang untuk menyiasat aktiviti antioksidan dan komposisi kimia strain *Schizophyllum commune* yang berbeza. Ekstrak mentah strain semula jadi (diasingkan dari Malaysia dan Thailand), strain yang terdedah kepada sinaran gamma (strain yang diperoleh apabila strain semula jadi mengalami dos radiasi gamma yang berlainan) dan hibrid (strain yang diperoleh daripada hibridisasi antara strain semula jadi) *S. commune* disediakan menggunakan 80% (v/v) etanol akues. Aktiviti antioksidan ekstrak kemudian diukur dengan menggunakan aktiviti merantas radikal 2,2 diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), kuasa penurunan ferik (FRAP) dan kapasiti antipengoksida penurunan ion kuprik (CUPRAC), dan berkorelasi dengan komposisi kimianya. Strain *S. commune* menunjukkan kandungan yang tinggi bagi fenolik (11.7-22.8 mg GAE/g ekstrak) dan protein (293.5-486.8 mg protein/g ekstrak). Ekstrak mentah strain *S. commune* menunjukkan aktiviti pembersihan yang setanding di kedua-dua aktiviti pengumpulan radikal DPPH antara 2.30 hingga 2.48 (mmol TE/g ekstrak) dan aktiviti pembersihan radikal ABTS berkisar antara 0.07 hingga 0.15 (mmol TE/g ekstrak). Dalam ujian FRAP (0.22 hingga 0.30 mmol Fe^{2+}/g ekstrak) dan CUPRAC (0.10 hingga 0.13 mmol TE/g

ekstrak), ekstrak mentah menunjukkan aktiviti penurunan yang setanding. Aktiviti antioksidan ekstrak mentah menunjukkan korelasi sederhana dengan jumlah kandungan fenolik (r = 0.625) dan flavonoid (r = 0.535). Penyelidikan lebih lanjut adalah mengenai pecahan cecair-ekstrak ekstrak mentah strain W, W2000 dan W4000 untuk menghasilkan pecahan heksana, etil asetat dan air. Untuk semua strain, pecahan air mengandungi kandungan gula, protein dan fenolik tertinggi manakala pecahan heksana memberikan kandungan flavonoid tertinggi. Pecahan etil asetat dari semua strain juga menunjukkan aktiviti antioksidan yang lebih kuat kira-kira satu kali lebih tinggi daripada pecahan air dan dua kali lebih tinggi daripada pecahan heksana dalam semua ujian antioksidan. Penemuan ini menunjukkan bahawa sebatian antioksidan dalam ekstrak mentah etanol akues *S. commune* berjaya dipekatkan dalam pecahan etil asetat dan aktiviti pengumpulan dan pengurangan radikal yang kuat mungkin disebabkan oleh banyaknya fenolik dan flavonoid berdasarkan kajian korelasi. Penemuan kami menunjukkan bahawa strain *S. commune* dan pecahan etil asetat dari strain terpilih (W, W2000 dan W4000) adalah sumber antioksidan semula jadi yang baik dan oleh itu ia berpotensi digunakan untuk aplikasi perubatan dan makanan.

Kata kunci: *Schizophyllum commune*, cendawan, aktiviti antioksidan, komposisi kimia, kacukan, penyinaran gamma dan pecahan cecair-cecair.

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LIST OF SYMBOLS AND ABBREVIATIONS

β	beta
°C	degree centigrade
µg	microgram
µl	microliter
µM	micromolar
%	percentage
AB	*Agaricus bisporus*
ANOVA	analysis of variance
ABTS	2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
CAT	catalase
CUPRAC	cupric ion-reducing antioxidant capacity
DNA	deoxyribonucleic acid
DPPH	2,2 diphenyl-1-picrylhydrazyl
dw	dry weight
EA	ethyl acetate
e.g.,	for example
EQ	ethoxyquin
et al	and others
etc.	et cetera
FRAP	ferric reducing antioxidant power
Fe^{2+}	ferrous ion

GAE	gallic acid equivalent
GE	glucose equivalent
GSHPx	glutathione peroxidase
g	gram
Gy	gray
Hex	hexane
h	hour
•OH	hydroxyl radical
K gray	kilogram gray
LDL	low-density lipoprotein
mg	miligram
ml	milliliter
mmol	milimol
mM	milimolar
min	minute
MAPK	mitogen-activated protein kinase
M	molar
nm	nanometers
Pp	*Pleurotus pulmonarius*
ROS	reactive oxygen species
rpm	round per minute
RE	rutin equivalent
SOD	superoxide dismutase
SPSS	Statistical Package for Social Science
TBHQ	*tert*-butylhydroquinone
TE	trolox equivalent

USA	United States of America
UV	ultraviolet
v/v	volume/volume
v/v/v	volume/volume/volume
w/v	weight/volume
w/w	weight/weight

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CHAPTER 1: INTRODUCTION

Oxidative stress happens when there is disequilibrium between the production of reactive oxygen species (ROS) and the biological system involved in its detoxification. This imbalance affects essential biomolecules and consequently impairs the functionalities of organs and systems in the organism (Durackova, 2010). Biomolecules such as DNA, protein and lipids represent the major target of free radicals. Exogenous sources of free radicals include drugs, pollutants, foods, climate and pathogens (Rahal et al., 2014). Free radicals are also generated endogenously, particularly via enzymatic and non-enzymatic oxidative metabolism. The excessive levels of ROS can cause severe biomolecular damage which accelerates aging and leads to many other medical conditions such as diabetes mellitus, cancer, ischemia, inflammatory diseases as well as neurodegenerative disorders (Valko et al., 2007).

As a countermeasure to the damaging effects of free radicals, the positive influence of antioxidants has been recognized by many researchers all over the world (Rahal et al., 2014). Over the last few years, antioxidants have gradually become a very vital part of human nutrition. Antioxidants are molecules that neutralize or inhibit free radical injurious activities, owing to their radical scavenging property (Devasagayam et al., 2004). Therefore, it is necessary to equilibrate between the body's level of free radicals and the cellular antioxidant defense for the survival of organisms. Endogenous defense system mechanism can be insufficient against the unrestrained free radical creation; hence, antioxidants from food remains a vital alternative for the consumers to protect against oxidative damage (Ferreira et al., 2009).

Although fruits, vegetables, tea, and herbs are some common sources of antioxidants, mushrooms are now gaining popularity as a possible store of antioxidative compounds. Mushrooms have for long served as functional nourishment due to their beneficial medicinal properties. Recently, the antioxidative qualities possessed by

medicinal mushrooms have been studied extensively and also documented to improve the human health and enhance the quality of life (Vamanu, 2014). The array of biological actions discovered in mushrooms is due to a vast variety of primary and secondary metabolites contained in the fungus. The natural compounds found in mushrooms are structurally diverse and therefore represent a potential target for drug discovery and development for various ailments (Russo et al., 2006). The primary metabolites of mushrooms are protein and carbohydrates, while the secondary compounds include phenolics, vitamins, tocopherols, etc. (Rathore et al., 2017). The chemical make-up of the fruiting body determines the quality of its related biomolecules. Particularly among the main group of less important nutritional constituents are phenol-based compounds, which antioxidative potential has been observed as a result of their abilities in diminishing of low-density lipoprotein (LDL) oxidation (Teissedre & Landrault, 2000) as well as inhibition of cancer and arteriosclerosis (Williams & Iatropoulos, 1997). Furthermore, some studies have also established a relationship between the antioxidant potential of mushrooms and their phenolic contents (Devi et al., 2014; Hu et al., 2019).

Generally, production of mushrooms with higher yield and quality characteristics is dependent on the genetic make-up of the strains (Kumara & Edirimanna, 2009). Simple strategies have been adopted to obtain a mushroom strain with improved characteristics, such as selection (monospore and multispore) and cross breeding (hybridisation) as well as gamma radiation (Lam et al., 2000; Martin & Thomsen, 2007). Besides, it has been observed that the bioactive components of mushroom can be further enhanced by improving the cultivation process and breeding new strains (Shah et al., 2004).

The split-gill mushroom, *Schizophyllum commune* Fr. is a widely distributed basidiomycete that is recognized for its medicinal properties as well as nutritive value in the Southeastern part of Asia (Han et al., 2005). Hence, it is now being cultivated commercially in both Malaysia and Thailand. As part of continuing work, hybridisation

and gamma-irradiation were applied on *S. commune* in order to obtain new strains. Natural strains (W and R) were isolated from Malaysia and Thailand respectively, hybrids (WR and RW) were obtained by hybridisation, whereas gamma irradiated strains (W2000, W4000, R2000 and R4000) were obtained by exposing the natural strains to different doses (2000 and 4000 Gy) of gamma rays. While several previous studies have established on the antioxidant activity of *S. commune* (Dulay et al., 2016; Emsen et al., 2017 Mirfat et al., 2010), very little is known about the variation of antioxidant activities between different strains of *S. commune* and the chemical compounds nature of the antioxidants in the fruiting bodies. Therefore, the aim of this research is to evaluate the antioxidant activities and chemical composition of the natural, gamma-irradiated and hybrid strains of *S. commune* in a comparative manner.

This study has the following specific objectives:

1. To evaluate the radical scavenging and reducing activity of aqueous ethanol extracts of *S. commune* strains, and the fractions derived from the extracts of selected strains.
2. To determine the chemical composition of aqueous ethanol extracts of *S. commune* strains and the fractions derived from the extracts of selected strains.
3. To correlate the antioxidant activity of the extracts and fractions of *S. commune* with their chemical composition.

CHAPTER 2: LITERATURE REVIEW

2.1 Oxidative stress

Oxidative stress reflects imbalance between the production of free radicals and the human body's defense system of antioxidants. Unsettling influences in the ordinary redox condition of cells can lead to the generation of peroxides and free radicals which result in toxic impacts that harm the major molecules of the cell such as damaging nucleic acid, oxidizing protein and lipid peroxidation causing cell death and tissue injury (Holst & Williamson, 2008). When cells are under excessive oxidative stress due to external stimulus such as exercise, radiation or pathogen, they produce extra amounts of enzymes associated with free radical's generation (such as lipogenase, xanthine oxidase and cyclooxygenase), stimulate phagocytes and launch free iron and copper ions which also result in the generation of extra reactive oxygen species (Ji et al., 1998; Rahal et al., 2014).

Oxidative stress received a significance interest from researchers due to its involvement in various pathophysiological conditions. The incidence and complication of cardiac disease, Alzheimer's disease, diabetes mellitus, age-induced eye disease as well as Parkinson's and other neurodegenerative diseases are correlated to oxidative stress. Similarly, the initiation, progression and advancement of different types of cancers in addition to the side-effects of its treatment (radiation and chemotherapy) are associated with the occurrence of oxidative stress (Barrera, 2012). Although reactive oxygen species at low concentration have been implicated in several beneficial biological activities, such as modulating immune response and protecting the cell against damage via mitohormesis (Holst & Williamson, 2008), they are extremely harmful to human cells at a higher concentration (Phaniendra et al., 2015).

2.2 Free radicals

A free radical is any chemical compound with one or more single unpaired electron on its atomic or molecular structure, and can exist independently. Hydroxyl radical (•OH) is one of the most common radicals which own a single unpaired electron on its oxygen atom, causing it to be highly unstable and reactive (Engwa, 2018). The chemical reactivity of free radicals resulting from the presence of an unpaired electron is short-lived and can become stabilized in a fraction of a second. Free radical production can be either endogenously as a by-product of metabolism or exogenously from the external environment (Lobo et al., 2010). An extreme level of ROS exposes all organisms to the danger of oxidative stress, which may lead to an attack on important chemical compounds, thereby disturbing essential metabolic reaction. A common example is the attack of free radicals on fatty acid in membrane lipid and DNA molecules, resulting in their degradation and modification, which ultimately affect their function (Shahidi & Zhong, 2010).

In living organisms, the enzymes that generate radicals are also responsible for producing a large fraction of reaction products. In addition, some essential activities such as blood pressure control and vascular tone are regulated by superoxide, nitric oxide radicals and products of their reaction (Gavazzi et al., 2006). Furthermore, they are considered as key players in the intervention of catabolic reaction of several biological compounds, tissue injury healing process and also act as messengers in the redox signaling process (Di Meo et al., 2016). Therefore, free radicals are indispensable in the body (Sarma et al., 2010). However, the uncontrolled production and accumulation of free radicals in the body are harmful to human health due to their high reactivity. Their presence in both the nucleus and the cell membrane is responsible for most alteration of major macromolecules such as DNA, protein, lipids as well as carbohydrates, thus causing homeostatic disruption. The combined effect of these damaged cellular

components results in aging and incidence of wrinkles, general physical body weakness and increased susceptibility to disease (Alexander, 2004).

Free radicals can be generated by internal and external sources (Rahal et al., 2014). The internal sources include those radicals produced as a by-product during the breakdown of nutrients and oxidative phosphorylation in the mitochondria (Pham-Huy et al., 2008). The oxygen utilized in the metabolic process can generate free radicals when it reacts with the metabolic compounds (Rahman, 2007). On the other hand, external sources are increasingly responsible for the rising case of human exposure to free radicals. Common external sources of free radicals are tobacco smoke, ultraviolet radiation, toxins, ionizing radiation, heat, electrical discharges, pathogens, electrolysis, environmental and occupational chemicals (Sen et al., 2010).

2.3 Antioxidants

Antioxidants are molecules capable of passing electron to free radicals and inhibiting cellular damage resulting from the oxidation of biological molecules. The reaction of antioxidants and free radicals results in the removal of free radicals intermediates, which prevent the initiation of chain reactions (Kohen & Nyska, 2002). Natural processes that generate free radicals, resulting in an episode of oxidative stress include energy generation, lipid degradation, response of catecholamine under stress and inflammatory processes. In order to prevent oxidative stress and protect the tissues and organs, the body possesses defense systems which can be categorized based on their actions as enzymatic and non-enzymatic antioxidants (Shahidi & Zhong, 2010).

Enzymatic antioxidants like glutathione peroxidase (GSHPx), superoxide dismutase (SOD) and catalase (CAT) play direct roles in ROS neutralization and are considered the first line of defense against oxidative stress (Lu et al., 2010) due to their destruction and removal of free radicals. On the other hand, the non-enzymatic

antioxidants can be further categorized into metabolic (endogenous) antioxidants like lipoid acid, glutathione, uric acid and ubiquinol as well as nutrient (exogenous) antioxidants such as trace metals, phenolics, and carotenoid which can only be obtained from food and/or supplements. The action of non-enzymatic antioxidants is to interrupt free radicals chain reaction (Droge, 2002; Willcox et al., 2004).

