ANTIOXIDATIVE AND ANGIOTENSIN CONVERTING ENZYME INHIBITORY ACTIVITIES OF *Schizophyllum commune* MYCELIAL EXTRACT

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ANTIOXIDATIVE AND ANGIOTENSIN CONVERTING ENZYME INHIBITORY ACTIVITIES OF *Schizophyllum commune* MYCELIAL EXTRACT

ABSTRACT

Schizophyllum commune is an edible mushroom known as the split gill mushroom that possessed various nutritional and medicinal properties. In this study, *S. commune* mycelia biomass was cultivated in shake (SHFM) and static (STFM) flask culture conditions for 14 days in Glucose-yeast-malt-peptone media. Freeze dried mycelia biomass was then extracted by solvents, polysaccharide and protein extraction methods producing 11 extracts for each culture condition. Antioxidant assays of the extracts gave different effect on different assays, for SHFM, in Folin-Ciocalteu assay, cold water extract (CWE) showed highest phenolic content with 7.80±0.25 mg GAE/g extract. On the other hand, STFM protein fraction obtained by precipitation with 90% ammonium sulphate (F90) gave highest phenolic content with 15.04±0.39 mg GAE/g extract. In DPPH scavenging activity, CWE from both SHFM and STFM conditions gave highest scavenging activity (20.94±1.93% and 16.93±2.65%) and IC_{50} of 38.46 mg/ml and 17.24 mg/ml respectively. In cupric ion reduction antioxidant capacity (CUPRAC), protein fraction F90 of both SHFM and STFM conditions gave highest absorbance value of 0.420±0.00 and 0.064±0.00 at 450 nm respectively. CWE of SHFM condition gave highest percentage of metal chelating activity with 67.51±0.77% while PE of STFM scored 30.02±1.23%. For inhibition of lipid peroxidation assay, HWE from both culture conditions showed highest inhibition percentage with 26.40±0.57% and 20.07±0.78%. The LCMS/MS analysis of extracts with potent antioxidant activity of each assays showed that, CWE-SH contained compounds such as tryptophan, gluconic acid and phenolic acid, while in CWE-ST

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compounds such as 2(3,4-dihydroxyphenyl)-7-hydroxy-5-benzene propanoic acid, gluconic acid and quinic acid conjugate were present. HWE-SH contained compounds such as phenolic acid while HWE-ST contained compounds such as propanoic acid, gluconic acid, quinic acid conjugate, phenylvaleric acids and protocatechuic acid. Preliminary antihypertensive activity of *S. commune* using Angiotensin-Converting-Enzyme kit demonstrated that at 100 µg/ml, WRE-SH have the highest inhibition (58.20±1.81%) for SHFM, while PE-ST (56.67±1.79%) for STFM. LCMS analysis of WRE-SH exhibited the presence of compounds such as hydroxylated cinnamic acid, tryptophan, leucine and thiamine. Four extracts that showed high ACE inhibition activity (WRE-SH, CDME-ST, PE-ST and F90-ST) were selected for further separation using a ultracentrifugal filter unit with nomial molecular weight limit cut-off at 10 000 Da. Each >10 kDa and <10 kDa extracts produced were subjected to ACE inhibitory assay. Highest ACE inhibition at 50 µg/ml was demonstrated by F90-ST <10 kDa with 30.0% inhibition. This fraction was subjected to SDS PAGE analysis and resolved protein bands were processed for LCMS-QTOF protein analysis. Results revealed two putative antihypertensive proteins named carboxypeptidases and alpha/beta hydrolase proteins; and a few putative uncharacterized proteins that may have anti hypertensive property.

Keywords: *Schizophyllum commune*, mycelia biomass, antioxidant, ACE inhibitory activity, LCMS/MS, LCMS-QTOF, antihypertensive protein

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ANTIOKSIDA DAN AKTIVITI PERENCATAN ENZIM PENUKARAN ANGIOTENSIN DARI EKSTRAK MISELIA *Schizophyllum commune*

ABSTRAK

Schizophyllum commune merupakan cendawan yang boleh dimakan dan dikenali sebagai cendawan kukur atau sisir, ia memiliki pelbagai khasiat dan ciri-ciri perubatan. Dalam kajian ini, biomas miselia *S. commune* telah dikulturkan secara goncangan (SHFM) dan statik (STFM) selama 14 hari dalam media GYMP. Biojisim miselia kering-beku kemudiannya diekstrak dengan kaedah pengekstrakan pelarut-pelarut, pengekstrakan polisakarida dan pengekstrakan protein lalu menghasilkan 11 ekstrak untuk setiap jenis kultur. Ujian antioksida ekstrak memberi kesan yang berbeza pada jenis ujian yang dijalankan, untuk SHFM, dalam ujian Folin-Ciocalteu, ekstark air sejuk (CWE) menunjukkan kandungan fenolik tertinggi dengan 7.80±0.25 mg GAE/g ekstrak, manakala bagi STFM protein fraksi F90 memberikan kandungan fenolik tertinggi dengan 15.04±0.39 mg GAE/g ekstrak. Dalam aktiviti percapahan radikal DPPH, CWE dari kedua-dua SHFM dan STFM memberikan aktiviti tertinggi (20.94 ± 1.93% dan 16.93 ± 2.65%) dan nilai IC_{50} 38.46 mg/ml dan 17.24 mg/ml masing-masing. Dalam ujian antioksida pengurangan ion kuprik (CUPRAC), pecahan protein F90 dari kedua-dua SHFM dan STFM memberi nilai serapan tertinggi 0.420±0.00 dan 0.064±0.00 pada 450 nm masing-masing. CWE dari SHFM memberikan peratusan tertinggi dalam aktiviti pengkelat logam dengan 67.51±0.77% manakala ekstrak polisakarida (PE) dari STFM dengan 30.02±1.23%. Untuk ujian menghalang pengoksidaan lipid, ekstrak air panas (HWE) dari kedua-dua kultur menunjukkan peratusan perencatan tertinggi dengan 26.40±0.57% dan 20.07±0.78%. Analisa LCMS/MS bagi ekstrak dengan aktiviti antioksida tertinggi bagi setiap ujian menunjukkan bahawa, CWE-SH mengandungi sebatian seperti triptofan, asid

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glukonik dan asid fenolik, manakala CWE-ST mengandungi sebatian seperti 2(3,4-dihidroksifenil) asid propanoik-7-hydroksi-5-benzena, asid glukonik dan konjugat asid quinik. HWE-SH pula mengandungi sebatian seperti asid fenolik manakala HWE-ST mengandungi sebatian seperti asid propanoik, asid glukonik, asid konjugat quinik, asid ferilvalerik dan asid protokatekuik. Ujian aktiviti antihipertensi awal *S. commune* menggunakan kit Angiotensin-Converting-Enzyme menunjukkan bahawa pada 100 µg/ml, WRE-SH mempunyai perencatan yang paling tinggi (58.20±1.81%) untuk SHFM, manakala PE-ST (56.67 ± 1.79%) bagi STFM. Analisis LCMS WRE-SH menunjukkan kehadiran sebatian seperti asid sinnamik terhidroksi, triptofana, leusina dan tiamina. Empat ekstrak yang menunjukkan aktiviti perencatan ACE tinggi (WRE-SH, CDME-ST, PE-ST dan F90-ST) telah dipilih untuk proses pengasingan lanjut menggunakan unit penapis ultrasentrifugal dengan had berat nomial molekul 10 000 Da. Setiap ekstrak >10 kDa dan <10 kDa yang terhasil diuji dengan perencatan ACE. Perencatan ACE tertinggi pada 50 µg/ml ditunjukkan oleh F90-ST <10 kDa dengan 30.0% perencatan. Fraksi ini kemudian dianalisa dengan SDS PAGE dan jalur protein yang dihasilkan telah diproses untuk analisis protein menggunakan LCMS-QTOF. Keputusan mendapati dua protein berpontensi sebagai anti-hipertensi iaitu protein karbosipeptidasi dan alpha/beta hydrolase; dan juga beberapa protein berpotensi lain yang mungkin mempunyai fungsi antihipertensi.

Kata kunci: *Schizophyllum commune*, biojisim miselia, antioksida, perencatan enzim penukaran angiotensin, LCMS/MS, LCMS-QTOF, protein anti-hipertensi

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Noor Hasni Mohd Fadzil

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbols/ Abbreviation	Definition
%	Percentage
<	Less than
>	More than
±	Plus minus
&	And
˚C	Degree Celsius
α	Alpha
ACE	Angiotensin-I Converting Enzyme
CAN	Acetonitrile
β	Beta
BCA	Bicinchoninic Acid
CMDE	Crude methanol-dichloromethane extract
CUPRAC	Cupric-ion reducing antioxidant capacity
CWE	Cold water extract
Da	Dalton atomic mass unit
DCM	Dichloromethane
DCME	Dichloromethane extract
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DPPH	1,1-Diphenyl-2-Picrylhydrazyl radicals
EA	Ethyl Acetate
EAE	Ethyl Acetate Extract
et al.	And others
F30	Protein fraction at 30% ammonium sulphate salt concentration
F60	Protein fraction at 60% ammonium sulphate salt concentration
F90	Protein fraction at 90% ammonium sulphate salt concentration
g	Gram
GAE	Gallic acid equivalence
h	Hour
HE	Hexane extract
HWE	Hot water extract

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IC_{50}	Concentration required to inhibit 50% of activity
kDa	Kilo Dalton
L	Litre
LCMS	Liquid Chromatography Mass Spectrometry
LCMS-QTOF	Liquid Chromatography Mass Spectrometry of Quadrupole Time of Flight
M	Molar
MeOH	Methanol
mM	Millimolar
mg	Milligram
min	Minute
Milli-Q	Ultrapure water
ml	Millilitre
Mm	Millimetre
mmHg	Millimetres of mercury
m/z	Mass to charge ratio
NA	Not available
nm	Nanometre
NMWL	Nomial Molecular Weight Limit
No.	Number
p	Probability
PE	Polysaccharide extract
RAAS	Renin Angiotensin Aldosterone System
rpm	Rotation per minute
S	Second
SD	Standard deviation
SDS PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SH	Shake flask
SHFM	Shake flask mycelia biomass
sp	Species
ST	Static flask
STFM	Static flask mycelia biomass
TFA	Trifluoroacetic acid
µg	Microgram
µl	Microlitre
V	Voltage

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v/v	Volume over volume
w/v	Weight over volume
w/w	Weight over weight
WHO	World Health Organization
WRE	Water residue extract

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CHAPTER 1:INTRODUCTION

Cardiovascular disease (CVD) is one of the leading causes of mortality in many countries. An estimated 17.7 million people died from CVDs in 2015, representing 31% of all global deaths (World Health Organization, 2017). In Malaysia, chronic diseases accounted for 71% of all deaths in 2002, where 30% of it comes from CVD alone (Ramli & Taher, 2008). Seven out of 10 Malaysian adults have the risk factors of CVD or heart diseases such as hypertension, diabetes, obesity, high cholesterol and also atherosclerosis.

Urbanization, modern lifestyle, unhealthy diet, tobacco, alcohol use, and increased psychological stress are known to be associated with CVD (Mittal & Singh, 2010). In addition, less exercise, excess body weight and sedentary occupations may be important risk factors for hypertension, while high cholesterol and atherosclerosis can further complicate CVD (Rampal *et al.*, 2008).

Hypertension or high blood pressure can develop in anyone regardless of race, age or gender and often give no warning sign or symptoms beforehand. Hypertension generally means high pressure or tension in the arteries and can increase the risk of developing cardiac disease, renal disease, arteriosclerosis and stroke. The vasoconstriction of the blood vessels contributes to hypertension, where an enzyme known as Angiotensin I-converting enzyme (ACE) regulate the dilation and constriction of blood vessels.

Recently, there are increasing evidence suggesting that oxidative stress is also involved in the pathogenesis of many cardiovascular diseases, including hypercholesterolemia, atherosclerosis, hypertension, diabetes, and heart failure. Reactive oxygen species (ROS) has been implicated in cell damage, necrosis and cell

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apoptosis due to its direct oxidizing effects on macromolecules such as lipids, proteins and DNA. Therefore, a balance of endothelium-derived vasodilators, especially ROS are important in modulating endothelial function (Higashi *et al.*, 2009).

Mushrooms are nutritional and it contributes to human health as functional food. Mushrooms contain fairly high protein which encompasses almost all the amino acids essential to human nutrition, low in calories, chitin, iron, zinc, fibre, vitamins and minerals. Bioactive compounds of edible mushrooms have been studied extensively for their potential use in the treatment of hypertension, diabetes, inflammation, hypercholesterolemia, high oxidative stress and other ailments.

Schizophyllum commune Fr. is a widely distributed edible and medicinal mushroom. This mushroom usually grows abundantly during rainy season and can be found on dead logs. *Schizophyllum commune* has been reported to contain good amount of proteins, vitamins and minerals (Tripathi & Tiwary, 2013). Furthermore, the polysaccharide of this mushroom known as schizophyllan possessed antitumor, anticancer and immunomodulating activities (Smith *et al.*, 2002). Therefore, further study on this species would be useful to explore its potential use as agent in the prevention and treatment of CVD. Hence, the objectives of the present study are:

1. To prepare the solvent, protein and polysaccharide extracts from the mycelial biomass of *S. commune* grown by liquid fermentation.
2. To determine the *in vitro* antioxidative activity of the extracts and chemical profiles of active extract(s).
3. To determine the *in vitro* anti-ACE activities of the extracts and chemical profiles of active extract(s).
4. To isolate and identify the potential anti-ACE compound(s) in the potent extract(s).

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CHAPTER 2:LITERATURE REVIEW

2.1 Risk factors of cardiovascular disease (CVD)

Cardiovascular disease (CVD) is a disorder that affects the heart and blood vessels, characterized by hypertension, congestive heart failure, acute myocardial infarction, stroke and a few more associated causes. As the number one cause of mortality globally, it is estimated that 17.7 million people died from CVDs in 2015, representing 31% of all global deaths (World Health Organization, 2017). According to World Health Organization, 3.6 million death was recorded due to CVD in South-east Asia (World Health Organization, 2011). In Malaysia, CVD was accounted for 36% of total death and is the leading cause of death in the country (World Health Organization, 2014).

The common risk factors for CVD can be categorized into two groups; risk factors that can be controlled, treated or modified, such as high blood pressure, obesity, cholesterol level, tobacco use, lack of physical activity and diabetes. On the other hand, risk factors that cannot be controlled include age, gender and family history. The risk of CVD increases with age where men are more likely to develop CVD at earlier age compared to women, while a family's history of CVD signifies a person's risk (McCusker *et al.*, 2004).

High blood pressure or hypertension is the most important risk factor. If the blood pressure is too high, it can damage the artery walls hence increases the risk of developing a blood clot. Additionally, smoking can also damage and narrow the coronary arteries as tobacco contain toxins that increase the stickiness of blood and reduces the amount of oxygen in the blood that leads to coronary heart disease, therefore, tobacco cessation is important to prevent complication of the artery damage

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(Bath *et al.*, 2009). Similarly, high cholesterol level in blood can cause the arteries to narrow while increasing the risk of developing blood clot. Diabetes on the other hand is a condition where the blood sugar level become too high which can damage the artery wall and more likely to develop fatty deposits or atheroma (Nicholls *et al.*, 2008).

Some of the risks are interlinked for instance obese people can have higher systolic blood pressure and insulin levels (Goldman & Hatch, 2000). Moreover, having multiple risk factors can speed up the disease progression and finally develops into a new condition, for example, combination of hypertension, diabetes and high cholesterol level in blood can accelerates atherogenesis leading to blockage of artery eventually resulting with heart attack and stroke (Peplow & Adams Jr, 2015).

The most popular prevention method of CVD is to lower the blood pressure and cholesterol by alteration of lifestyle to reduce the risks. Low fat diet and regular exercise can control blood cholesterol levels thus also help patient to lose some weight. Furthermore, healthy lifestyle is able to reduce stress in most individuals. Modest alcohol intake and tobacco use can improve CVD risk, thus, blood pressure levels will be reduced and can lower the risks of stroke and congestive heart failure (Mukamal, 2006).

In addition to controlling the risk factors that can be managed, drug therapy is the other course of action. Pharmacological agents and antithrombotic drugs have been tremendously developed resulting in different variety and scope of treatment and prevention of CVD. Nevertheless, the role of dietary factors, herbal medicines and natural products with possibility of their use in CVD's treatment has gained a lot of attention. The use of antioxidant vitamins and the antioxidant properties of herbal materials and foods may improve common cardiovascular risk factors and may have antithrombotic effects (Walden & Tomlinson, 2011).

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2.2 Hypertension

Hypertension is a major public health problem and is also a leading cause of death in developing countries. One-quarter of the world's adult population has hypertension, and likely to increase in the coming years (Chockalingam *et al.*, 2006). The prevalence of hypertension is relatively high in Malaysia, the latest statistical data pins the figure at 40.5% amongst respondents aged 30 years old and above in 2004 (Rampal *et al.*, 2008). Lack of awareness, improper treatment and poor control of hypertension has left CVD to remain as the leading cause of death in Malaysia.