2.3.1 Types of antioxidants

The prevention or deletion of a chain of oxidative processes is the main feature of an antioxidant. This is achieved by stabilizing the generated radical, which results in a marked reduction of oxidative damage in the body. Antioxidants classified into two types are natural and synthetic antioxidants (Gordon, 1990). A natural antioxidant is a substance which is available in low concentrations when compared with an oxidizable catalyst and can considerably prevents the oxidation of the catalyst (Halliwell & Tferidge, 1999). This is also referred to as biological antioxidant. An ideal antioxidant is characterized by easy absorption by the body and production of a physiologically noticeable effect on the prevention of free radicals' formation. The examination, classification and uses of natural antioxidants are still the focal points of many research teams worldwide. This curiosity about natural antioxidants is triggered by the universality of their roles in varying redox systems.

Natural antioxidants such as carotenoids, phenolics, tocopherols etc., are commonly found in diet and possess broad biological activities such as anti-inflammatory, anti-cancer, and anti-diabetic (Xu et al., 2017). Due to promising functional activities of the antioxidants obtained from natural sources, their uses are increasingly considered for developing important ingredients for some products such as dietary supplements, pharmaceutical products, animal nutrition and cosmetics among others (Xu et al., 2017). An example is the remarkable increase in the use of natural

antioxidants to stabilize foods that contain lipids. This is due to the new information pertaining to the possible toxicity of synthetic antioxidants and the drift towards natural food additives by consumers (Khal, 1984).

Phytochemicals are secondary metabolites which are biologically synthesized from different natural sources. Several studies have been conducted on fruits, herbs, vegetables, cereals, seeds, mushroom and sprouts of many species in a bid to detect their antioxidant capabilities (Kumar et al., 2006). Natural products are classified based on four schemes viz. physiological activity, molecular skeleton, biogenesis and chemotaxonomy. They are divided into five major groups which include alkaloid, enzyme cofactors, non-ribosomal polypeptides, terpenoids and steroids as well as fatty acid–derived substances (Alamgir, 2018). Great attention has been given to phenolic compounds as natural antioxidants due to their ability to inhibit lipid peroxidation (Kristinova, 2009). Phenolic compounds like simple phenols, phenolic acids, flavonoids, coumarin, stilbenes, lignans, lignins as well as condensed and hydrolyzable tannins can be found in vegetables (Moon & Shibamoto, 2009) and mushrooms (Robaszkiewicz et al., 2010).

On the other hand, synthetic antioxidants have been introduced to meet the growing demand of antioxidant especially in the food and cosmetic manufacturing industries. The increase in demand for convenient food has brought about quick growth in the ready-to-eat food category (Silberbauer & Schmid, 2017). However, many of these food ingredients include unsaturated fatty acids which are very prone to decline in quality, especially when subjected to oxidative stress. Considerable efforts have been geared towards developing new synthetic antioxidants to reduce the impacts of damages resulting from free-radicals in many industries, especially the food, rubber, biomedical, plastic and petroleum industries (Hussain et al., 2003; Khaledi et al., 2011). However, the use of antioxidants is limited and differ depending on country (Reische et al., 2002). In the

European Union for example, many synthetic antioxidants are permitted to be used as food additives (Lundebye et al., 2010).

Some synthetic phenolic compounds that are commonly used as antioxidants are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) as well as others such as propyl gallate, ethoxyquin (EQ) and *tert*-butylhydroquinone (TBHQ), all of which perfectly limit oxidation (Venkatesh & Sood, 2011). Butylated hyroxyanisole (BHA) which is mostly used as an additive in the food industry can negatively impact the regulation of mitogen-activated protein kinase (MAPK) activity, depending on the applied quantity (Kozarski et al., 2014). As a result of this, there is a need for testing and approval before the use of synthetic antioxidants in food or food product. Based on their functional properties, synthetic antioxidants are classified as primary and secondary antioxidants. Primary antioxidants such as phenol molecules and their derivatives are also known free radicals scavengers or metal chelators as they are involved by direct elimination (neutralization) of the reactive species such as peroxy and hydroxy radicals. However, secondary antioxidants such as phosphites are referred to as peroxide scavengers, owing to their ability to inhibit the generation of free radicals from hydroxyperoxides by converting them into non-reactive components (Schwetlick, 1990).

Phenolic antioxidants make up an important group of synthetic compounds that work to hinder oxidation, and their action is of both economic and biological significance. Many food additives actually play antioxidative roles (Hertog et al., 1993). The variation in the structure of phenolic antioxidants are likely to have significant effects on their physical properties, thus leading to disparities in their antioxidative roles. As an instance, BHA and BHT are phenols with di-tert-butyl groups on their phenolic ring and are considered as an effective primary antioxidants (Reische et al., 2002). It should be noted however that some synthetic antioxidants have the likelihood of triggering adverse harmful effects in some specific situations (Kozarski et al., 2014).

Recently, there are legal restrictions on the use of synthetic antioxidants due to the number of undesirable physical properties. These compounds are carcinogenic and are both unstable and evaporate at high temperature. As a result, consumers are now directing their attention towards natural antioxidants (Kumar et al., 2015). The research studies of antioxidants derived from natural sources have begin to flourish following the increasing safety concerns for synthetic antioxidants (Mendiola, 2010).

2.3.2 Mechanism of antioxidants

Organisms come under oxidative stress through different conditions which triggers the body response to avert the destructive impacts of ROS. One of the main body responses is up-regulation of antioxidants and allied enzymes. This response mechanism involves three levels namely the sensing of ROS, signal transmission via particular routes and up-regulation of desired genes to improve the levels of their products (Lushchak, 2014). However, the mechanism of action of antioxidants can be illustrated by two concepts (Rice & Diplock, 1993).

The first mechanism which is also known as chain-breaking involves the donation of electron by antioxidants to the free radicals. The other mechanism is done by secondary antioxidants which is based on the concept of removing ROS initiators via destruction of chain-initiating catalyst (Krinsky, 1992). Antioxidants are important in preserving cell membrane structure and function by averting lipid peroxidation resulting from enzymatic action of GSHPx, CAT and SOD (Santos-Sanchez et al., 2019).

2.4 Edible mushrooms

Mushrooms have been used by humans as a traditional food for decades as they are considered as a nutritious food. The recent years have also witnessed increasing in mushroom consumption due to their recognition in sustaining human health. Mushroom is estimated to have about 12,000 species, out of which about 2,000 species are considered edible. In contrast to nearly 200 wild mushroom species used for medicinal purpose, the edible species which are commercially cultivated are 35 in number and are widely accepted by consumers in most countries (Aida et al., 2009).

Most Asian countries included edible wild mushrooms in their diet as a traditional food and medicine due to their rare flavor and texture (Manzi et al., 1999). Currently, mushrooms have become a functional food, owing to their chemical composition which is responsible for several important biological activites such as antioxidant, anti-diabetic, anti-microbial and anti-inflammatory (Elmastas et al., 2007). Among the bioactive compounds present in mushroom are polysaccharides, proteins, terpenes, polyphenols, tocopherols and vitamins. Furthermore, mushrooms are being utilized as an additive in pharmaceutical, food and cosmetic industries (Dai & Mumper, 2010).

Medicinal mushrooms are also composed of specific bioactive compounds which give them high therapeutic value with a huge advantage for the human health. They strengthen the immune system and play an essential part in curing and preventing serious diseases like heart disorder, hypertension, cerebral stroke and cancers, all of which are considered threats to human life (Stefanis et al.,1997).

Various parts of many medicinal mushrooms such as the fruiting body, mycelia and culture media have been extracted to obtain different bioactive compounds (Wasser, 2005). Many previous studies proved that mushrooms exhibit anti-fungal, anti-inflammatory, anti-cancer, anti-bacterial, anti-viral, anti-diabetic, hepatoprotective, hypolipidemic, hypotensive and anti-thrombotic activities (Wasser & Weis, 1999).

2.4.1 Mushrooms as a source of antioxidants

Mushrooms are considered as an effective source of nutrition to human food in addition to their health-promoting benefits, which is related to the presence of bioactive substances such as phenolic acids (Muthangya et al., 2019). Due to their antioxidative properties, they are capable of preventing damages related to excessive production of free radicals and oxidative stress. The level of mushrooms in human diet is correlated with the reduction of oxidative stress-related diseases and disorders (Vikineswary & Chang, 2013).

Various edible mushrooms (mostly basidiomycetes) represent reliable carbohydrate sources like β-glucans; phenolics (e.g., tocopherols); vitamin B-complexes (e.g., niacin, flavin and pyridoxine); organic acids (e.g., ascorbate, shikimate, malate and fumarate); monoterpenoid and triterpenoid; lipids; proteins (e.g., hydrophobins) as well as micro elements (e.g., selenium). All of these have been seen as being liable for the anti-cancer and anti-aging properties possessed by mushrooms (Chen et al., 2012). Therefore, mushrooms could be a boon against aging and age-related diseases. Moreover, mushrooms have high amounts of two important antioxidants, namely ergothioneine and glutathione. Although the two antioxidants are present in varying amount in different species of mushrooms, it has been demonstrated that mushrooms are the richest source of the two antioxidants (Kalaras et al., 2017).

There are many studies on mushrooms as a source of bioactive components, particularly the phenol-based compounds and polysaccharides with different bioactivities such as antioxidants, anti-microbial and anti-carcinogenic (Bach et al., 2017; Li et al., 2017). Similarly, several mushroom species exhibit potential benefits for consumption in different laboratory studies such as *Agaricus bisporus* (Barros et al., 2008; Kozarski et al., 2011), *Pleurotus pulmonarius* (Ramesh & Pattar, 2010) and others.

2.4.2 Types of antioxidant compounds in mushrooms

Secondary metabolites are the group of compounds responsible for natural product chemistry and are produced from primary metabolites for protection and survival of the plants (Zhong & Xiao, 2009). Mushrooms accumulate many secondary metabolic substances which include phenolic compounds, polyketides, steroids and terpenes together (Cheung et al., 2003). Owing to their structural variety and biological actions, some strong secondary metabolites have been modified for medical treatment and play an important part in drug discovery (Newman & Cragg, 2016). Antioxidant role of mushrooms has drawn significant interest from researchers seeking to explore them as a means of helping the human body to reduce oxidative damage. Most of the fungal metabolites in mushrooms have shown potency against some human diseases.

Mushrooms are a source of a vast range of molecules that act as free radical scavengers such as polysaccharides and polyphenols (Mau et al., 2002), dietary antioxidants like vitamins C and E, as well as carotenoids, whose role in the defense of degenerative illnesses is of great importance (Byers & Guerrero, 1995). Phenols belong to the main group of non-essential dietary compositions which have been implicated in the inhibition of free radicals in a bid to prevent cardiovascular conditions and certain cancers.

Phenolic compounds: These are aromatic hydroxylated compounds with known antioxidant properties and are most abundant in food (Scalbert et al., 2005). The difference of phenolic compounds subclasses depends on the number of the aromatic rings (one or more) with one or more hydroxyl groups. This diversity of structure creates a distinction between phenolic acids, stilbenes, lignans and flavonoids. Phenolic acids are considered as the main compounds in mushrooms. The antioxidant activity of phenol-based compounds is represented by stimulating the synthesis of endogenous antioxidant molecules in the cells, promoting their capacity (Cote J et al., 2010; D'Archivio et al.,

2010), quenching free radical's species (chain breaker), decomposing peroxide and inactivating metal (Dziezak, 1986). Study on five *Agaricus* sp. revealed that phenolic compounds (8.95-2.72 mg/g) constitute the largest antioxidant component in all extracts. Besides, *A. silvaticus* possessed the lowest EC_{50} values in both chemical and biochemical assays and the highest antioxidant activity in the electrochemical power which may be attributed to its high phenolic contents (Barros et al., 2008).

Ascorbic acid: It is known as vitamin C and functions to protect the body against diseases like heart disease, stroke and cancer which are caused by oxidative stress (Naidu, 2003). Vitamin C is found in several mushroom species such as *Agaricus* sp. (Barros et al., 2008) and *Pleurotus ostreatus* (Mattila et al., 2001) and is implicated in the radical scavenging activity of mushrooms via a direct or synergistic effect (Barros et al., 2008; Lung & Chang, 2011; Ramesh & Patter, 2010). Ascorbate is considered potent against superoxide, hydrogen peroxide, hydroxyl radical, peroxyl radical and singlet oxygen (Lung & Chang, 2011; Sies et al., 1992). It also shields biological membranes against lipid peroxidation by eliminating peroxyl radicals in the aqueous state before exhibiting its activity (Ferreira et al., 2009).

Carotenoids: A carotenoid is a natural pigment which is found in several fruits. The well-known carotenoids that are found in the body and diet of human beings are β-carotene, α-carotene, lutein, lycopene and β-cryptoxanthin. Lycopene has been detected in various mushroom species in varying amount ranging from 0.38 µg/g in *A. romagnesii* to 4.70 µg/g in *A. arvensis* (Barros et al., 2008). In another study, the lowest (20 µg/g) and highest lycopene (183.2 µg/g) concentration were recorded in *Lentinus sajor-caju* and *L. squarrosulus* respectively (Hussein et al., 2015). β-carotene and lutein were particularly found in several mushroom species (Kozarski et al., 2015). Bai et al. (2005) revealed that different researches show the presence of antioxidative and anti-inflammatory actions in β-carotene. Carotenoids function as chain-breaking antioxidant in lipids, particularly in

situations of low oxygen partial pressure (Paiva & Russell, 1999).

Polysaccharides: Generally, *β*-glucan is most common polysaccharides found in mushroom and is responsible for the several bioactivities of the fungi including antioxidative properties (Badalyan, 2003). Study on *Boletus edulis* revealed that the polysaccharides isolated from the fruiting bodies of the mushroom is responsible for the chelating activity and inhibitory effects on hydroxyl and superoxide radicals (Zhang et al., 2011). Similarly, it was demonstrated that the four polysaccharides obtained from *Ganoderma lucidum* exhibited scavenging activities (Shi et al., 2103).

Tocopherols: Vitamin E represents a general name for family of related tocopherol compounds (Ferreira et al., 2009). They are natural antioxidants found in several mushroom species and are known for their ability to scavenge free radicals and prevent against degenerative diseases resulting from oxidative stress (Sanchez, 2017; Valverde et al., 2015). Tocopherols function by donating a hydrogen atom to peroxyl radical to form a hydroperoxide and itself becomes a less reactive radical which can stabilized by reacting with other radicals (Ferreira et al., 2009). Examples for vitamin E found in mushrooms include α-tocopherol and β-tocopherol in *Agaricus* sp. (Barros et al., 2008) and *B. edulis* (Ferreira et al., 2009) respectively.

Steroids: These are organic substances which possess a distinct arrangement in which four cycloalkane rings being joined together. The dietary fat cholesterol and dexamethasone, an anti-inflammatory medication, are two examples of steroids. According to Zhang et al. (2003), two known steroids with new cyclooxygenase inhibiting and antioxidative properties were recently separated from the fruiting bodies of *Agrocybe aegerita* which is an edible mushroom.

2.5 Strain improvement in mushroom

With the population of the world expected to keep increasing, there is the likelihood of a decrease in available food and proper medical care which individuals might be able to access. For many years, mushrooms have been consumed and cherished for their economic, ecological, nutritional and medicinal properties. Recently, mushroom production has been improved to a new level, especially in countries with vibrant economies like China, Poland, Hungary and India (Vikineswary & Chang, 2013). Edible mushroom cultivation is of absolute relevance in present times especially with the advancement in cultivation techniques which increases the potential to obtain improved strains. Subsequently, researches have been carried out on mushrooms to discover ways to improve its yield, nutritional value and disease resistant ability (Chakravarty, 2011; Sanchez, 2004). These represent the major goal for research on breeding mushroom production. Secondary objectives of mushroom breeding include reduction of the cost of production and efficient compost use for growth. There are several techniques that have been applied in strain improvement of mushrooms. These include sexual hybridisation, somatic crossing (protoplast fusion), random mutagenesis and genetic engineering (Nevalainen, 2001).

Sexual hybridisation involves the identification of parent lines with desired traits for the isolation of its spores for hybridisation using dilution plate or spore print technique. The homokaryons are verified by the observation of slowed growth, lack of clamps and absence of fruiting bodies during cultivation. Subsequently, the selected homokaryons are crossed or mated to form a hybrid which can be confirmed by tests such as fructification test, restriction enzymes and DNA markers (Baral et al., 2018). Peng et al. (2001) have successfully produced hybrid strains of oyster mushroom with varying morphological advantages using intraspecific hybridisation method. Cross-breed strains have brought about mushroom that are not only resistant to disease and pest, but can also

withstand environmental and cultural stresses. As an instance, a lot of older strains which never mature despite growing massively and producing a large number of pins are cross-breed with less prolific strains to produce high-yielding cross-breed strains with increased thickness and mushroom cap density (Chakravarty, 2011).

Protoplast fusion, a somatic crossing technique involves several steps to develop hybrid with properties of interest. According to Horgen et al. (1991) and Kerrigan et al. (1992), two successful laboratory breeding methods are the protoplast culture and spore germination. In the 90s, a boost was recorded in efforts to improve commercially grown fungi strains via development of methods of protoplast isolation (Chang, 1993). These include isolation of single spore from the parent strains via spore sprint, followed by the evaluation of the monokaryotic mycelia subsequent to the isolation of their protoplast using lytic enzyme. The hybrid strains are obtained by mixing both protoplasts, after which they are evaluated for desired properties (Aswini et al., 2014). In the study by Oropeza et al. (2018), four hybrid strains of *Pleurotus djamor* from monokaryotic components (neohaplonts) were evaluated for their antioxidant capacity. The hybrid strains presented more total phenols than their respective mycelia and consequently exhibited better antioxidants activity. In the work done to improve the strain of *Pleurotus* by Aswini et al. (2014), they used protoplast fusion technique between protoplast isolated from a wild type and UV mutant strain. The produced hybrid proved that their mycelia were significantly faster in growth and larger size than their parental strain.

Common chemical mutagens used to effect point mutations and deletions. However, due to safety concerns of the chemical mutagenic agents the use of physical mutagens such as UV light, X-ray and gamma irradiation are generally favored. According to Djajanegara, (2008), five mutants were obtained when *Pleurotus florida* mycelia were exposed to gamma irradiation at dose of 0.75 KGray but only one of the mutants showed higher antioxidant activity, based on the DPPH assay, than the control.

2.6 *Schizophyllum commune*

Schizophyllum commune is a white color fungus, which looks just like the waves of packed corals which are undulating when observed and possesses stemless fruiting bodies. This fungus is also referred to as split-gill mushroom due to the uniquely longitudinal division of the 'gills' under the cap and can be found worldwide with the exception of Antarctica (De Mattos et al., 2016).

Figure 2.1: Morphology of *S. commune* fruiting bodies.
Beneath the cap is radial gill-like folds, each of which is centrally split.

These mushrooms are found on decaying trees in the wild after the wet season. However, they are naturally collected during the dry spells that follow the rainy season. During the monsoon, *S. commune* is opened up and soft, but returned to being hard and

white when the season changed (Sim, 2014). In fact, *S. commune* is edible and currently consumed in many tropical nations in Asia (Petcharat, 1995; Mongkontanawat, 2013). West Africa (Osemwegie et al., 2014) and some parts of Latin America, especially Mexico (Guzman, 2008).

Figure 2.2: *S. commune* is cultivated in sawdust substrate.

Schizophyllum commune is of huge significance in the food and pharmaceutical industries because of its ability to produce metabolites which are useful in various industrial production processes (Tripathi & Tiwary, 2013). It is also recognized for its high medicinal value because of its anti-fungal, anti-viral and immunomodulatory activities (Taylor et al., 2006).

2.7 Antioxidant activities of *S.commune*

Schizophyllum commune is an edible mushroom of significant interest to both consumers and researchers due to its high nutritional and medicinal value. Thus, it was selected for evaluation and examination. Moreover, the production of the mushroom has been scaled up resulting from its easy cultivation (Wasser, 2002). The antioxidant activity of *S. commune* has been previously studied and are summarized in Table 2.1.

Table 2.1 Previous studies on the antioxidant activities of *S. commune*

Mushroom part	Location	Extraction solvent	Chemical compositions	Major findings	References
Fruiting bodies (cultivated)	Malaysia	Methanol	Total phenolic content (1.72 mg GA/g extract).	DPPH radical scavenging activity (IC_{50}: 0.145 mg/ml).	Mirfat et al. (2010)
Fruiting bodies (wild)	Serbia	Hot water and hot alkali	The total phenolic contents of HWE, HWP and HWAE of *S. commune* are 0.9, ,1.0, and 0.2 g/100 g, respectively. α-glucan and β-glucan	DPPH radical scavenging activities, reducing power and chelating ability.	Klaus et al. (2011)
Mycelia (cultivated)	Malaysia	Ethanol	Total phenolic content (40.99 mg TAE/g extract) and flavonoids 4.58 mg QE/g extract.	At 10 mg/ml reducing power 0.44 mg BHA/g extract, DPPH radical scavenging activity (IC_{50}: 3.16 mg/ml).	Arbaayah & Umi (2013)
Fruiting bodies (cultivated)	Malaysia	Methanol:dichloro methane	Total phenolic content (82.42 mg GAE/g extract)	DPPH radical scavenging activity (IC_{50}: 70 µg/ml), reducing power activity and inhibition of lipid peroxidation.	Mayakrishnan et al. (2014)

Table 2.1 Continued

Mashroom part	Location	Extraction solvent	Chemical compositions	Major findings	References
Fruiting bodies (wild)	India	Water, ethanol and methanol	The water extracts: total phenolic content was 81.97 µg/µl then followed by methanol extract (75.11 µg/µl) and ethanol extract (47.08 µg/µl).	DPPH radical scavenging activity (IC_{50}: 18.56 µg/ml), reducing power, chelating activity and hydrogen peroxide scavenging activity.	Chandrawanshi et al. (2017)
Cap and stipe (wild)	Turkey	Water, methanol and chloroform	Total phenolic content (2.51 to 22.57 µg/mg), total flavonoid content (0.12 to 0.22 µg/mg), β-carotene and lycopene.	DPPH radical scavenging activity (IC_{50}: 7.652 mg/ml), reducing power and metal chelating.	Emsen et al. (2017)
Cap and stipe	Thailand	Hexane, ethyl acetate, dichloromethane, 2-butanol, ethanol, methanol and water	ND	DPPH radical scavenging activity (IC_{50}: 23 µg/ml)	Srichanun et al. (2018)
Fruiting bodies (cultivated)	Malaysia	Ethanol, methanol, aqueous ethanol , aqueous methanol.	Total phenolic content (9.49 mg GAE/g extract), total polysaccharide content (75.68 mg GE/g extract) and β-glucan (6.08 %).	DPPH radical scavenging and reducing activities.	Abd Razak et al. (2019)

ND: not determined

CHAPTER 3: MATERIALS AND METHODS

3.1 Chemicals

Iron(II) sulphate ($FeSO_4$) and sodium carbonate (Na_2CO_3) were obtained from R&M Chemicals, Malaysia. Folin-Ciocalteu's phenol reagent and D-glucose were obtained from Merck, Germany. Iron(III) chloride ($FeCl_3$), sodium hydroxide pellets (NaOH), neocuproine ($C_{14}H_{12}N_2$), sodium nitrite ($NaNO_2$), ammonium acetate ($C_2H_7NO_2$) and sulphuric acid (H_2SO_4) (Friendemann Schmidt, Australia). Sodium acetate ($C_2H_3NaO_2$), acetic acid, 2,4,6-tripyridyl-s-triazine(TPTZ), potassium persulphate ($K_2S_2O_8$), 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), aluminium chloride ($AlCl_3$) were purchased from Sigma-Aldrich, Germany. Positive controls such as trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and butylated hydroxyltoluene (BHT) were obtained from R&M Chemicals, Malaysia. The Micro BCA^{TM} Protein Assay Kit was purchased from Thermo Scientific, Rockford, IL, USA. Antioxidant standards like ascorbic acid, gallic acid and rutin were obtained from Sigma-Aldrich, Germany. Analytical grade solvents used in this study including ethanol, methanol, n-hexane, ethyl acetate and hydrochloric acid (Friendemann Schmidt Chemical, Australia).

3.2 Mushroom samples

The mycelium cultures of *S. commune*, including selected natural strains from Malaysia and Thailand, strains subjected to two dosages of gamma irradiation, 2000 and 4000 Gy (representing the IC_{25} and IC_{50} of the treatment) for 10 minutes and strains derived from hybridisation were obtained from the Mycology Laboratory, Institute of Biological Sciences, Faculty of Science, University of Malaya. The cultures were subsequently used in the production of fruiting bodies via the solid-substrate fermentation

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 fruiting bodies of *Pleurotus pulmonarius* (grey oyster mushroom) and *Agaricus bisporus* (white button mushroom) were obtained from a local supermarket in Petaling Jaya, Selangor, Malaysia. All mushroom samples were cleaned, dried in a commercial food dehydrator (WAKimart, Malaysia) and then powdered using a Warring blender. The mushroom samples used in this study are listed in Table 3.1.

Table 3.1: Mushroom samples used in this study

Samples	Description
Schizophyllum commune strains	
W	Natural strain isolated from Malaysia
W2000	Strain W subjected to gamma irradiation (2000 Gy)
W4000	Strain W subjected to gamma irradiation (4000 Gy)
R	Natural strain isolated from Thailand
R2000	Strain R subjected to gamma irradiation (2000 Gy)
R4000	Strain R subjected to gamma irradiation (4000 Gy)
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.

3.3 Preparation of crude aqueous ethanol extracts

The powdered fruiting bodies 4 g 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 on an orbital shaker (Lab Companion, SK-300) at 130 rpm. The extracts then were filtered and the procedure was repeated twice. Excess ethanol was removed using a rotary evaporator (40°C) before the extracts were freeze-dried. The dried crude extracts were kept at -20°C until further analysis.

3.4 Determination of chemical composition of the extracts

3.4.1 Total sugar content

Total sugar content of the extracts was determined using the phenol-sulphuric assay according to the method by Masuko et al. (2005). A 150 µl of concentrated sulphuric acid was added to 50 µl of samples (crude 2.5 mg/ml) and (fractions 5 mg/ml) in a 96-well plate. Then, 30 µl of 5% (w/v) phenol in water was added. The mixtures were heated for 5 min at 90°C in a static water bath. After cooling to room temperature, the absorbance was measured at 490 nm. D-glucose (0-1 mg/ml) was used as the standard. Results were expressed as mg glucose/g extract.

3.4.2 Total Protein content

Total protein content was analysed using the Micro BCA^{TM} Protein Assay Kit according to the manufacturer's protocol. Briefly, 200 µl of reagent A and B in the ratio of 50:1 (v/v) was added to 25 µl of sample (1 mg/ml). The mixture was incubated in water bath at 37°C for 30 min. The absorbance was read at 562 nm. Bovine serum albumin (0-2 mg/ml) was used as the standard. 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 & Rossi (1965) with some modification. A 25 µl of 1 N Folin-Ciocalteu reagent was added into 50 µl of sample (1 mg/ml). After 5 min, 100 µl of saturated sodium carbonate solution (0.57 M) was added to the mixture. Then final volume was completed to 250 µl by adding MiliQ water. After 2 h of incubation, the absorbance was read at 760 nm (Tecan, Infinite M1000 Pro, GmbH, Austria). Gallic acid (0-0.1 mg/ml) was used to plot a standard calibration curve. Results were expressed as mg gallic acid equivalents (GAE)/g extract.

3.4.4 Total flavonoid content

Total flavonoid content of the extracts was measured using a modified aluminium chloride colorimetric method described by Liu et al. (2008). A 100 µl of sample (5 mg/ml) was mixed with 10 µl of 5% sodium nitrite. After 5 min of incubation, 10 µl of 10% (w/v) aluminium chloride was added. Followed by another 6 min of incubation to the mixture before 100 µl of 1M sodium hydroxide was added to the mixture. MiliQ water was added to make the final volume of the reaction mixture to 250 µl. The absorbance was read at 510 nm. A standard calibration curve of rutin (0-0.1 mg/ml) was plotted. Results were expressed as mg rutin equivalents (RE)/g extract.