Hypertension or high blood pressure happens when the force of the blood pumping through the arteries is too strong. Hypertension occurs when the measurement value of blood pressure are above normal reading which are below 140/90 millimetres of mercury (mm Hg) (Carretero & Oparil, 2000). Increase in blood pressure will increase the risk of heart attack, heart failure, stroke and kidney disease. Moreover, the presence of additional risk factors such as smoking, diet rich in salt and saturated fat, high cholesterol levels and diabetes increase the CVD risk from hypertension.

Hypertension usually occurs unnoticed, it can quietly damage the arteries for years before symptoms develop. If the condition is extremely high, symptoms like dizziness and trouble seeing can be experienced. Uncontrolled high blood pressure gradually can cause variety of problems, including aneurysm, angina, heart attack, heart failure, kidney failure, eye damage and stroke. From the study conducted to determine the prevalence, awareness, treatment and control of hypertension in Malaysia, Rampal *et al.* (2008) reported that only 34.6% of the subjects were aware that they had hypertension with female being more aware (40.6%) than the male subjects (29.4%). Awareness among the patient is important in achieving controlled blood pressure thus reduced the risk of CVD.

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Hypertension prevention is important to reduce the high frequency of its occurrences, changing the unhealthy life style which is the major modifiable risk factor can hinder cardiovascular and kidney diseases (Kearney *et al.*, 2005). Primary prevention of hypertension is needed; focusing also on the inter-related risks such as cholesterol level, tobacco use, high body mass index, physical inactivity, poor diet and diabetes. Population-based strategies can be implemented in order to modify the social norm towards increasing the healthy behaviour, targeting risks via legislation, tax, financial incentives, health-promotion campaigns or engineering solutions (World Health Organization, 2009).

2.2.1 Hypertension treatment and pharmacology

Reducing or eliminating the modifiable risks described previously could reduce more deaths by three quarters or more of leading diseases such as ischaemic heart disease (World Health Organization, 2009). According to Wang and Vasan (2005), antihypertensive therapy can reduce the risk of stroke by approximately 30%, coronary heart disease by 10 to 20%, congestive heart failure by 40 to 50%, and total mortality by 10%. Nevertheless, drug therapy may also be an important approach to treat patient with hypertension.

Hypertension medication may work differently based on their modes of action. For some cases, combination of drugs is needed in order to keep the blood pressure under control. Among the classes of antihypertension medications include, diuretics, beta-blockers, angiotensin converting enzyme inhibitors (ACE), angiotensin II receptor blockers, calcium channel blockers, alpha blockers, alpha-2 receptor agonist, central agonists, peripheral adrenergic inhibitors and vasodilators. The classes of antihypertensive medications, mode of actions, example of the drugs used and possible side-effects or symptoms are tabulated in Table 2.1.

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The antihypertensive drug prescribed should be selected based on the greatest hypotensive effect and suited for various accompanying condition for each hypertensive patient, rather than the class of antihypertensive drug basis (Japanese Society of Hypertension, 2014). Condition-matched antihypertensive drugs should be selected corresponding to the accompanying condition such as diabetes and post myocardial infarction. This is in agreement with each class of the drug, there are compelling indications, contraindications and conditions which require careful drug usage.

Table 2.1: Antihypertensive medications. Adapted from American Heart Association (2015).

Anti-hypertension medication class	Mode of action	Example drugs	Possible side-effects/ symptoms
Diuretics	Help the kidneys get rid of excess water and sodium. This can reduces the volume of blood that needs to pass through the blood vesssels thus bringing the blood pressure down.	• Thiazide diuretics • Potassium-sparing diuretics • Loop diuretics • Combination diuretics	• Some of the drugs may decrease body's supply of potassium • Diabetic patient may have increase in blood sugar level
Angiotensin-converting enzyme (ACE) inhibitors	ACE inhibitors inhibit the formation of angiotensin II that causes the blood vessels to narrow. Blood pressure will decrease by the expanding blood vessels.	• Benazepril hydrochloride • Captopril • Enalapril maleate • Fosinopril sodium • Lisinopril	• Skin rash • Loss of taste • Chronic dry, hacking cough • Dangerous for pregnant woman
Angiotensin II reseptor blockers	This drug blocks the effect of angiotensin that causes the arteries to become narrow by blocking the receptors and reduce the blood pressure.	• Candesartan • Eprosartan mesylate • Irbesarten • Losartan potassium • Telmisartan	• Dizziness • Dangerous for pregnant woman

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Table 2.1, continued,

Calcium channel blockers	Keep calcium from entering the smooth muscle cells of the heart and blood vessels thus make the heart beat less forcefully and helps blood vessels to relax.	• Amlodipine besylate • Felodipine • Isradipine • Verapamil hydrochloride	• Palpitations • Swollen ankles • Constipation • Headache • Dizziness
Alpha blockers	Block the binding of hormone that constrict blood to the alpha reseptors so blood can flow more freely and blood pressure falls.	• Doxazosin mesylate • Prazosin hydrochloride • Terazosin hydrochloride	• Fast heart rate • Dizziness • Drop in blood pressure when stand up
Alpha-2 receptor agonist	Reduce the sympathetic nervous system activity which decreases blood pressure. First choice treatment during pregnancy.	• Methyldopa	• Drowsiness • Dizziness
Central agonists	Keep the brain from sending signals to the nervous system that would speed up heart rate and tighten blood vessels.	• Alpha methyldopa • Clonidine hydrochloride	• Drop in blood pressure when stand up • Drowsiness • Constipation
Peripheral adrenergic inhibitors	Block neurotransmitters in the brain thus block the smooth muscles from getting the message to constrict. Used only if other medications aren't effective	• Guanadrel • Guanethidine monosulfate • Reserpine	• Insomnia • Diarrhea • Heartburn • Drop in blood pressure when stand up
Vasodilators	Relax the muscle in the blood vessels especially arterioles thus widen vessel and allows blood to flow through better	• Hydralazine hydrocholoride • Minoxidil	• Headaches • Swelling around the eyes • Heart palpitations • Aches and pains in the joints

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2.2.2 Renin-angiotensin-aldosterone system (RAAS)

The blood pressure is controlled by the biochemical pathways interaction and predomination in a human body (Hong *et al.*, 2008). Hypertension typically occurs because of an increase of either excess of arteriolar vasoconstriction mostly related to excessive activation of the sympathetic nervous system and/or the renin-angiotensin-aldosterone system (RAAS). The RAAS regulate the blood volume and systemic vascular resistance cooperating in regulating the cardiac output and arterial pressure in addition to the regulation of fluid and electrolyte balance (Atlas, 2007).

The RAAS components are made of 1) renin, 2) angiotensin and 3) aldosterone. The juxtaglomerular apparatus in kidneys release renin into the circulation which is formed by a proteolytic cleavage of the renin precursor or proenzyme. Renin secretion is stimulated by a fall in perfusion pressure or in sodium chloride delivery and by an increase in symphatetic activity. This will then stimulate the formation of angiotensin in blood and tissues, which in turn stimulates the release of aldosterone from the adrenal cortex (Klabunde, 2011).

Renin functioned as an enzyme on a protein substrate known as the angiotensinogen to produce a peptide that mediates the vasopressor effect of renin known as the angiotensin (Li, 2015). After being release into blood, renin will stimulate the proteolytic cleavage of N-terminal portion of a large molecular weight globulin, angiotensinogen, to form a decapeptide angiotensin I (Figure 2.1 and Figure 2.2) (Streatfeild-James *et al.*, 1998). The majority of the circulating angiotensinogen is derived from the liver while low but detectable levels are found in the kidney, adrenal, lung, large intestine, stomach and spleen (Griendling *et al.*, 1993).

Angiotensin I produced from the angiotensinogen cleavage has very little activity, however it serves as an important precursor in the RAAS. The angiotensin I is then cleaved further by angiotensin converting enzyme (ACE) that is found on the

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vascular endothelium particularly in the lung to form the octapeptide angiotensin II. The cleavage occurred through removal of two C-terminal residues histidine (His) and leucine (Leu).

Angiotensin II is a potent vasoconstrictor thereby increasing systemic vascular resistance and arterial pressure. Angiotensin II also can increase the heart workload if it's profusion is high thus causing complications to the CVD. Moreover, angiotensin II stimulates the adrenal cortex to release aldosterone and sodium transport at renal tubular sites which regulate the sodium and water retention (Klabunde, 2011). Having more fluid in the body in a restricted space will cause the blood pressure to rise. Another role played by angiotensin II is it act as substrate for enzyme aminopeptidases that produce angiotensin III and angiotensin IV (Rang *et al.*, 2014).