3.5 Assessment of the antioxidant activity of aqueous ethanol extracts of mushrooms

The crude ethanol extracts of mushrooms were dissolved in 20% (v/v) aqueous ethanol to produce stock solutions of 20 mg/ml. Different *in vitro* antioxidant assays were used to determine the antioxidant activity of the extracts, such as the 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS), ferric reducing antioxidant power (FRAP), and cupric ion-reducing antioxidant capacity (CUPRAC). Results were expressed as trolox equivalent antioxidant capacity.

3.5.1 2,2 diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH) assay

The DPPH radical scavenging activity was measured using a method of Brand-Williams et al. (1995) with slight modifications. Briefly, 195 µl of DPPH solution in methanol (100 µM) was added into 50 µl of extracts (10 mg/ml). The mixture was incubated for 30 min prior to absorbance reading at 515 nm. The DPPH free radical scavenging activity was calculated using the following equation:

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

$A_{control}$ = Absorbance of DPPH radicals without sample or standard.

$A_{sample\ or\ standard}$ = Absorbance of DPPH radicals with sample or standard.

Results were expressed as trolox equivalents (mmol TE/g extract). Ascorbic acid, gallic acid, rutin, and butylated hydroxytoluene (BHT) at 1 mg/ml were used as positive controls.

3.5.2 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) or ABTS radical scavenging assay

The 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) or ABTS radical cation decolourisation assay (Re et al., 1999) was used to determine the trolox equivalent antioxidant capacity (TEAC) of the extracts. Firstly, 7 mM ABTS radical and 2.45 mM potassium peroxodisulphate were mixed and prepared in 10 ml of distilled water then incubated for 12-16 h in the dark at room temperature to generate ABTS radical cation. Later, the stock solution was further diluted to obtain an absorbance of 0.70 ± 0.05 at 734 nm. A 300 µl of ABTS solution was added into 3 µl extract (10 mg/ml) and incubated for 6 min prior to absorbance measurement at 734 nm. The ABTS radical scavenging activity was calculated using the following equation:

ABTS radicals scavenging activity (%) = $(A_{control} - A_{sample}) / A_{control} \times 100$

$A_{control}$ = Absorbance of ABTS radicals without sample or standard

A_{sample} = Absorbance of ABTS radicals with sample or standard

Results were expressed as trolox equivalents (mmol TE/g extract). Ascorbic acid, gallic acid, rutin, and butylated hydroxytoluene (BHT) at 1 mg/ml were used as positive controls.

3.5.3 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was done according to the method described by Benzie & Strain (1996) with slight modifications. The FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM hydrochloric acid and 20 mM ferric chloride at a ratio of 10:1:1 (v/v/v), respectively. Briefly, 300 µl of FRAP reagent was mixed with 5 µl of mushroom extracts (5 mg/ml). The mixture was incubated for 30 min at 37°C before measuring the absorbance at 595 nm. Results were calculated based on the calibration curve of iron(II) sulphate (0-1 mM) and expressed as

mmol Fe^{2+}/g extract. Ascorbic acid, gallic acid, rutin, and butylated hydroxytoluene (BHT) at 1 mg/ml were used as positive controls**.**

3.5.4 Cupric ion-reducing antioxidant capacity (CUPRAC) assay

The CUPRAC assay described by Apak et al. (2005) was used with some modifications. The CUPRAC reagent was freshly prepared by mixing 50 µl of each solution in the following order: copper(II) chloride (10 mM), neocuproine (7.5 mM) and acetate buffer (1000 mM, pH 7). Then, 150 µl of the reagent was added to 50 µl of extracts (5 mg/ml). The mixtures were then incubated in the dark at room tempereature for 30 min. The absorbance was measured at 450 nm. Trolox (0-1 mg/ml) was used to plot a calibration curve. Results were expressed as trolox equivalents (mmol TE/g extract). Ascorbic acid, gallic acid, rutin, (0.1 mg/ml) and butylated hydroxytoluene (BHT) (1 mg/ml) were used as positive controls.

3.6 Fractionation of the selected *S. commune* strains

The crude aqueous ethanol extracts of the following *S. commune* strains, including W, W2000 and W4000 were selected for further study. The powdered fruiting bodies (20 g) of these strains were subjected to extraction procedure as described in Section 3.3. The resulting crude aqueous ethanol extracts of these three strains were further extracted with hexane (500 ml) to yield the hexane-soluble fraction. The hexane-insoluble fraction was dissolved in 100 ml of ethyl acetate and partitioned with 50 ml water for three times to yield the ethyl acetate and water fractions. The hexane and ethyl acetate fractions were dried using the rotary evaporator while the water fractions were freeze-dried. Figure 3.1 shows the preparation of *S. commune* fractions. The fractions were kept in -20°C prior to analysis.

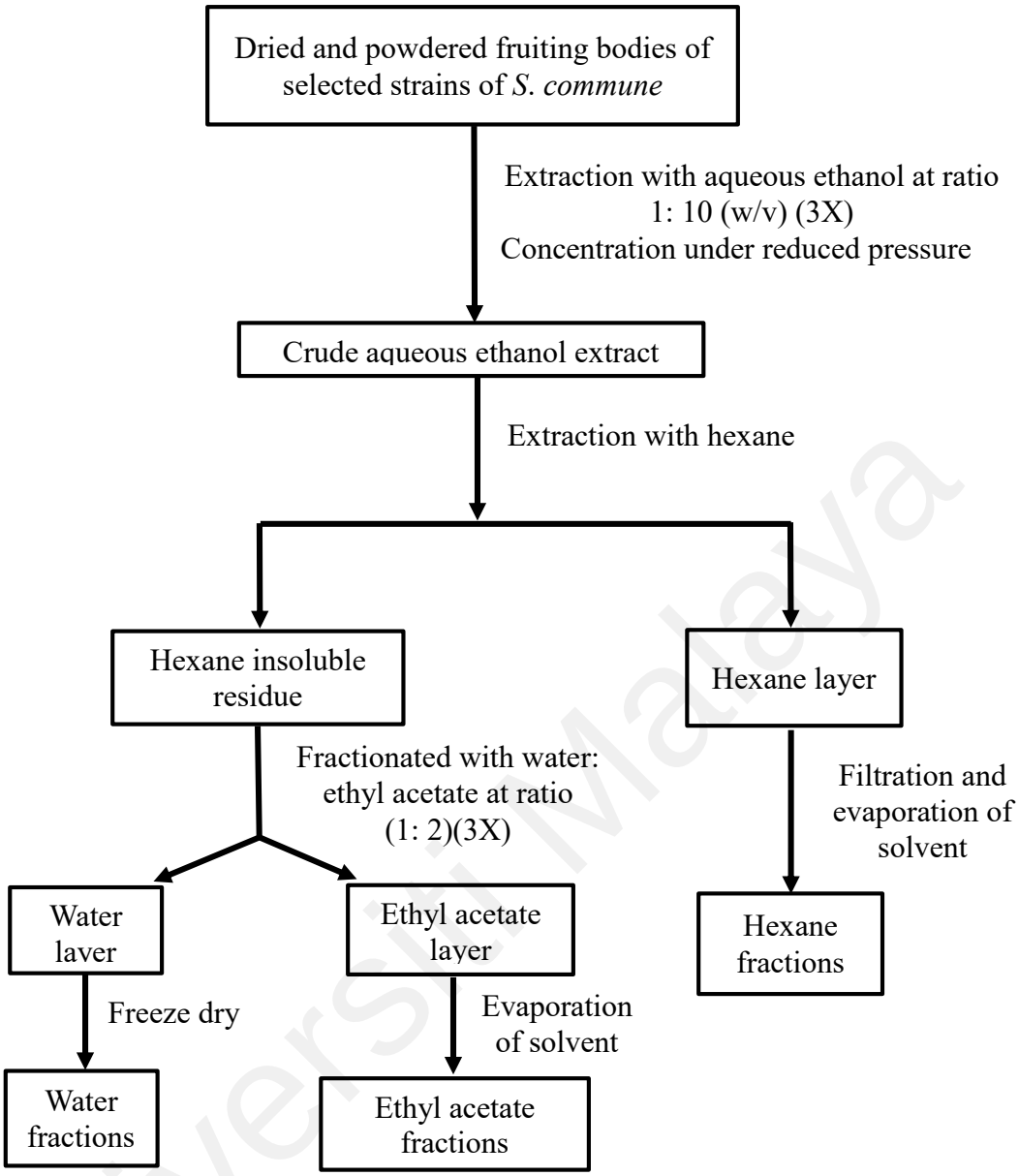

Figure 3.1: Flow chart of extraction and liquid-liquid fractionation of *S. commune* selected strains.

The strains W, W2000 and W4000. From the three crude aqueous ethanol extracts of each strain, a total of nine fractions (hexane-soluble, ethyl acetate-soluble and water-soluble fractions) were obtained.

3.7 Statistical analysis

All experiments were performed in triplicates and results were expressed as mean ± standard deviation. Statistical analysis was performed by using the Statistical Package for the Social Sciences software version 25 (SPSS Inc., Chicago, Illinois, USA). One-way analysis of variance (ANOVA) was used to compare means among groups. The Pearson's correlation test was used to accomplish the relationships between the chemical compositions and antioxidant activities. p value < 0.05.

CHAPTER 4: RESULTS

4.1 Extraction yields of mushroom samples

The yields for the crude aqueous ethanol extracts of the natural, gamma-irradiated and hybrid strains of *S. commune*, and two other edible mushrooms, *P. pulmonarius* and *A. bisporus* are shown in Table 4.1. The yields of the extracts of *S. commune*, expressed on a dried weight (dw) basis, ranged from 18.24 % to 30.83%. The three highest yields obtained from the *S. commune* samples were from the W, W4000 and R4000 strains (28.32-30.83%). These values were lower than *P. pulmonarius* (32.23%) and *A. bisporus* (52.78%).

Table 4.1: Total yield of the crude aqueous ethanol extracts of selected mushroom

Samples	Yield (%)
W	29.39
W2000	24.12
W4000	28.32
R	18.24
R 2000	24.65
R 4000	30.83
WR	18.52
RW	25.87
Pleurotus pulmonarius	32.23
Agaricus bisporus	52.78

W, R (natural strains); W2000, W4000, R2000, R4000 (strains obtained when the natural strains were subjected to different doses of gamma radiation); WR, RW (strains obtained from hybridization between the natural strains)

4.2 Chemical compositions of crude mushroom extracts

The chemical compositions of the crude extracts of *S. commune* strains including total sugar content, total protein content, total phenolic content and total flavonoid content have been determined in this study. The results are presented in Table 4.2.

4.2.1 Total sugar content

The total sugar contents of the crude extracts of *S. commune* ranged from 31.78 to 109.74 mg glucose/g extract (Table 4.2). All strains contained sugar content lower than that of *P. pulmonarius* (135.97 mg glucose/g extract) but higher than *A. bisporus* (14.79 mg glucose/g extract). The natural strain R and its gamma-irradiated strains, R2000 and R4000 were observed to have higher sugar content than that of the natural strain W and its gamma-irradiated strains, W2000 and W4000.

4.2.2 Total protein content

The extracts of all strains of *S. commune* contained higher protein content (225.60-486.81 mg protein/g extract) than both *P. pulmonarius* (172.27 mg protein/g extract) and *A. bisporus* (219.54 mg protein/g extract) (Table 4.2). The gamma-irradiated strains R2000 and R4000 recorded higher protein content than the natural strain R but similar pattern was not observed for the natural strain W and its gamma-irradiated strains.

Table 4.2: Chemical compositions of crude aqueous ethanol mushroom extracts

	Chemical compositions			
Mushroom samples	Total sugar content (mg glucose/g extract)	Total protein content (mg protein/g extract)	Total phenol content (mg GAE/g extract)	Total flavonoid content (mg RE/g extract)
W	31.78 ± 0.16^{h}	486.81 ± 2.40^{a}	20.94 ± 0.42^{b}	3.25 ± 0.15^{cd}
W2000	73.97 ± 3.84^{f}	447.12 ± 21.82^{ab}	22.87 ± 0.14^{a}	5.36 ± 0.19^{b}
W4000	33.08 ± 2.27^{h}	293.48 ± 2.28^{d}	11.78 ± 0.21^{e}	6.85 ± 0.84^{a}
R	109.74 ± 1.01^{b}	225.60 ± 17.86^{e}	6.77 ± 0.06^{f}	3.77 ± 0.26^{cd}
R2000	77.52 ± 0.25^{ef}	409.54 ± 9.57^{bc}	17.20 ± 0.23^{d}	3.26 ± 0.11^{cd}
R4000	87.65 ± 7.29^{c}	375.90 ± 55.98^{c}	17.62 ± 0.49^{d}	2.36 ± 0.07^{e}
WR	49.49 ± 0.70^{g}	390.75 ± 22.64^{bc}	17.75 ± 0.41^{d}	3.19 ± 0.11^{d}
RW	79.93 ± 2.17^{d}	439.54 ± 56.99^{ab}	19.07 ± 0.72^{c}	3.30 ± 0.25^{cd}
Pb	135.97 ± 3.66^{a}	172.27 ± 29.91^{e}	5.46 ± 0.20^{g}	4.04 ± 0.20^{c}
Ab	14.79 ± 0.74^{i}	219.54 ± 27.64^{e}	6.77 ± 0.08^{f}	3.13 ± 0.92^{d}

$^{a, b, c \cdots h}$means values with different superscripts within the same column differ significantly ($p < 0.05$). Results are expressed as mean ± standard deviation (n = 3). GAE, gallic acid equivalent; RE, rutin equivalent. W, R (natural strains); W2000, W4000, R2000, R4000 (strains obtained when the natural strains were subjected to different doses of gamma radiation); WR, RW (strains obtained from hybridization between the natural strains); Pp, ***Pleurotus*** *pulmonarius*; Ab, *Agaricus bisporus*.

4.2.3 Total phenolic content

The total phenolic contents of the extracts of *S. commune* ranged from 17.20 to 22.87 mg GAE/g extract with the exception of strain R and W4000 with the values of 6.77 and 11.78 mg GAE/g extract, respectively (Table 4.2). These values were still higher than that for *P. pulmonarius* (5.46 mg GAE/g extract) and *A. bisporus* (6.77 mg GAE/g extract). Notably, the total phenolic content of gamma-irradiated strains, R2000 and R4000, were almost three times higher than the natural strain R.

4.2.4 Total flavonoid content

With the exception for W2000 and W4000, the total flavonoid contents of the other crude extracts of *S. commune* strains were ranged from 2.36 to 3.77 mg RE/g extract, comparable to that of *P. pulmonarius* and *A. bisporus* with 4.04 and 3.13 mg RE/g extract, respectively (Table 4.2). The gamma-irradiated strains of W showed higher flavonoid content than the natural strain but an opposite trend was observed for the natural strain R in which the gamma-irradiated strains have lower flavonoid content.

4.3 Antioxidant activities of crude mushroom extracts

The antioxidant activities of the crude extracts of the natural, gamma-irradiated and hybrid strains of *S. commune* was evaluated using the DPPH and ABTS radicals scavenging assays as well as the FRAP and CUPRAC assays. The results were compared with those obtained from two common edible mushrooms *P. pulmonarius* and *A. bisporus*. Ascorbic acid, gallic acid, rutin and BHT were used as positive controls.

4.3.1 DPPH radicals scavenging activity

The DPPH radicals scavenging activities of the extracts of *S. commune* strains, expressed as trolox equivalents, ranged from 2.30-2.48 mmol TE/g extract. The antioxidant activities of all the strains were higher than that of *P. pulmonarius* (1.82 mmol TE/g extracrt) but comparable to that of *A. bisporus* (2.41 mmol TE/g extract) (Figure 4.1). The scavenging activities of the positive controls including ascorbic acid, gallic acid, rutin and BHT, ranged from 2.51 to 2.64 mmol TE/g, were higher than all crude extracts.

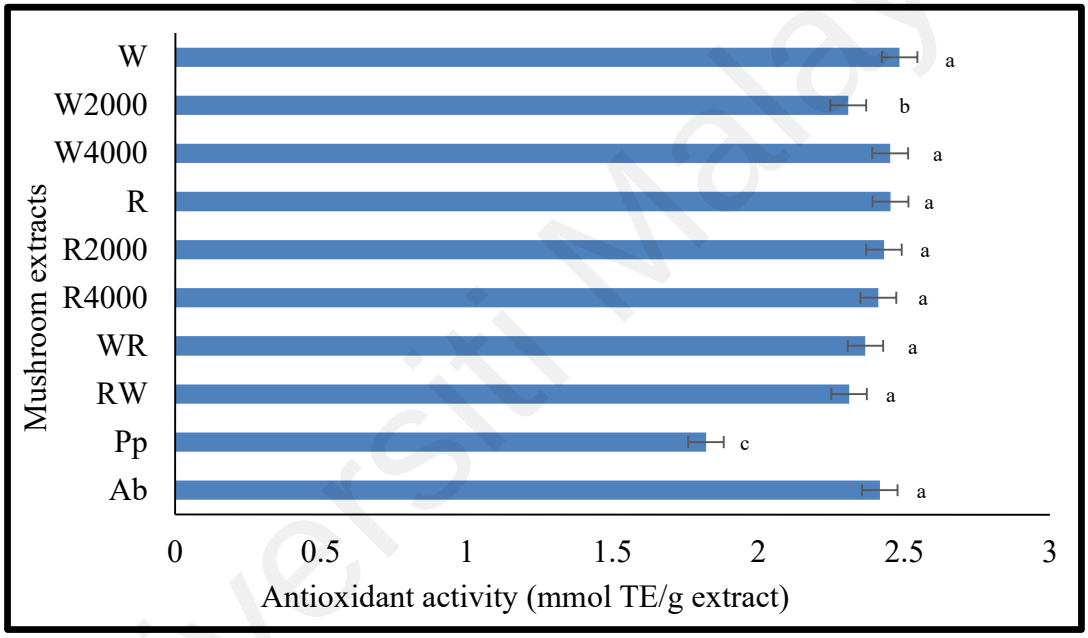

Figure 4.1: DPPH free radical scavenging activities of mushroom extracts

[a, b, c] means with different superscripts on bars differ significantly ($p < 0.05$). The extracts were tested at 10 mg/ml. Results were expressed as mean ± standard deviation (n = 3). W, R (natural strains); W2000, W4000, R2000, R4000 (strains obtained when the natural strains were subjected to different doses of gamma radiation); WR, RW (strains obtained from hybridization between the natural strains); Pp, *Pleurotus pulmonarius*; Ab, *Agaricus bisporus*.

4.3.2 ABTS radicals scavenging activity

Figure 4.2 shows the ABTS radicals scavenging activities of the crude extracts of *S. commune* strains expressed as trolox equivalents. The antioxidant activities of all strains (0.07-0.15 mmol TE/g extract) were higher than that of *P. pulmonarius* (0.04 mmol TE/g extract) and *A. bisporus* (0.06 mmol TE/g extract). The scavenging activities of the all the strains were lower than positive controls (mmol TE/g) namely ascorbic acid (2.57) and gallic acid (4.5) but comparable with rutin (1.84) and higher than BHT (0.003).

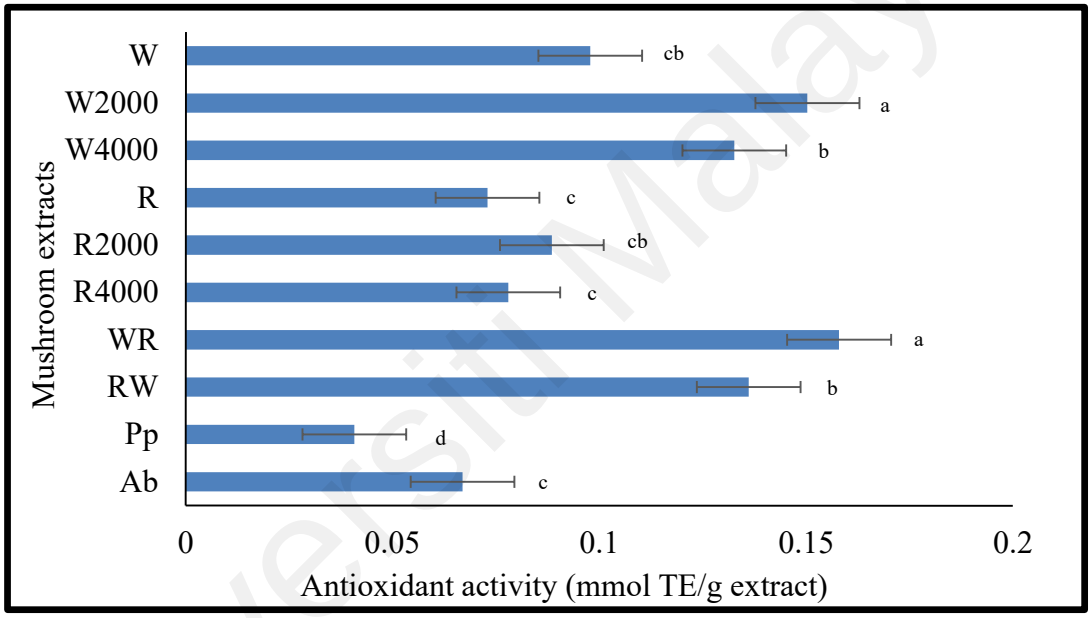

Figure 4.2: ABTS free radical scavenging activities of mushroom extracts

[a, b, c, d] means with different superscripts on bars differ significantly ($p < 0.05$). The extracts were tested at 10 mg/ml. The results were expressed as mean ± standard deviation (n = 3). W, R (natural strains); W2000, W4000, R2000, R4000 (strains obtained when the natural strains were subjected to different doses of gamma radiation); WR, RW (strains obtained from hybridization between the natural strains); Pp, *Pleurotus pulmonarius*; Ab, *Agaricus bisporus*.

4.3.3 Ferric reducing antioxidant power (FRAP)

The reducing activities of the crude extracts of *S. commune* based on the FRAP assay are shown in Figure 4.3. All *S. commune* strains have the ability to reduce ferric ions with values ranging from 0.22 - 0.30 mmol Fe^{2+}/g extract, higher than those of *P. pulmonarius* (0.13 mmol Fe^{2+}/g extract) and comparable with *A. bisporus* (0.19 mmol Fe^{2+}/g extract). Both gamma irradiated strains (W2000 and R2000) exhibited slightly higher reducing activity than their respective natural strains (W and R).

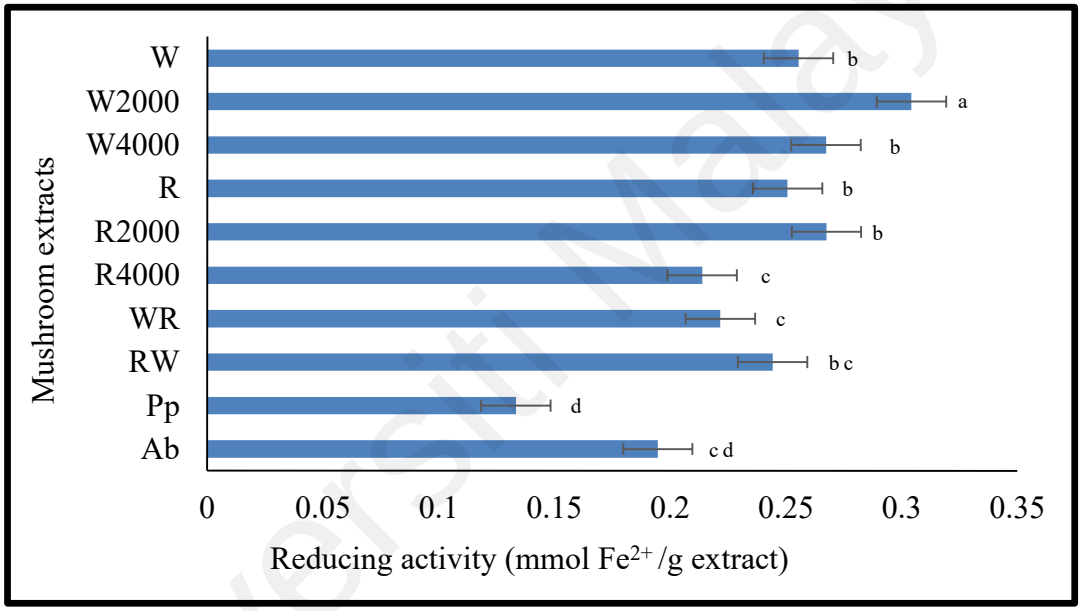

Figure 4.3: Ferric reducing activities of mushroom extracts

[a, b, c, d] means with different superscripts on bars differ significantly ($p < 0.05$). The extracts were tested at 5 mg/ml. The results were expressed as mean ± standard deviation (n = 3). W, R (natural strains); W2000, W4000, R2000, R4000 (strains obtained when the natural strains were subjected to different doses of gamma radiation); WR, RW (strains obtained from hybridization between the natural strains); Pp, *Pleurotus pulmonarius*; Ab, *Agaricus bisporus*.

4.3.4 Cupric ion-reducing antioxidant activity (CUPRAC)

The reducing activities of the extracts of *S. commune* strains, as determined by the CUPRAC assay, are presented in Figure 4.4. The cupric reducing activities of the extracts (0.10-0.13 mmol TE/g extract) were higher than that of *P. pulmonarius* (0.09 mmol TE/g extract) but comparable to that of *A. bisporus* (0.1 mmol TE/g extract). Both gamma irradiated strains (W2000 and R2000) displayed higher reducing activities than their respective natural strains (W and R). The reducing activities of the positive controls (mmol TE/g) including ascorbic acid (6.97), gallic acid (11.95), rutin (5.16) and BHT (0.56) were higher than all crude extracts.

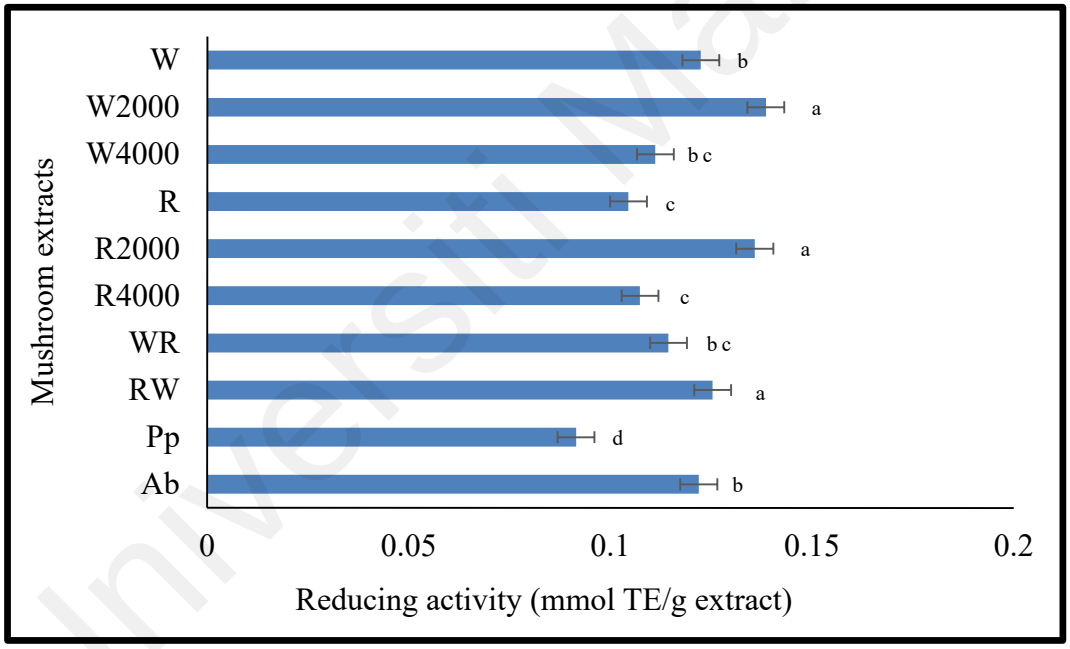

Figure 4.4: CUPRAC of mushroom extracts

[a, b, c, d] means with different superscripts on the bars differ significantly ($p < 0.05$). The extracts were tested at 5 mg/ml. The results were expressed as mean ± standard deviation (n = 3). W, R (natural strains); W2000, W4000, R2000, R4000 (strains obtained when the natural strains were subjected to different doses of gamma radiation); WR, RW (strains obtained from hybridization between the natural strains); Pp, *Pleurotus pulmonarius*; Ab, *Agaricus bisporus*.