2.2.3 Angiotensin-I converting enzyme (ACE)

Angiotensin converting enzyme is a membrane-bound enzyme found on the surface of endothelial cells particularly in the lung, renal proximal tubular epithelium, ciliated intestinal epithelium and also smaller germinal form found in testis (Bernstein *et al.*, 2013; Fleming, 2006). ACE and its activity were discovered in the mid-1950s by Skeggs and his colleagues where at that time, it is known as hypertension-converting enzyme (Skeggs *et al.*, 1954). The discovery has led to investigation of its amino acid sequence, its laboratory synthesis, and also facilitates the understanding the pharmacology and physiology of the RAAS.

ACE is a dipeptidyl carboxypeptidase that converts angiotensin I to angiotensin II and inactivates the vasodilator bradykinin and kallidin in the kallikrein-kinin system (Guang *et al.*, 2012). The RAAS pathway contributes to the pathogenesis of heart failure, thus therapeutic manipulation of this pathway is very important in

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treating hypertension and heart failure for example, ACE inhibitor, angiotensin II receptor blockers and aldosterone receptor blockers (Klabunde, 2011).

Besides catalyzing the formation of angiotensin II, ACE also catalyzes the degradation of bradykinin and several other peptides. Kininases II, the enzyme that inactivate kinins is identical to ACE, thus kininase II inactivates a vasodilator and activates a vasoconstrictor (Rang *et al.*, 2011). Inhibition of ACE will help bradykinin functions normally in promoting vasodilation by stimulating the production of arachidonic acid metabolites, nitric oxide and endothelium-derived hyperpolarizing factor in vascular endothelium, while in specific tissues or organ such as in the uterine, it causes the smooth muscle contraction and also increased the vascular permeability (Hornig *et al.*, 1997). In short, ACE regulates the balance of vasodilatory of bradykinin and vasoconstrictive property of angiotensin II (Brown & Vaughan, 1998).

ACE and its peptide substrate and products in RAAS affect many physiologic processes other than blood pressure control such as hematopoiesis: process of creating new blood cells, reproduction, renal development, renal function, and also immune response (Bernstein *et al.*, 2013). ACE has also been investigated for its importance in fertilization processes, where the influence of ACE and angiotensins on sperm functions and the sperm-egg interaction were studied (Kohn *et al.*, 1998).

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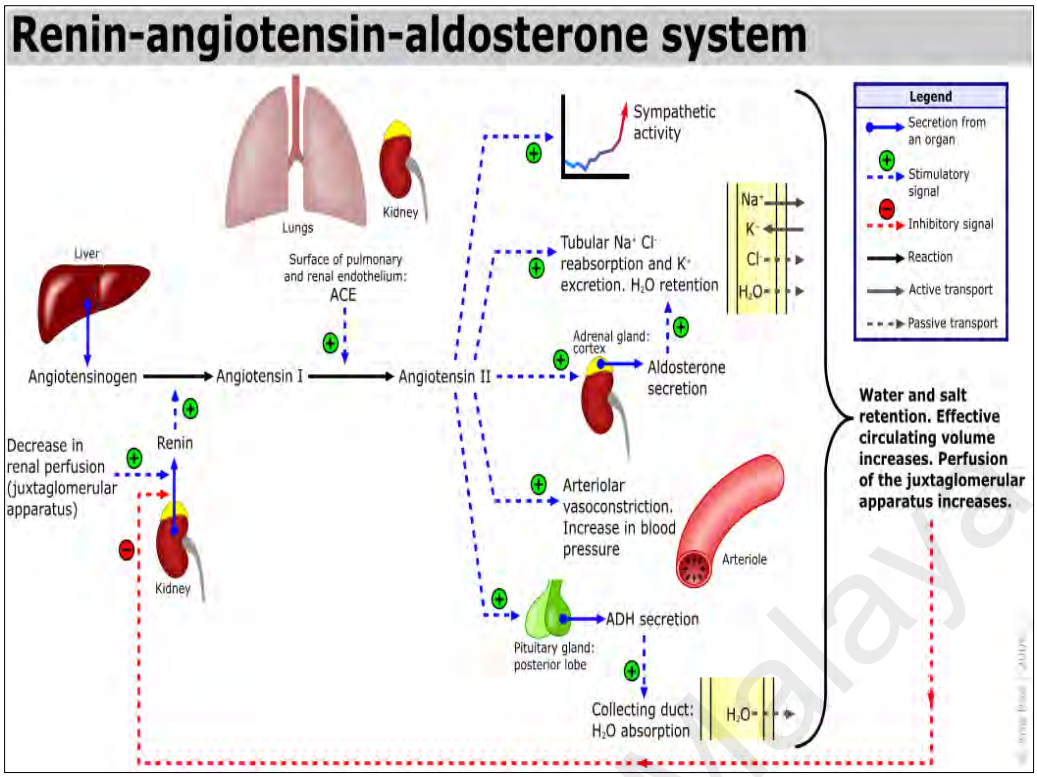

Figure 2.1: RAAS System. (Wikipedia, https://en.wikipedia.org/wiki/Renin-angiotensin_system)

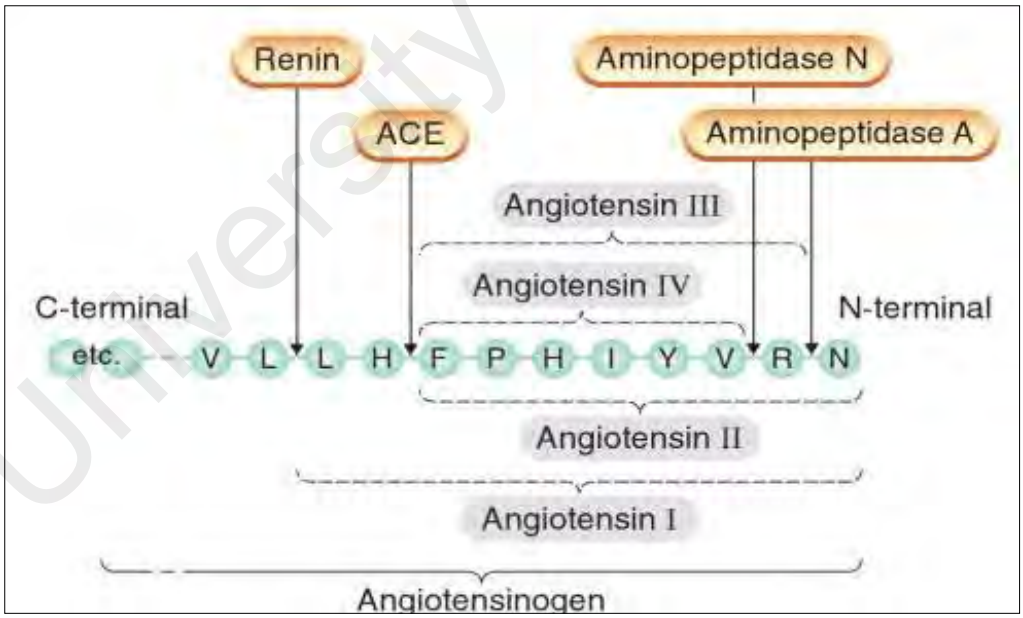

Figure 2.2: Formation of angiotensin I-IV from the N-terminal of the precursor protein angiotensinogen (Rang *et al.*, 2014).

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2.2.4 Angiotensin-I converting enzyme (ACE) inhibitors

In order to prevent conversion of angiotensin I to angiotensin II which is the potent vasoconstrictor, the converting enzyme has to be inhibited. ACE inhibitors have the ability to suppress the enzymatic conversion and inhibit the degradation of bradykinin thus giving the blood pressure lowering effect. Through this, blood vessels will be more relaxed and widened, making it easier for blood to flow. Furthermore, it also lowers the body water retention thus lowering the blood pressure.

ACE inhibitor was first isolated from snake venom in 1960s where its peptides were found to inhibit kinase II, an enzyme that facilitates degradation of bradykinin. Ever since the discovery, synthetic analogues of the venom peptide were developed and orally effective drug was produced (Bryan, 2009). The first ACE inhibitor, captopril or D-3-mercapto-2-methylpropanoyl-L-proline was developed in 1977 while enalapril followed in 1980 (Strube & Strube, 1992). The clinical effects of ACE inhibitors were then investigated involving a large number of patients to measure its ability in lowering blood pressure.

ACE inhibitors not only provide the tools to further investigate ACE function, it also has been proven to be beneficial in the management of hypertension (Esther Jr *et al.*, 1997). In several large clinical studies, it is shown that ACE inhibitor drugs reduce mortality in congestive heart failure and myocardial infarction (Swales, 1994). Moreover, ACE inhibitors have been found to decrease renal damage because of diabetes and have positive effects in the treatment of patients with atherosclerosis (Curzen & Fox, 1997; Lewis *et al.*, 1993).

According to the guidelines for the management of hypertension proposed by WHO and the International Society of Hypertension, ACE inhibitors are recommended as suitable for first-line treatment, alongside with diuretics and beta-blockers (Madhur, 2014). Currently, there are more than ten ACE inhibitors marketed

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and are widely being used. Among the differences in ACE inhibitors includes the molecular structure, potency, bioavailability, plasma half-life and tissue affinity (Hernandez & Harrington, 2008).

ACE inhibitors are effective with low incidence of side effects. Among the side effects recorded are dry cough that affects 5 – 20% of patients and angioedema affects 0.1 – 0.5% of patients (Israili & Hall, 1992). Other side effects include dizziness, allergies and taste disturbance depending on the patient's tolerance and compatibility. In addition, ACE inhibitor is not suitable for pregnant woman because of their association with fetus growth retardation, renal failure and death (Guang *et al.*, 2012). Thus, the search for ACE inhibitor from natural sources is important to act as an alternative to the chemically synthesized drugs.

ACE inhibitors from natural sources have been reported to possess antihypertensive activities with no side effects. Among the group of bioactive compounds that have been reported to possess ACE inhibitory activity are proteins and peptides (De Leo *et al.*, 2009), carbohydrates (Endringer *et al.*, 2014) and plant phytochemicals such as anthocyanins, flavonols, and triterpenes (Balasuriya & Rupasinghe, 2011). ACE inhibitors from microbial sources, food proteins, marine resources, dairy products and plants bioactive compounds are widely been studied. Besides having no side effects and are harmless, natural sources drugs are cost effective and may contains other health promoting compounds.

ACE inhibitory peptides derived from food proteins are the major group of compounds investigated and have attracted particular attention for their ability to prevent hypertension. Compared with chemosynthetic drugs, peptides derived from food proteins may have reduced toxic effects in humans; therefore, these food-derived peptides could be used as potent functional food additives and represent a healthier and more natural alternative to ACE inhibitor drugs (Ni *et al.*, 2012).

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ACE inhibitory peptides have been isolated from numerous sources such as dairy products (Mullally *et al.*, 1997; Pihlanto-Leppälä, 2000), plant-derived peptides such as soybean and rapeseed (Pedroche *et al.*, 2004; Wang *et al.*, 2008), meat and fish for example protein from beef (Jang & Lee, 2005), salmon and tuna protein hydrolysate (Ewart *et al.*, 2009; Lee *et al.*, 2010), peptides of mushrooms for instance fruit body of *Pleurotus florida* and *Agrocybe* sp. (Abdullah *et al.*, 2012), *Tricholoma giganteum* and *Grifola fondosa* (Choi *et al.*, 2001; Lee *et al.*, 2004b), *Pleurotus cystidiosus* and *Agaricus bisporus* (Lau *et al.*, 2012).

2.3 Oxidative stress

Oxidative damages to DNA, lipids, proteins and other molecules can occur when the antioxidant defences are inadequate to scavenge the Reactive Oxygen Species (ROS) (Aruoma, 1998). Besides the naturally generated endogenous antioxidant, externally supplied antioxidant through foods such as vitamin C, vitamin E, β-carotene, selenium and zinc can also contributes in neutralizing the excess of free radicals. Antioxidant is important as it helps to fight the oxidative damage which is associated with the pathology of atherosclerosis and vascular dysfunction (Halliwell, 2000).

Oxidative stress plays an important role in the pathogenesis and development of cardiovascular diseases including hypertension, dyslipidemia, diabetes mellitus, atherosclerosis, myocardial infarction, angina pectoris and heart failure. There are increasing evidences that suggests that increased oxidative stress account for a significant proportion of endothelial dysfunction (Heitzer *et al.*, 2001). Endothelial dysfunction may represent an early development of atherosclerosis and has been observed in patients with established coronary artery disease or coronary risk factors, both in the coronary and peripheral vasculature (Drexler, 1997). Therefore, there is

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growing research in the role of antioxidants on endothelial function as a new therapeutic approach for reducing the risk of cardiovascular disease.

Most researches on antioxidant try to determine the effect of antioxidant capacity from a compound by measuring the thermodynamic conversion efficiency of an oxidant probe upon reaction with an antioxidant (Apak *et al.*, 2013). Generally antioxidant assays can be grouped into two categories, hydrogen atom transfer (HAT) reaction assays and electron transfer (ET) reaction assays. The HAT-based assays quantify hydrogen atom donating capacity while the ET-based assays measure an antioxidant's reducing capacity. HAT-based involves in the breaking of the radical chain reaction and monitor competitive reaction kinetics, and the quantitation is derived from the kinetic curves (Huang *et al.*, 2005).

Natural antioxidant that contains bioactive compounds has long been used to help reducing damage to human body due to oxidation process. Natural antioxidants such as from fruits, vegetables, plants, teas and foods have been widely studied as they contain effective free radical scavengers. Antioxidant from natural sources is known to be less toxic when compared to the synthetic antioxidant such as butylated hydroxyanisole (BHA) and butylated hydroxyltoluene (BHT), which have been suspected to be carcinogenic and may damage the liver, kidney and other organs (Ito *et al.*, 1985).

2.4 Antioxidants

The basic definition of an antioxidant is a molecule that has the ability to inhibit the oxidation of other molecules. For instance, an enzyme or other organic substances that counteracting the damaging effects of oxidation in animal tissues (Huang *et al.*, 2005). It is also the components which prevent fats from becoming rancid in food but most importantly, it adverse the effects of free radicals on normal human body

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function. There are relationship between free radical reaction and cardiovascular tissue injury involving oxidative damage which is similar to the common mechanisms of molecular and cellular damages in human (Mimić-Oka *et al.*, 1999).

2.4.1 Free radicals

Free radicals in human body are formed when a molecule's bond split leaving odd and unpaired electron thus became unstable. These free radicals are very reactive as they are trying to gain stability by attacking other molecules to form a covalent bond or, donate its electron to or take one electron from it, resulting new radical and initiate a series of chain reactions (Mimić-Oka *et al.*, 1999). Free radicals can also be found in food components, capable of oxidizing the biomolecules and leads to cell death and tissue damage.

Free-radical mechanisms have been implicated in the pathology of diseases, such as cancer, atherosclerosis, malaria, and rheumatoid arthritis and neurodegenerative diseases. At high concentration of reactive species, a process called oxidative stress generated which will damage cell structure by adversely altering lipids, proteins and DNA thus leads to the development of diseases (Pham-Huy *et al.*, 2008). Oxidative stress is the term referred to as the imbalance of reactive oxygen species and the activity of the antioxidant defences which will cause cell damage and death (Aruoma, 1998).

Free radicals are derived from normal essential metabolic processes in human body. Besides that, environment factors too can contribute to free radical formation including environment pollutants, radiation, cigarette smoking, certain drugs, industrial solvents and chemicals, and also pesticides and herbicides (Lobo *et al.*, 2010; Riley, 1994; Valavanidis *et al.*, 2009). These exogenous substances can

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penetrate into the body by different routes, and then decomposed or metabolized by the body into free radicals.

The major free radical species are oxygen free radicals, example: superoxide anion free radical ($O_2^{\bullet -}$) and hydroxyl radical ($OH^{\bullet}$); reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), and lipid peroxide (LOOH); and other free radicals such as carbonyl, thiyl and nitroxyl radicals (Mimić-Oka *et al.*, 1999). Hydroxyl radical ($OH^{\bullet}$) is the most reactive free radical *in vivo* formed through the Fenton reaction where superoxide anion radical reacted with hydrogen peroxide in the presence of Fe^{2+} or Cu^{+} as a catalyst (Pham-Huy *et al.*, 2008).

At low or moderate concentration, free radicals are necessary in maturation of cellular structures and in induction of a mitogenic response which proved its importance to human (Valko *et al.*, 2007). Despite its harmful effect, free radicals are essential for aerobic cells as it is constantly produced during metabolic processes when oxygen reduced to water in mitochondrial electron transport chain reaction. Moreover, phagocytes, the white blood cells that protect the body release free radicals to destroy invading foreign pathogenic microbes as the defence mechanism (Challem, 2003). In short, free radicals in a controlled condition are vital to human health.

These free radicals will become highly reactive if not tightly controlled; therefore, aerobic organisms have an antioxidant protection system to prevent excess in free radicals. Defensive activity of aerobic organisms includes: preventive and control of primary radical species formation, control of the proliferation of secondary radicals in chain breaking reactions such as lipid peroxidation and a few more defence activity (Mimić-Oka *et al.*, 1999). Thus, oxidant and antioxidant balance is critical as they maintains cell membrane integrity and functionality, besides controlling signal transduction and gene expression (Knight, 2000).