4.3.5 Correlation analysis between antioxidant activities of *S. commune* crude extracts and their chemical compositions

Table 4.3 shows the correlation between the antioxidant activities of the extracts of *S. commune* and their chemical compositions. Total phenolic content showed a significant and moderate correlation ($r = 0.625$, $p < 0.01$) with CUPRAC assay. Total phenolic contents also showed moderate correlation ($r = 0.427$, $p < 0.05$) with ABTS assay. Total flavonoid content of the extracts showed significant and moderate correlation ($r = 0.535$, $p < 0.01$) with FRAP assay. Moderate correlation ($r = 0.420$, $p < 0.05$) was also observed between total flavonoid contents and ABTS assay. Furthermore, total sugar and total phenolic contents showed moderate correlation with ABTS and DPPH assays respectively but the correlations were negative.

Table 4.3: Correlation analysis of the antioxidant activities of the *S. commune* crude extracts and their chemical compositions

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	- 0.234	- 0.463*	- 0.099	- 0.109
Total protein content	- 0.346	0.313	0.105	0.498*
Total phenolic content	- 0.494*	0.427*	0.182	0.625**
Total flavonoid content	- 0.002	0.420*	0.535**	0.044

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

4.4 The yields of fractions of the selected *S. commune* strains

As the crude aqueous ethanol extracts of *S. commune* strains W, W2000 and W4000 consistently exhibited high antioxidant activities, the three strains were selected for further liquid-liquid fractionation to yield the hexane, ethyl acetate and water fractions. As shown in Table 4.4, the yields of the water fractions (61.85-67.54%) were the highest followed by the hexane (6.02-7.13%) and ethyl acetate (3.77-5.92%) fractions.

Table 4.4: The yields of different fractions derived from the selected *S. commune* strains

Mushroom strains	Yield (%)		
	Hexane fractions	Ethyl acetate fractions	Water fractions
W	7.07	3.77	61.85
W2000	7.13	5.02	67.54
W4000	6.02	5.92	63.65

W (natural strain), W2000 and W4000 (strains obtained when the natural strains were subjected to different doses of gamma radiation).

4.5 Chemical compositions of selected *S. commune* fractions

The total sugars, proteins, phenolics and flavonoids of the fractions derived from the crude extracts of selected *S. commune* by liquid-liquid fractionation are shown in Table 4.5.

4.5.1 Total sugar content

The total sugar contents of all fractions ranged from 3.92-33.50 mg glucose/g extract (Table 4.5). The water fractions for the strains W2000 and W4000 possess significant ($p < 0.05$) higher total sugar content about six times than the ethyl acetate and hexane fractions.

Table 4.5: Chemical composition of fractions derived from selected *S. commune* extracts

		Chemical compositions			
Samples	Fractions	Total sugar content (mg glucose/g extract)	Total protein content (mg protein/g extract)	Total phenolic content (mg GAE/g extract)	Total flavonoid content (mg RE/g extract)
W	Crude	8.63 ± 0.58^{bc}	114.39 ± 8.14^{c}	14.09 ± 0.22^{c}	2.58 ± 1.16^{c}
	Hexane	6.58 ± 0.14^{c}	63.33 ± 9.19^{d}	9.62 ± 0.49^{d}	21.31 ± 1.13^{a}
	Ethyl acetate	10.50 ± 0.32^{b}	253.93 ± 18.37^{b}	22.08 ± 0.54^{b}	16.33 ± 0.85^{b}
	Water	19.47 ± 0.65^{a}	360.30 ± 7.94^{a}	38.27 ± 0.14^{a}	1.48 ± 0.20^{d}
W2000	Crude	26.51 ± 0.17^{b}	478.93 ± 30.22^{a}	21.51 ± 1.19^{c}	2.29 ± 0.13^{c}
	Hexane	3.92 ± 0.07^{d}	68.18 ± 1.81^{d}	2.25 ± 0.05^{d}	9.01 ± 0.65^{b}
	Ethyl acetate	6.98 ± 0.49^{c}	285.15 ± 2.09^{c}	23.23 ± 0.59^{b}	36.55 ± 1.86^{a}
	Water	31.44 ± 0.49^{a}	356.36 ± 11.02^{b}	31.04 ± 1.00^{a}	1.59 ± 0.016^{d}
W4000	Crude	21.94 ± 0.61^{b}	375 ± 4.16^{a}	18.62 ± 0.55^{c}	1.63 ± 0.30^{c}
	Hexane	5.72 ± 0.40^{d}	14.84 ± 5.00^{d}	6.04 ± 0.31^{d}	27.33 ± 1.79^{a}
	Ethyl acetate	6.92 ± 0.21^{c}	295.45 ± 6.36^{b}	30.95 ± 0.69^{b}	11.73 ± 1.21^{b}
	Water	33.50 ± 0.38^{a}	334.54 ± 5.52^{c}	34.25 ± 0.28^{a}	1.78 ± 0.20^{c}

$^{a, b, c, d}$ means values with different superscripts within the same column differ significantly ($p < 0.05$) Results were expressed as mean ± standard deviation (n = 3). GAE: gallic acid equivalent; RE: rutin.

4.5.2 Total protein content

The total protein contents of the fractions are tabulated in Table 4.5 which ranged from 14.84 to 478.93 mg protein/g extract. The highest amount of the protein content was quantified in water fractions followed by the ethyl acetate and hexane fractions.

4.5.3 Total phenolic content

The total phenolic contents of the fractions ranged from 2.25 to 38.27 mg GAE/g extract (Table 4.5). The water fractions showed significantly higher total phenolic content than ethyl acetate and hexane fractions. Among the fractions, hexane fractions of the selected strains showed the lowest phenolic content. Notably, the water and ethyl acetate fractions showed higher phenolic content than their respective crude extracts.

4.5.4 Total flavonoid content

The total flavonoid contents of all the fractions were ranged from 1.48 to 36.55 mg RE/g extract (Table 4.5). The hexane fractions showed significantly higher total flavonoid content than the ethyl acetate and water fractions except for W2000 ethyl acetate fraction which showed the highest flavonoid content 36.6 mg RE/g extract among all fractions.

4.6 Antioxidant activities of selected *S. commune* fractions

The crude extracts and fractions (hexane, ethyl acetate, water) of *S. commune* strains W, W2000 and W4000 were subjected to the DPPH and ABTS radical scavenging assays as well as the FRAP and CUPRAC assays.

4.6.1 DPPH radicals scavenging activity

Figure 4.5 shows the antioxidant activities of *S. commune* extracts and fractions based on their abilities in scavenging the DPPH radicals. In this assay, all fractions (0.15-0.26 mmol TE/g extract) consistently demonstrated a comparable scavenging activity. The scavenging activities of the positive controls (mmol TE/g) including both ascorbic acid and gallic acid (0.40), rutin (0.38) and BHT (0.37).

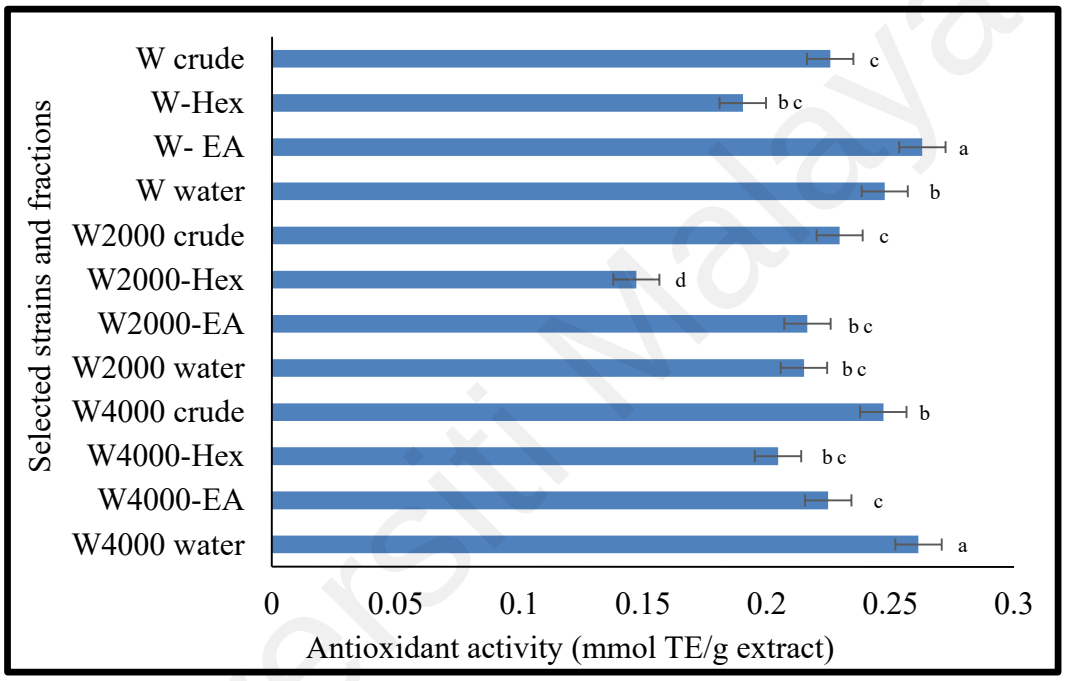

Figure 4.5: DPPH free radical scavenging activities of fractions derived from selected *S. commune* extracts

[a, b, c, d] means with different superscripts on bars differ significantly ($p < 0.05$). The extracts were tested at 10 mmol TE/g extract. The results were expressed as mean ± standard deviation (n = 3). Hex, hexane; EA, ethyl acetate.

4.6.2 ABTS radicals scavenging activity

Figure 4.6 shows the antioxidant activities of *S. commune* extracts and fractions based on their abilities in scavenging the ABTS radicals. The ethyl acetate fractions (0.07-0.09 mmol TE/g extract) displayed significantly higher antioxidant activity than the other fractions (0.03-0.05 mmol TE/g extract). The antioxidant activities of all the ethyl acetate fractions were approximately two-fold higher than that of their respective crude extracts. The scavenging activities of the positive controls (mmol TE/g) including ascorbic acid (3.02), gallic acid (3.04), rutin (1.47) and BHT (0.25) were higher than all fractions.

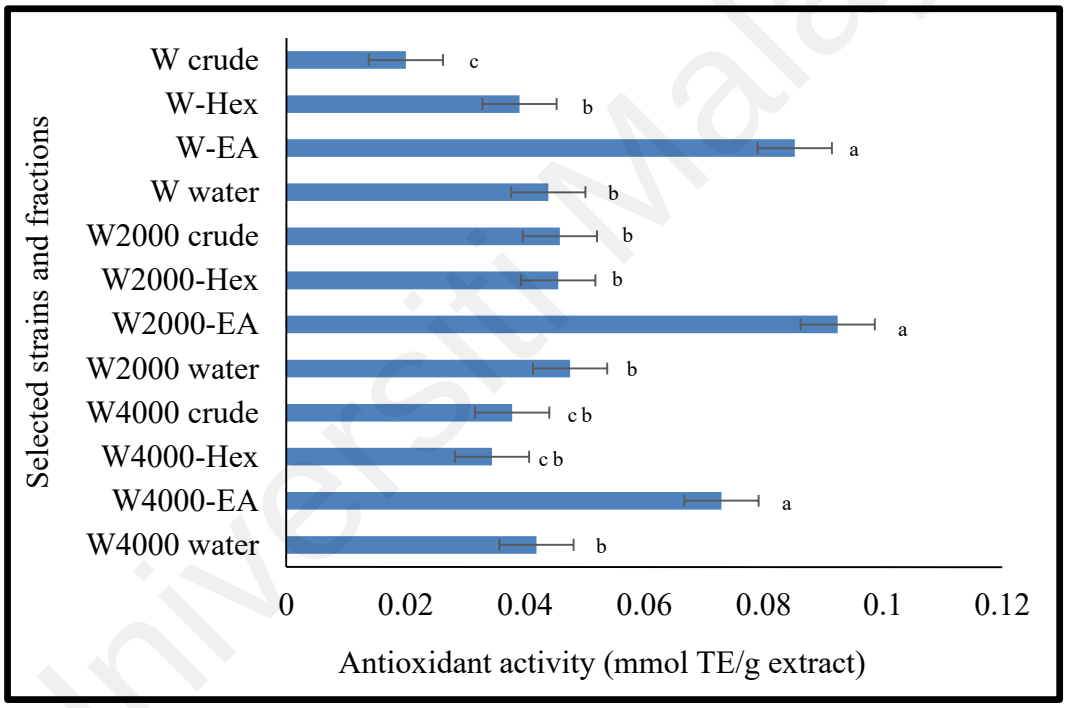

Figure 4.6: ABTS radicals scavenging activities of fractions derived from selected *S. commune* extracts

[a, b, c] means with different superscripts on bars differ significantly ($p < 0.05$). The extracts were tested at 10 mg/ml. The results were expressed as mean ± standard deviation (n = 3). Hex, hexane; EA, ethyl acetate.

4.6.3 Ferric reducing antioxidant power (FRAP)

Figure 4.7 shows the reducing activities of *S. commune* extracts and fractions based on the FRAP assay. For all strains, the fractions demonstrated higher reducing activity than the crude extracts. Amongst the fractions, the ethyl acetate fractions (0.35-0.40 mmol Fe^{+2}/g extract) displayed significantly higher reducing activity than water fractions (0.19 to 0.27 mmol Fe^{+2}/g extract) and hexane fractions (0.13-0.16 mmol Fe^{+2}/g extract). The positive controls (mmol Fe^{2+}/g) including ascorbic acid (7.94), gallic acid (7.73), rutin (7.11) were higher than all fractions but the activity of BHT (0.83) was comparable with some fractions.