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In the prevention of primary radical species formation, an antioxidant enzyme such as superoxide dismutase, catalase and glutathione prevent oxidation by reducing the rate of chain initiation either by scavenging initiating free radicals or by stabilizing transition metal radicals. On the other hand, chain breaking defence system works when radical releases or obtains an electron forming a second radical, the new radical exerts the same action on another molecule and continues until it is stabilized by an antioxidant such as vitamin C, or it simply disintegrates into inactive product (Young & Woodside, 2001).

Initiation of lipid peroxidation is one of the most destructive effects of oxygen free radicals. It can cause the destruction of the cell membranes and leads to cell autolysis (Machlin & Bendich, 1987). It happened when hydroxyl radical and peroxynitrite are in excess thus initiate the process. Free radicals-induced DNA damage involves single or double stranded DNA breaks thus resulting in either arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, which all are involved with carcinogenesis (Valko *et al.*, 2007).

2.5 Antioxidants and Hypertension

2.5.1 Natural antioxidants and ACE inhibitors from foods

Health promoting foods are getting spotlight over the recent decade as people now believed that food can contribute directly to their health. Maintaining good health through diet and exercise can reduce the risk of the non-communicable diseases such as cardiovascular diseases, cancers, chronic respiratory diseases and diabetes. Food's roles are now not only to satisfy hunger and provide nutrients, but also to promote health and prevent diseases.

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As people value the quality of life, foods that have the benefit beyond the basic nutrition needed were chosen to fulfil the healthy diet. This is where the functional foods play a very significant role as it has properties such as bioactive compounds that can help in hindering and remedy for diseases. Functional foods enable the consumer to lead a healthier life without changing eating habits since the bioactive compounds are present in the food itself.

For instance, oat contains beta-glucan that can significantly reduce the total and low density lipoprotein (LDL) cholesterol thereby reducing the risk of coronary heart disease (Hasler, 1998). In addition, animals also offer physiologically-active components for example milk from dairy products provide calcium which can prevent osteoporosis while omega-3 fatty acid from fish oil particularly the DHA, are fundamental for the neurological and visual pre- and post-natal development (Soccol & Oetterer, 2003). Fruits and vegetables contain properties that can enhance quality of health for example; the bioactivity of their phenolic compounds.

Natural antioxidants are important because of their effect on inhibiting the deterioration of foods and their significant role in the treatment of different diseases such as arthrosclerosis, cancer, and diabetes (Sarmadi & Ismail, 2010). Endogenous and exogenous reactive oxygen species and free radicals also have been implicated in the occurrence of hypertension and other degenerative diseases. The amount of these reactive species is controlled by endogenous antioxidants until it reaches a level when the antioxidants are overwhelmed, a state known as oxidative stress. The combination of ACE inhibitory and antioxidant activities in food could be very helpful for the control of cardiovascular diseases by synergies of different regulatory mechanisms (Rao *et al.*, 2012).

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2.5.2 Medicinal properties of mushroom

Mushrooms have unique flavours and have been consumed by many communities around the world. It was highly regarded by the ancient Greeks, Romans and Egyptians besides the Chinese who treasure mushrooms as elixir to prolong life (Chang & Buswell, 1996). Chinese people have documented over 100 mushroom species used by practitioners of traditional Chinese medicine for a wide range of ailments. It has been used for food or to cater traditional medicine where it was prepared as tonic or by mixing the extract with tea or drinks (Smith *et al.*, 2002).

Many of the mushroom-derived medicinal products are now produced by major Japanese, Korean and Chinese pharmaceutical companies. Lack of acceptable pharmaceutical purity makes it to be less used in medicinal practices by the Western countries. However, in the recent decades, the Western region has started to appreciate mushroom because of the expanding body of scientific research supporting numerous health benefits from it.

Mushrooms are highly appreciated for its taste, flavour and texture in addition to their medicinal properties such as antibacterial, antifungal, antioxidant, antiviral, antitumor, immunosuppressive, antiallergic, antiatherogenic hypoglycemic, anti-inflammatory and hepatoprotective activities (Ferreira *et al.*, 2010). Examples include *Auricularia* spp. which is traditionally used as treatment for haemorrhoids and stomach ailments. *Tramella fusiformis* or the white jelly fungus could be used in maintaining healthy lung tissue and have hypocholesterolemic activity (Cheung, 1996), *Hericium erinaceus* used to treat gastric ulcers and have immune-modulating properties (Khan *et al.*, 2013), *Volvariella volvacea* used for lowering blood pressure and wound healing (Breene, 1990), *Lentinus edodes* used in prevention of several diseases including cancer, heart disease, diabetes and hepatitis (Bisen *et al.*, 2010).

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The compounds that are responsible for the observed medicinal properties can be isolated and identified from fruiting bodies, cultured mycelia biomass or broth, and also in certain species from its sclerotium. Various important nutrients such as poly-saccharides, proteins, fibre and various low molecular weight metabolites including phenolic compounds, polyketides, triterpenoids, and fatty acids can be found in the mushrooms (Elisashvili, 2012). There are many bioactive compounds from mushrooms that have already been commercialized including polysaccharides from *L. edodes,* Lentinan; Schizophyllan from *Schizophyllum commune* and Grifon-D from *Grifola frondosa* which have undergone clinical trials and neither of these compounds show any significant side effects (Smith *et al.*, 2002).

The most cultivated edible mushroom worldwide is the button mushroom, (*Agaricus bisporus*), shitake (*L. edodes*), oyster mushrooms (*Pleurotus* sp.) and enoki (*Flammulina velutipes*) with China being the biggest producer in the world (Valverde *et al.*, 2015). In addition, lingzhi or reishi or *Ganoderma lucidum* is a medicinal mushroom cultivated in large scale in China, found to be rich in β-glucan, heteropolysaccharides and glycoproteins (Lee *et al.*, 2003). China's mushrooms cultivation and production in 2011 has reached 24 billion USD and accounted for 40% of total world mushroom export in recent years (Zhang *et al.*, 2014).

In recent years, pharmaceutical potential of the mushroom have been studied extensively and is promoted as alternative in prevention and treatment of diseases. Mushroom extracts are now commercialized in the form of dietary supplements and also used in medical and biotechnological applications such as lignocellulose-degrading enzymes and proteases (Erjavec *et al.*, 2012). Regular intake of the mushroom nutriceuticals can enhance the immune responses and increase the resistance to diseases (Lindequist *et al.*, 2005).

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Mushroom contains major nutritional importance such as protein with essential amino acid, fibre, low in fat and high in important fatty acids such as linoleic, oleic and palmitic acids, besides vitamins (B1, B2, B12, C, D and E) and carbohydrate. The moisture content of mushroom is high between the range of approximately 80 to 95 g per 100 g (Valverde *et al.*, 2015). In addition, mushrooms contain the most significant amount of selenium which is an antioxidant that can help to prevent cell damage and also ergothioneine which is an amino acid that contains sulphur, important in protecting the DNA from oxidative damage.

As for the nutraceuticals properties, mushrooms comprise good quantity of various bioactive compounds. β-glucan is one of important polysaccharides found in mushrooms. It has anticancer, immunomodulating, anticholesterolemic, antioxidant and neuroprotective activities from many edible mushrooms. β-glucan stimulates the human immune system and protect from pathogenic microbes, environmental toxins and carcinogens, infectious diseases and cancer. It also helps cancer patients recover from the effects of chemotherapy and radiotherapy treatments.

2.6 Submerge cultivation of mushrooms mycelia

About 80–85% of all medicinal mushroom products are derived from the whole mushrooms or known as fruiting bodies, which have been either commercially farmed or collected from the wild (Lindequist *et al.*, 2005). That leaves about 15% of products that are based on extracts from mycelia. A small percentage of mushroom products are obtained from culture filtrates and the remaining are from culture broth and sclerotium. However, the production of medicinal mushrooms' fruiting bodies usually will take several months, and it is difficult to control the quality of the final product.

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For this reason, the submerged cultivation of medicinal mushrooms has received a great deal of attention as a promising and reproducible alternative for the efficient production of mushroom mycelium and metabolites. Mushroom mycelium is important in its growth phase as mushrooms spend more than 90% of their life cycle in the mycelial, or vegetative stage. Under favourable conditions, the mycelium will matures and produces the fruiting structure (Miles & Chang, 2004). The mycelium provides extracellular compounds and metabolites that function to promote the longevity and vitality of the living mushroom.

2.6.1 Advantages of mycelium as a source of bioactive compounds

The mushroom mycelium, originated from the spores that germinate to produce a mass of interwoven, single-cell wide structures known as hyphae, and masses of hyphae are known as the mycelium. Mycelia are a valuable food component, and can be used for all purposes to which the fruiting body is adapted. Although mycelium is primarily useful as human food, it can be as well adapted to be used as an animal feed given its high protein content (Humfeld, 1954).

Mycelia cultivation is rapid and under sterile condition, compared to fruiting body cultivation. Furthermore, fungal fermentation in liquid medium have been reported to comprise of a highly uniform quantitative biomass production and it can be an alternative source of obtaining potential medicinal products (Vamanu, 2014). Costs and expenses in production can be reduced by using mycelia cultivation compared to the fruiting body. The equipment required for submerged process is much smaller compared to the composting method, besides that, less man power required for handling the process.

In addition, mushroom fruiting bodies are fragile, require cleaning after harvesting and may not be in a uniform size and condition. Mycelia culture on the

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other hand, can be produced using a controlled system and eliminate the problems faced by the fruiting bodies. The equipment for mycelia culture can occupy a compact space with an advantage of low chances of contamination (Friel & McLoughlin, 2000). Mycelia cultivation can utilize media from various sources, for instance, waste from farming communities, food industries and agricultural sectors which contain sugars, nitrogenous materials, minerals and salts (Humfeld, 1954).

In terms of storage, the mycelia culture can be kept frozen or canned like the canned fruits and vegetables. Mycelia biomass can also be freeze dried and stored for a long time. Besides using mycelia culture product as food and animal feed, it can be use as a spawn to inoculate beds for the mushrooms fruit bodies production. Studies showed that the chemical composition of the cultivated mycelia biomass using submerged cultures are in varying composition based on selected media and physiochemical conditions (Griensven, 2000). Nevertheless, submerged culture condition for mycelium biomass production has been long adapted in research for obtaining various compounds and valuable bioactive metabolites from mushrooms (Lee *et al.*, 2004a; Zhong & Tang, 2004).

2.7 *Schizophyllum commune* Fr.

Schizophyllum commune is the most widely distributed fungi throughout the world except in Antarctica region (Ohm *et al.*, 2010). *S. commune* belongs to *Shizophyllaceae* family. The greyish-white mushroom can be found year round on a dead log especially during rainy season where the gills are open, soft and pliable (Figure 2.3). Meanwhile, in a hot and dry weather, it will harden and shrink from its original form but revived back after rains (Salahuddin, 2008).

This mushroom is also known as the split gill mushroom because it's gills are split longitudinally. It has a beautiful shell-like or fan-like shape, about 1 to 5 cm in

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diameter. The fruit body has no stem, the flesh is a bit tough and thin while the spore print is white. Frequently, the fruiting body are lobed or fused at the base with other brackets, scattered to cluster on hardwood logs and branches. The *S. commune* fruit body are drought resistant where it can spring back to life when favourable condition available and producing spores again (Vellinga, 2013). *S. commune* mycelium appeared as cottony white colony on the petri dish.

Although there are reports on *S. commune* being a pathogen to human and animals, it is edible and its nutritional perspective have been studied widely. This mushroom have been consumed by many communities for instance, the people in Malaysia, Thailand, Indonesia, Madagascar, and tribes in the south-western Nigeria and north India either they prepare it as dishes or concoctions (Jonathan & Fasidi, 2003; Longvah & Deosthale, 1998). In Malaysia, *S. commune* is also known as cendawan kukur or kulat sisir or kulat sisik and is popular among the Malay community. In South-East Asia, currently Thailand and Malaysia are commercially cultivating this mushroom due to ease of cultivation and increase in demand (Chang & Buswell, 1996; Salahuddin, 2008).

Since the early twentieth century, *S. commune* has been the subject of genetic analysis where it became the model system for studying mating-type gene function and also mushroom development (Ohm *et al.*, 2010). Previous studies showed that *S. commune* has significant medical importance such as antioxidant, antimicrobial and anti human papilloma virus activities, has positive effect on patients with chronic hepatitis B and most importantly it has antitumor and immunomodulatory activities (Kakumu *et al.*, 1994; Klaus *et al.*, 2011; Mirfat *et al.*, 2010).

S. commune's polysaccharide called schizophyllan is known for its antitumor, anticancer and immunomodulating activities. Schizophyllan is a non-ionic water soluble homopolysaccharide of β-D-(1→3)-glucopyranosyl groups liner chain with β-

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D-(1→6)-glucopyranosyl groups attached to it. It was first found by a group of researcher which had successfully isolated it from the precipitation of submerged culture of *S. commune* (Kikumoto *et al.*, 1970). Clinical trials that have been carried out in Japan showed that schizophyllan increased the survival rate in patients with recurrent and inoperable gastric cancer, increased overall survival of head and neck cancers, and also prolonged the overall survival of Stage II cervical cancer (Kimura *et al.*, 1994; Okamura *et al.*, 1989).

Among the nutrition value reported by previous study on *S. commune* includes 16% crude protein, 5.3% moisture content, 68% carbohydrates and fiber. It also contains higher minerals content when compared to *L. edodes* (Longvah & Deosthale, 1998). According to Okwulehie *et al.* (2007), *S. commune* possessed 3.8% flavonoids, 0.7% phenols, 0.7% tannins and 0.015% alkaloids. The high bioactive content of *S. commune* makes it pharmaceutically important.

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Figure 2.3: *Schizophyllum commune* in the wild (Image courtesy of Mycology Laboratory, University of Malaya)

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CHAPTER 3:MATERIALS & METHODS

3.1 *Schizopyllum commune* culture

Culture of *S. commune* (KUM 50016) was obtained from the Mycology Laboratory, Institute of Biological Sciences, University of Malaya. Mycelial cultures were maintained on Malt Extract Agar (MEA) (Oxoid).

3.2 Liquid Fermentation of *S. commune*

Liquid Glucose-yeast-malt-peptone (GYMP) medium was prepared as in Appendix 1.1.1 and sterilized in the 500 ml Erlenmeyer flasks as described by Mhd Omar *et al.* (2011). Ten seven days old, about 8 mm in diameter mycelial plugs were inoculated into 100 ml of medium and stoppered with non-adsorbent cotton plugs. Incubation was subjected for 14 days, and for static culture, the flasks were incubated at 25 ˚C; while for shake culture, the flasks were incubated at 25˚C on the rotatory shaker shaking at 150 rpm. Harvested mycelial biomass was freeze dried and kept in -20 ˚C freezer.

3.3 Preparation of *S. commune* extracts

3.3.1 Preparation of solvent extracts

For solvent extraction, freeze dried mycelia were soaked in methanol:dichloromethane (MD) (2:1 v/v) mixture for 3 days at 1:20 (w/v) ratio at room temperature. The mixture was filtered using Whatman No. 1 filter paper; the filtered mycelia were collected and the soaking and filtrating processes were repeated for 3 times, the solvent extract obtained was pulled together and then rotary evaporated. The crude extract obtained was denoted as crude methanol-dichloromethane extract (CMDE).

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To prepare hexane extract (HE), at room temperature, about 2 g CMDE was dissolved in 90% methanol; the mixture was vigorously shaken for 15 min with hexane (1:1) (v/v) in the separating funnel; the hexane mixture and aqueous methanol mixture were collected respectively and the step was repeated for 3 times using the same aqueous methanol mixture with fresh hexane solvent. The HE was obtained after the hexane mixture was rotary evaporated.

The aqueous methanol mixture was rotary evaporated to obtain a semisolid form and then dissolved with water to be partitioned with dichloromethane (DCM) (1:1) (v/v) at room temperature and later with ethyl acetate (1:1) (v/v) repeated 3 times, then rotary evaporated to obtain DCM extract (DCME), ethyl acetate extract (EAE) and water residue extract (WRE) respectively.