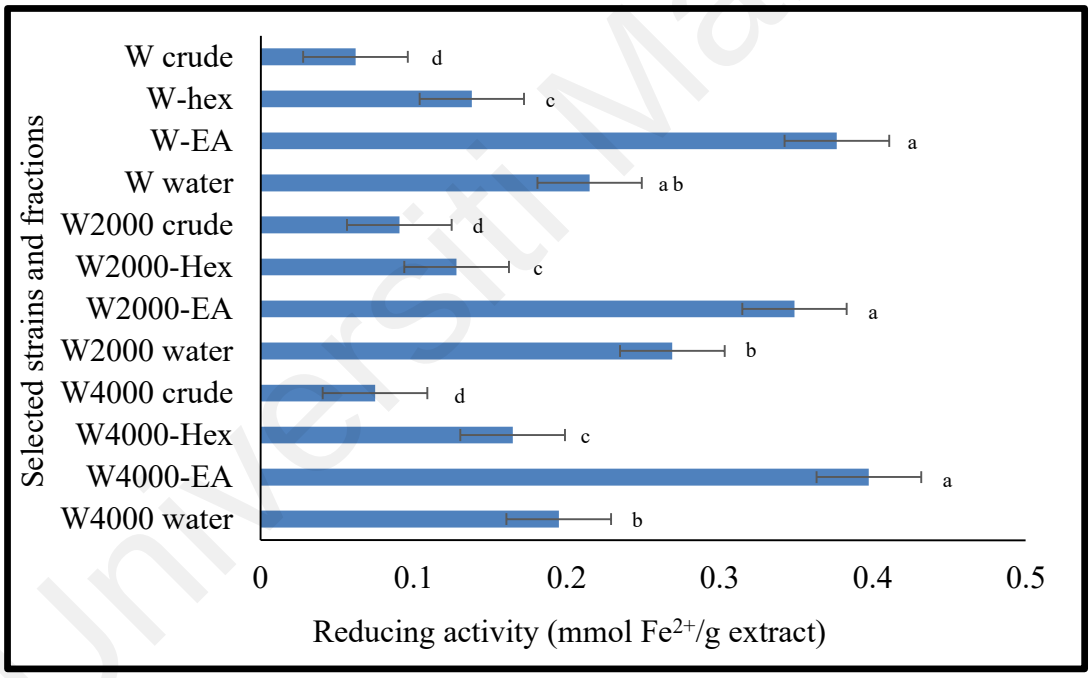

Figure 4.7: Ferric reducing activities of fractions derived from selected *S. commune* extracts

[a, b, c, d] means with different superscripts on bars differ significantly ($p < 0.05$). The extracts were tested at 5 mg/ml. The results were expressed as mean ± standard deviation (n = 3). Hex, hexane; EA, ethyl acetate.

4.6.4 Cupric ion-reducing antioxidant activities (CUPRAC)

Figure 4.8 shows the reducing activities of *S. commune* extracts and fractions based on the CUPRAC assay. Among the fractions of the selected strains, ethyl acetate fractions (0.09-0.16 mmol TE/g extract) showed higher reducing activities than water and hexane fractions. The reducing activities of the fractions were lower than the positive controls (mmol TE/g) including ascorbic acid (6.98), gallic acid (11.96), rutin (5.16) and BHT (0.56).

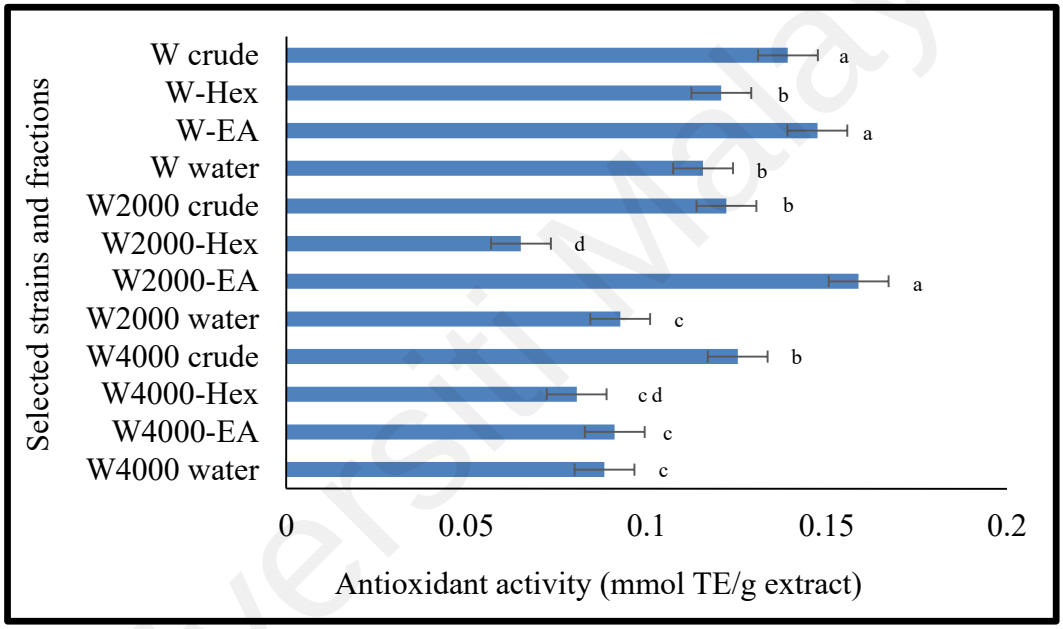

Figure 4.8: CUPRAC of fractions derived from selected *S. commune* extracts

[a, b, c, d] means with different superscripts on the bars differ significantly ($p < 0.05$). The extracts were tested at 5 mg/ml. The results were expressed as mean ± standard deviation (n = 3). Hex, hexane; EA, ethyl acetate.

4.6.5 Correlation analysis between antioxidant activities of selected *S. commune* strains' fractions and their chemical compositions

The Pearson correlation analysis between the antioxidant activities of the fractions (derived from the crude extracts of the selected strains W, W2000 and W4000) with their chemical compositions including total phenolic content, total flavonoid content, total sugar content and total protein content is shown in Table 4.6.

Total sugar contents showed moderate correlation ($r = 0.426$, $p < 0.05$) with DPPH. Total protein contents showed significant ($p < 0.01$) and moderate correlation with DPPH ($r = 0.499$) assay. Similarly, total phenol contents showed significant and positive moderate correlation with DPPH ($r = 0.642$) assay. Furthermore, significant and moderate correlation was observed between the total protein contents and FRAP ($r = 0.569$) assay. Total flavonoid content showed significant and moderate correlation with CUPRAC ($r = 0.494$) assay. Additionally, moderate correlation ($r = 0.420$, $p < 0.05$) was noted from total flavonoid contents and ABTS assay.

Table 4.6: Correlation analysis of the antioxidant activity of the selected *S. commune* strains' fractions and their chemical compositions

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	0.426*	- 0.283	- 0.039	- 0.151
Total protein content	0.499**	0.369	0.569**	0.289
Total phenolic content	0.642**	- 0.62	0.088	0.291
Total flavonoid content	- 0.104	0.420*	0.159	0.494**

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

CHAPTER 5: DISCUSSION

5.1 Extraction yields of *S. commune* crude extracts and fractions

Optimised extraction conditions such as temperature, particles size, time of extraction and extraction solvents are important to achieve high yield of antioxidants (Hijazi, 2015). Using aqueous ethanol instead of absolute ethanol as the extraction solvent facilitates to obtain different types of secondary metabolites and increase the yield since no single solvent is capable of extracting all compounds (Ferrari et al., 2012). Hence, a combination of water and ethanol may facilitate the extraction of a wide range of compounds that are soluble in water and/or organic solvent (Do et al., 2014). Several studies reported that aqueous ethanol is an efficient solvent for the extraction of polar antioxidants from mushrooms (Ferrari et al., 2012; Pumtes et al., 2016; Abd Razak et al., 2019; Shabir et al., 2011). The extraction yield and antioxidant activity of any extract depends on the extraction solvent (Marinova & Yanishlieva, 1997). In the present study, all eight strains of *S. commune* showed high yield (18.24-30.83%, w/w). As a comparison, the natural strain from Malaysia W (29.39%) showed higher yield than the natural strain from Thailand R (18.24%). On the other hand, higher yields were noted for strains R that have been subjected to gamma irradiation. Overall, the yields of *S. commune* strains were lower than *P. pulmonarius* (32.23%) and *A. bisporus* (52.78%). The yields obtained from the strains W, W4000 and R4000 are higher than those extracted using aqueous ethanol from *S. commune* (22.02%) (Abd Razak et al., 2019) and *Pleurotus flabellatus* (*23.52*%) (Pumtes et al., 2016). In addition, the overall yield obtained in the current study was higher than those yield obtained using absolute ethanol from eight edible Korean mushrooms that ranged from 3.9 to 19.6% (Choi et al., 2005), and methanol and ethanol extracts of *S. commune* (14%) (Chandrawanshi et al., 2017). This indicates that a large proportion of the metabolites in *S. commune* is polar in nature and aqueous alcohol is more efficient as an extraction solvent compared to alcohol alone.

To gain insights into the nature of compounds found in the crude aqueous ethanol extracts of *S. commune*, the extracts were subjected to liquid-liquid partition with solvents of increasing polarity to yield the fractions. Certain antioxidants tend to be more soluble in polar solvents such as water and moderate polar solvents such as ethyl acetate, while lipophilic compounds tend to be soluble in non-polar solvents such as hexane. Among the fractions of the selected strains (W, W2000 and W4000) the highest yield was obtained from the water fractions, followed by the hexane and ethyl acetate fractions. This trend was in agreement with the fractions yielded from *Pleurotus eryngii* and *Agrocybe aegerita* (Man, 2003), *Auricularia polytricha* (Teoh et al., 2018) and *Inonotus sanghuang* (Liu et al., 2017). This indicates that significant proportion of the compounds in the crude extracts of the selected strains (W, W2000 and W4000) was polar.

5.2 Chemical compositions and antioxidant activities of crude mushroom extracts

This study set out with the aim of assessing the chemical compositions in the *S. commune* strains. In the current study, strain W showed higher total protein and total phenolic contents than R strain, whereas strain R showed higher total sugar content than W strain. Overall, among the chemical compositions, total protein contents represent the highest compound followed by total sugar contents in both natural strains. This trend is similar to a previous study done on aqueous ethanol extract of *S. commune* (Basso et al., 2020).

The crude aqueous ethanol extract of *S. commune* can be a possible source of proteins and some amino acids. Paice et al. (1978) analysed amino acids in *S. commune*, whereby 18 amino acids were detected but the most abundant were glycine, serine, aspartic acid, threonine, tyrosine, alanine and glutamic acid. Studies also available on the amino acids in other mushrooms, for instance Chirinang & Intarapichet (2009) reported 18 amino acids in *P. ostreatus* and *P. sajor-caju* with the most abundant amino

acids in both mushrooms' species were glutamic acid, aspartic acid, and arginine. Bach et al. (2017) reported eight essential amino acids in nine edible mushrooms namely arginine, phenylalanine, histidine, isoleucine, lysine, methionine, threonine, tryptophan and valine.

In general, the total phenolic contents of all eight *S. commune* strains recorded in this study higher than total phenolic contents obtained from the methanol extract of *S. commune* (1.72 mg GAE/g extract) (Mirfat et al., 2010), aqueous ethanol extract of *S. commune* (9.49 mg GAE/g extract) (Abd Razak et al., 2019), ethanol extract of *Ramaria flava* (12.95 mg GAE/g extract) (Liu et al., 2013), aqueous ethanol extract of *Agaricus brasiliensis* (12.50 mg GAE/g extract) (Gan et al., 2013) and methanol extracts of six different species of mushrooms from Nepal (1.19-5.18 mg GAE/g extract) (Adhikari et al., 2019). On the other hand, the total phenolic contents ranged from 17.20 to 22.87 mg GAE/g extract and total flavonoid contents ranged of 2.36 to 6.85 mg RE/g extract in the samples of this study were higher than the total phenolic contents (0.01 to 0.07 mg GAE/g extract) and total flavonoid contents (0.02 to 0.13 mg QE/g extract) of ten Malaysian wild mushrooms collected from the forests around Selangor and Sabah (Azieana et al., 2017). Additionally, the flavonoid contents of samples in this study were higher than aqueous ethanol extract of *A. bisporus* (1.75 mg QE/g extract) (Gan et al., 2013) and *P. ostreatus* (0.069 mg QE/g extract) (Gonzalez-Palma et al., 2016). Generally, *S. commune* natural strains exhibited a moderate amount of total phenolic content but trace amount of total flavonoid content which is in agreement with the findings of Emsen et al. (2017) and Devi et al. (2014) on the same mushroom species.

Overall, the two natural strains of *S. commune*, collected from different geographical locations, showed obvious difference in the distribution of chemical compositions. Environmental factors can be the main reason for the variation in their chemical compositions (Li et al., 2019). The variation of chemical composition and

antioxidant activities of mushroom samples from different geographical locations were previously reported in another mushroom species, *Tuber indicum* (Li et al., 2019; Wu et al. 2020).

The gamma irradiation at two doses, 2000 and 4000 Gy, had different impacts on the chemical compositions of both natural strains. The sugar content in the natural strain W (31.8 mg glucose/g extract) was significantly lower than the gamma irradiated strain W2000 (73.9 mg glucose/g extract) whereas the total sugar content in natural strain R (109.7 mg glucose/g extract) was higher than that gamma irradiated strains R2000 and R4000 with 77.5 and 87.6 mg glucose/g extract, respectively. However, the total protein and total phenolic contents significantly decrease approximately to the half in the gamma irradiated strain W4000 when compared to the natural strain. In contrast, strains R2000 and R4000 showed higher total protein and total phenolic contents than natural strain R. Furthermore, gamma irradiated strains showed higher total flavonoid content compared to natural strain. The effects of gamma irradiation have been studied earlier by Weng et al. (2004) who reported the mutant strain of *Agaricus blazei* Murrill showed higher total protein content than its original strain. A possible explanation for this inconsistency in some chemical composition content might be that gamma radiation induced oxidative stress (Al-Rumaih & Al-Rumaih, 2008). The cells counteract ROS damage by altering the pattern of gene expression which led to modulation of certain metabolic pathways (Kiong et al., 2008). In an investigation into the effects of gamma radiation, Borzouei et al. (2010) reported an increase in proline content in gamma irradiated *Triticum aestivum* seedlings compared to non-irradiated seedlings.

Hybridisation may offer potentially large variation in concentration of chemical compositions. There was an increase in total sugar content in both hybrids when compared to the parental strain W. Likewise, there was an increase in total protein and total phenolic contents in both hybrids compared to the parental strain R. These results

are similar to previous findings on *Pleurotus djamor* (Oropeza et al., 2018) and *P. pulmonarius* (Ummu Humaira, 2017).

As a consequence of hybridisation, the chemical compositions exhibited in both hybrid strains were similar to the respective parental strains but the amounts of sugars, proteins, phenolics, and flavonoids varied. According to Orians (2000), there are several categories to describe the quantitative variation of the concertation of secondary metabolites of the hybrids relative to their parents. O-overexpression occurs when hybrids have higher chemical composition than in either parent. In this study both hybrids showed sugar content (49.49 and 79.93 mg glucose/g extract) higher than the parental strain W (31.78 mg glucose/g extract) while both hybrids showed protein and phenolic content higher than R. On the other hand, D-dominance expression occurs when hybrids not different in chemical composition from either parent when the parents show similar concentrations. That can be applied on both hybrid strains which the amount of flavonoid content (3.19 and 3.30 mg RE/g extract) in this study were similar to flavonoid content (3.25 and 3.77 mg RE/g extract) of both parental strains W and R respectively.

Generally, aqueous ethanol extracts of *S. commune* strains could be important sources of macronutrients, sugars, proteins, and phenolics since these chemical components were much higher than the two common edible mushrooms, *P. pulmonarius* and *A. bisporus*.

The various chemical constituents detected in *S. commune* strains may contribute to their antioxidant activities. According to the findings of this study, there was no significant difference in the radicals scavenging activities and reducing activities between the two natural strains W and R in spite of being originated from different locations. In general, the aqueous ethanol extracts of natural strains showed moderate scavenging activities and reducing activities. The radical scavenging activity (79.82-85.32%) of *S. commune* strains in this study (at 10 mg/ml) is higher compared to those of *A. brasiliensis*

85.44% and *A. bisporous* 82.39% at 25 mg/ml (Gan et al., 2013), but comparable to that of *A. blazei* (83.6% at 10 mg/ml) (Huang & Mau, 2006). On the other hand, *S. commune* strains showed higher antioxidant potential than the two commonly consumed mushrooms, *P. pulmonarius* and *A. bisporous*, analysed in this study. Previous studies have reported moderate scavenging and reducing activities of *S. commune* (Arbaayah & Umi, 2013; Chandrawanshi et al., 2017; Devi et al., 2014; Emsen et al., 2017; Mirfat et al., 2010; and Abd Razak et al., 2019).

As far as the effect of gamma irradiation in *S. commune* strains is concerned, there is no increase or decrease in radical scavenging and reducing activities were detected in all four gamma irradiated strains at both doses. These findings are not in agreement with previous studies on the effectiveness of the gamma irradiation in *Pleurotus osteratus* (San et al., 2019) and *Pleurotus florida* (Djajanegara, 2008). They observed that the gamma irradiated (750 Gy) *P. osteratus* and *P. florida* displayed higher scavenging activity than the non-irradiated control. The reason for this is not clear but it may be related with the dose of gamma irradiation and the response of individual mushroom species (Tsai et al., 2014).

Our results showed that hybridisation did not improve the scavenging activities or reducing activities in the hybrid strains. The trend of the antioxidant activities in this study do not agree with Ummu Humaira (2017) who reported that *P. pulmonarius* hybrids showed lower scavenging activities and reducing activities than the parental strain. Another study by Oropeza et al. (2018) reported that *P. djamor* hybrid showed higher antioxidant activities than its parental strain.

Overall, the *S. commune* strains mostly showed higher antioxidants activities than the two common edible mushrooms, *P. pulmonarius* and *A. bisporus*. Furthermore, the strains performed two times higher than the positive control BHT in ABTS radicals scavenging activities. Thus, due to the high levels of antioxidant activities the crude

extracts of *S. commune* strains, this mushroom might be another potential source for natural antioxidants.

The correlation coefficient was used to measure the degree or strength between the antioxidant activities of *S. commune* extracts and their chemical compositions. Overall, no correlation was found between any of the chemical compositions analysed in this study and DPPH radical scavenging activities. From Pearson correlation analysis, a moderate correlation ($p < 0.01$) was observed between TFC and FRAP assay. Phenolics showed a moderate correlation ($r = 0.625$, $p < 0.01$) with CUPRAC assay. This indicates TPC and TFC might play a role in the reducing activity with the ability to donate an electron to free radicals which becomes a cation radical (Vuolo et al., 2019). The increasing in phenolics and flavonoids will increase the reducing activity in CUPRAC and FRAP assays, respectively. Additionally, a moderate correlation ($p < 0.05$) was observed between phenolics ($r = 0.427$) and flavonoids ($r = 0.420$) with ABTS assay. In addition, the high level of total protein contents in the samples may have influenced the antioxidants activities since a moderate correlation ($p < 0.05$) was found between total protein contents and CUPRAC assay. The findings in this study were consistent with a previous study by Islam et al. (2016) who documented total phenolic contents and total flavonoid contents correlated with scavenging activities. Besides, Azieana et al. (2017) reported a moderate correlation between the total flavonoid contents and reducing activities.

5.3 Chemical compositions and antioxidant activities of fractions of the selected strains

In the current study, fractionation of the crude extracts of the selected strains, namely W, W2000 and W4000, was done by liquid-liquid partition with solvents of increasing polarity. When comparison is made between the fractions, our results indicated that there was a steady increase in total sugars, proteins and phenolic contents with the increase in solvents' polarity. Proteins represent the most abundant component in the ethyl acetate and water fractions of the three strains. Similarly, phenolics are the second major components in the ethyl acetate and water fractions. The levels of sugar, protein and phenolic contents in the hexane fractions were several times lower than ethyl acetate and water fractions. Phenolic acids are polar compounds so they tend to be soluble in polar and moderate polar solvents such as water and ethyl acetate (Cheung et al., 2003). The hexane fractions contained the highest flavonoid contents amongst the fractions and this may be due to the presence of some flavonoids with weak polarity and tend to be soluble in non-polar solvents (Marston & Hostettmann, 2006). Emsen et al. (2017) reported high flavonoid content in the hexane extract of *S. commune*. Also, previous studies have reported high flavonoid content in hexane extracts of different plant samples (Hazli et al., 2019; Nawaz et al., 2020). There are weak polar flavonoids such as genistin, genistein, and apigenin which might be presence in the hexane extracts (Xu et al., 2019).

Our results have demonstrated that the highest amounts of sugars, proteins and phenolics were observed in water fractions of all the selected strains. Fractionation of the crude extracts may have concentrated the various chemical components in the fractions such as TPC and TFC in the crude extracts which have been separated and then concentrated in the respective fractions. Hence, the values of TPC and TFC of the fractions were higher than the crude extracts. Similar observation has been reported by Teoh et al. (2018). Phenolic compounds are well-known secondary metabolites

recognized as excellent antioxidants because of their ability to scavenge free radicals by single electron transfer (Cheung et al., 2003). The water fractions of the selected strains showed total phenolic contents ranging from 31.04 to 38.27 mg GAE/g extract and these were higher than phenolic contents in the water fractions of *Auricularia polytricha* (15.10 mg GAE/g extract) (Teoh et al., 2018) and in *Agrocybe aegerita* (12.9 μg GAE/mg extract) (Man, 2003) but lower than that in *R. flava* (61.01 mg GAE/g extract) (Liu et al., 2013). The ethyl acetate fractions of all the selected strains showed total phenolic content ranging from 22.08 to 30.95 mg GAE/g extract; these are lower than phenolic contents in ethyl acetate fractions of *A. polytricha* (105.50 mg GAE/g extract) (Teoh et al., 2018) but higher than that in *R. flava* (14.52 mg GAE/g extract) (Liu et al., 2013) and *A. aegerita* (51.2 μg GAE/mg extract) (Man, 2003). Notably, the major total flavonoid contents observed in hexane fractions of W and W4000 (21.31 and 27.33 mg RE/g extract, respectively) were lower than total flavonoid contents in hexane fraction of *Cantharellus cibarius* (40.01 mg QE/g extract) (Ebrahimzadeh et al., 2015).

The antioxidant activities of the fractions of selected strains were analysed together with the crude extracts. The antioxidant activities are affected by factors like mushroom strain, solvents polarity and the assay mechanism. DPPH, ABTS, FRAP and CUPRAC were applied for comprehensive assessment of the antioxidant activities of the fractions obtained with solvents of different polarity.

Far too little attention has been paid to investigate the antioxidant activities of *S. commune* fractionated extracts. Previous studies on the antioxidant activities of *S. commune* focused mainly on the crude extracts except for the work of Mayakrishnan et al. (2013) who investigated the activities of the fractions of the methanol-chloroform crude extracts; however, the assays employed in their work are different from those in the present study.

Overall, the results indicated that all fractions of the selected strains had antioxidant activity both in terms of scavenging activities and reducing activities. The ethyl acetate and water fractions showed the highest antioxidant activities and similar trend was observed for all three strains. This suggests that both the semi-polar and polar compounds in *S. commune* are most likely responsible for the observed antioxidant activity. These findings are in agreement with many previous studies that reported that the ethyl acetate fractions showed high antioxidant activities in *R. flava* (Liu et al., 2013), *C. cibarius* (Ebrahimzadeh et al., 2015), *Lignosus rhinocerotis* (Nallathamby et al., 2016), *Ganoderma lucidum* (Utami et al., 2107) and *A. polytricha* (Teoh et al., 2018). Whereas the hexane fractions showed the weakest antioxidant activities despite the presence of high amount of flavonoids. This may due to the effect of the chemical structure of the compound on the antioxidant activities (Bendary et al.,2013). The number and positions of the hydroxyl group (-OH) may affect antioxidant activities and the presence of 3-OH in flavonoid compounds may contribute in the suppression of antioxidant activities (Wang et al., 2018).

Based on previous studies, Lu et al. (2010) fractionated ethanol crude extract of *Inonotus obliquus* to obtain petroleum ether and ethyl acetate fractions. Five compounds belong to the classes steroids and triterpenoids were isolated and identified from the ethyl acetate fraction namely lanosterol, 3*β*-hydroxy-lanosta-8,24-diene-21-al, 3*β*,22Rdihydroxy-lanosta-8,24-diene(inotodiol), ergosterol peroxide and 3*β*-hydroxy-lanosta-8,24-diene-21-acid (trametenolic acid). Inotodiol and trametenolic acid were found to have DPPH radicals scavenging activities. Kaur et al. (2019) who sequentially fractionated hydromethanol extract of *Ganoderma mediosenence*. Gallic acid was isolated from ethyl acetate fraction. It is well-known phenolic acid and it was responsible for DPPH radicals scavenging activities in their study. There is a possibility some or one

of the compounds isolated in the previous studies might present in the ethyl acetate fractions of *S. commune* and involved in antioxidant activities.

Overall, the contribution of various chemical components of *S. commune* to the scavenging and reducing activities in this study was proved by Pearson correlation analysis. Proteins ($r = 0.499$) and phenolics ($r = 0.642$) moderately correlated ($p < 0.01$) with DPPH assay, as well as proteins ($r = 0.569$, $p < 0.01$) showed a moderate correlation with FRAP assay while flavonoids ($r = 0.494$, $p < 0.01$) correlated moderately with CUPRAC assay. This indicates that the compounds in the fractions derived from the extracts have the potency of antioxidants to donate electrons or hydrogen atoms to deactivate radical species as well as, contribute to the reducing activities.

This study demonstrated that the crude aqueous ethanol extracts and fractions of selected strains possessed radical scavenging and reducing activities. However, each extract showed different trends in the assays, probably due to the different mechanisms such as SET-based assays measure the antioxidant's reducing capacity (Ozyurek et al., 2011). Overall, *S. commune* crude extracts and fractions of the selected strains showed DPPH radicals scavenging activities and FRAP reducing activities higher than ABTS radicals scavenging activities and CUPRAC reducing activities. As discussed above, there was a moderate correlation between the chemical compositions exhibit in both crude and fractions extracts and the assays ABTS, FRAP and CUPRAC. Notably, in the crude extracts there was no correlation between the chemical compositions and the DPPH radicals scavenging activities. There is a possibility that the crude extracts may either contain more non-phenolic compounds or possess phenolic compounds that contain a smaller number of active groups (Do et al., 2014). Since phenolic compounds are classified in a range of groups according to their structure. Such variations give them diverse characteristics and the antioxidant activities that is likely linked to the molecular structure of phenolic compound (Vuolo et al., 2019). DPPH assay showed a correlation

with total sugar, protein and phenolic content in the fractions. This may due to the fractions concentrated and presented active compounds that exhibited hydrogen-donating capacity to scavenge DPPH radicals as possible mechanism for their antioxidant activities (Vijai & Sriram, 2010). From the results above, it can be deduced that the determined chemical compositions present in the crude extracts and the fractions of the selected strains play a role in the antioxidant activities in different ways. Furthermore, the active antioxidant compounds in aqueous ethanol crude extracts were then concentrated in the ethyl acetate fractions.

CHAPTER 6: CONCLUSION

The present study investigated and compared the chemical compositions and antioxidant activities of aqueous ethanol extracts of natural, gamma-irradiated and hybrid strains of *S. commune*. The results of the present study showed that the chemical compositions vary among the aqueous ethanol extracts of *S. commune* strains. However, all the strains contain high amount of proteins and moderate amount of sugars and phenolics as well as trace amount of flavonoids. Our findings have shown that *S. commune* strains are a good source of antioxidants that may even be comparable to other commonly consumed mushrooms such as the *P. pulmonarius* and *A. bisporus*. This study extends our knowledge that the major antioxidants in the crude aqueous ethanol extracts are likely to be compounds of intermediate polarity. Phenolics and flavonoids are the potential compounds that may be responsible for the antioxidant activities of aqueous ethanol extracts of *S. commune* strains. This hypothesis is supported by a moderate correlation between phenolics and flavonoids contents and antioxidant activities.

The strains W, W2000 and W4000 were selected for liquid-liquid fractionation. Three fractions namely hexane, ethyl acetate and water fractions were obtained from the aqueous ethanol extracts of the selected strains. Generally, water and ethyl acetate fractions of the selected strains contained high amount of proteins and moderate amount of sugars and phenolics. It was also shown that hexane fractions contained the highest flavonoids among the fractions. The ethyl acetate fractions of the selected strains exhibited the highest antioxidant activities amongst all fractions and this might be attributed to the phenolics that are abundant in the ethyl acetate fractions.

Based on all the findings, aqueous ethanol extracts of *S. commune* strains and the ethyl acetate fractions of the selected strains showed antioxidant potential and can be considered as sources of protein, sugars and phenolics. Nevertheless, further chemical

profiling is required to identify and characterize the compounds in the ethyl acetate fractions of *S. commune* that are responsible for the observed antioxidant activity. Then, the variation in the levels of those bioactive compounds in different strains of *S. commune* can be compared in order to decide which strain has higher potential to be commercialized.

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