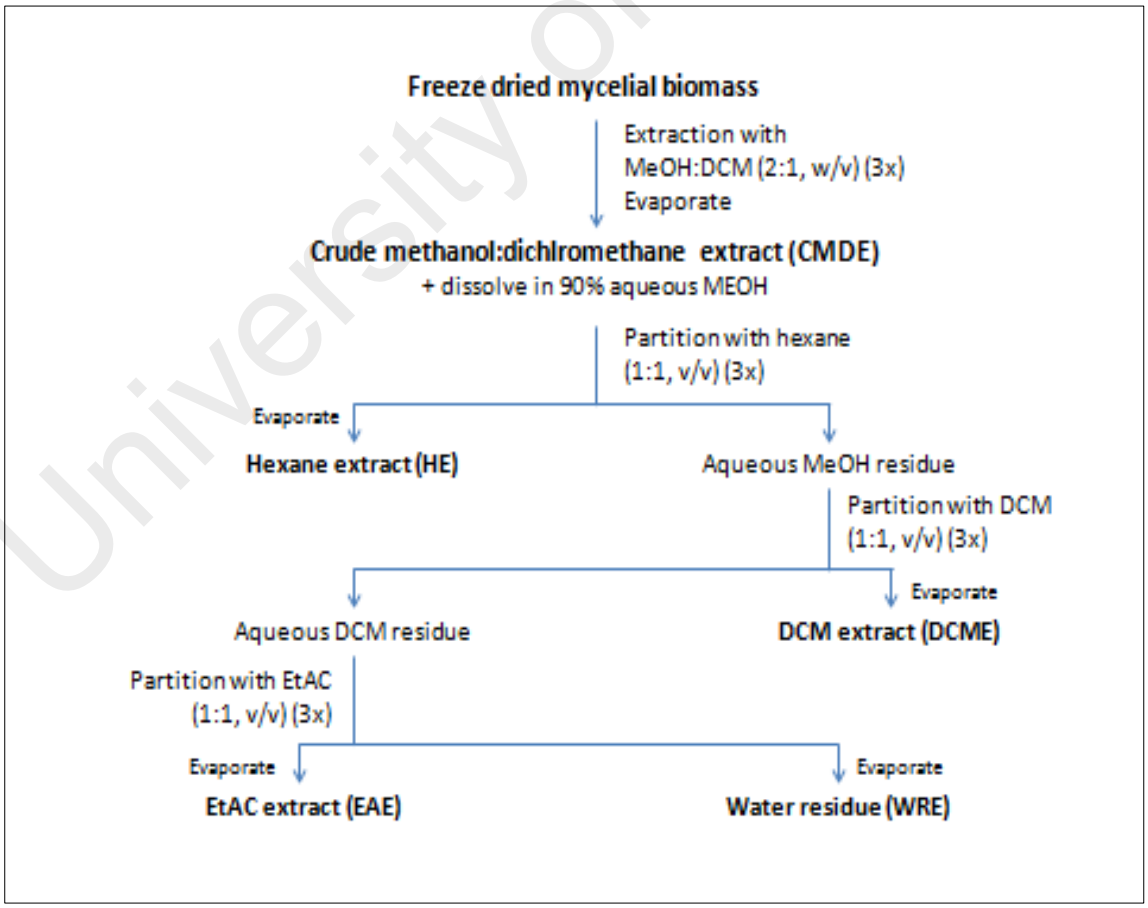

Figure 3.1: Solvent extraction workflow

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3.3.2 Preparation of water extract

Freeze dried mycelium was blended using commercial Waring blender for 10 seconds with distilled water at a ratio of 1:20 (w/v). The mixture was filtered using muslin cloth and vacuum filtered to separate the water extract from the biomass. The water extract was freeze dried, stored at 4˚C for further analysis and denoted as crude water extract (CWE).

3.3.3 Preparation of *S. commune* protein fractions by ammonium sulphate precipitation

For protein fractionation, 2.0 grams of freeze dried water extract was re-dissolved in 100 ml of distilled water at a ratio 1:5 (w/v). Ammonium sulphate $(NH_4)_2SO_4$ was weighed appropriately according to the desired saturation of 30-90% as tabulated in Table 3.1.

Small portion of ammonium sulphate was added gradually into the water extract while stirring with a magnetic stirrer on ice bath until the salt was fully dissolved before adding the next portions and left to achieve equilibrium with continuous stirring. The water extracts with dissolved salt were then centrifuged at 10 000 rpm for 15 minutes at 4˚C. The precipitates were then collected and re-suspended in 4 ml of distilled water, while the supernatants were further used for precipitation of protein at higher salt concentration (60% and 90%).

Dialysis was then conducted to further purify the protein fractions collected from ammonium precipitation using SnakeSkin™ Pleated Dialysis Tubing (Thermo Scientific) with molecular cut-off of 3500 Da. This step will remove the impurities such as salt and residual that is bound to the protein molecules.

The required length of tubing was cut and 2-3 inches of one end of the tubing was briefly dipped into distilled water to soften the tube. A knot was securely tied at

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one end while protein samples were added into the other end of tubing and then tightly tied. These protein fractions were dialysed against distilled water with continuous stirring at 4˚C for 48 hours. Distilled water was changed every 12 hours throughout the dialysis process. Dialysed protein fractions denoted as F30, F60 and F90 were then stored at -20˚C for further analysis.

Table 3.1: Amount of ammonium sulphate salt (g) to be added in 100 ml solution

	Final salt concentration (%)										
Initial salt concentration (%)		10	20	30	40	50	60	70	80	90	100
	0			17.6							
	10										
	20										
	30						19.8				
	40										
	50										
	60									22.7	

3.3.4 Preparation of hot water and polysaccharides extracts

Freeze dried mycelia were boiled in distilled water at the ratio of 1:10 (w/v) at 100˚C for 3 hours. The supernatant was filtered using Whatman No. 1 filter paper and was freeze dried to obtain hot water extract (HWE).

One-third of the supernatant was soaked with ethanol 95% overnight at 4˚C at 1:5 ratio (v/v), centrifuged and de-proteinated according to the method described by

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Jahanbin *et al.* (2011) (Appendix 1.1.2) to prepare the polysaccharide extract (PE). Pellet collected was freeze dried and kept at 4˚C.

3.4 Estimation of Protein Content

Protein content was estimated by using Pierce® Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific) based on the protocols recommended by the manufacturer. Standard curve of Albumin Standard (BSA) was used for the protein estimation of all extracts (Appendix 1.1.3).

This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing Bicinchoninic acid (Smith *et al.*, 1985). The chelation between two molecules of BCA with one cuprous ion produces purple colour reaction and exhibits a strong absorbance at 562 nm.

A volume of 25 µl of BSA standard solutions and *S. commune* extracts were pipetted into the microplate wells followed by 200 µl of working reagent. The microplate was incubated at 37˚C for 30 minutes. The absorbance readings were taken using Sunrise™ ELISA reader (Tecan, Switzerland) at 562 nm after the plate was cooled to room temperature. Standard curve of albumin was generated and protein content of the protein fractions was then estimated.

3.5 Estimation of Carbohydrate Content

Carbohydrate content of the samples were estimated using protocol by DuBois *et al.* (1956), where a volume of 500 µl (100 µg/ml) of *S. commune* extracts and standard solution of glucose (3.125-100 µg/ml) were added with 300 µl phenol and 3 ml sulphuric acid in the test tubes incubated at 30˚C for 15 min before the absorbance

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was taken at 490 nm. A standard curve of glucose was generated and the carbohydrate content of the extracts was determined from the graph.

3.6 Estimation of Total Phenolic Content

To determine the total phenolic contents in *S. commune* extracts, Folin-Ciocalteu assay was performed according to the method of Abdullah *et al.*, (2012). For the assay, 250 µl (100 µg/ml) of *S. commune* extracts and standard solution of gallic acid (2–10 µg/ml) was pipetted in the test tube, followed by the addition of 250 µl of 10% Folin-Ciocalteu solution as prepared in Appendix 1.1.4.1. The mixture was incubated for 2 minutes at room temperature and followed with the addition of 500 µl of saturated sodium carbonate (10% aqueous solution). Reactions were incubated in the dark for 1 hour and absorbance was taken at 750 nm using the spectrophotometer (Shimadzu). Total phenolic contents of the mushroom extracts were expressed as mg Gallic Acid Equivalents (GAE) per gram extract (Appendix 1.1.4.1).

3.7 Determination of Antioxidant Capacities

It is known that the antioxidant properties depend on the type of solvent used in the extraction and the complexity of compounds exhibiting antioxidant effects via different mechanisms, hence, different methods must be used to assess their antioxidant activity. In this study, scavenging effect on 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) radicals, inhibition of lipid peroxidation, cupric-ion-reducing antioxidant capacity (CUPRAC) and metal chelating activity assays were employed.

3.7.1 Scavenging Effect on 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Radicals

The DPPH free radical scavenging activity of the extracts was measured based on the method of Chatatikun (2013) with some modifications. For the test, 195 µl of 1 mM DPPH radical (Appendix 1.1.4.2) was added to the 5 µl (100 µg/ml) of *S. commune* extracts in 96 well plate. Reaction mixture was incubated for 30 minutes at

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room temperature in the dark and absorbance was measured at 515 nm using a spectrophotometer with methanol as the blank. The percentage of radical scavenging activity was calculated as follows:

$$\text{Radical scavenging activity} = \left(\frac{A_0 - A_s}{A_0} \right) \times 100,$$

Where A_0 is the absorbance of 1 mM methanolic DPPH only whereas A_s is the absorbance of the reaction mixture.

3.7.2 Inhibition of Lipid Peroxidation

The lipid peroxidation effects of *S. commune* extracts were determined by the method of Abdullah *et al.* (2012) using the egg yolk as the rich lipid medium. Briefly, 1 ml of buffered fowl egg yolk was mixed with 100 µl of 1 mM ferrous sulphate ($FeSO_4$) solution followed with 100 µl (100 µg/ml) of extracts in a test tube. The mixture was incubated at 37℃ for 1 h before being treated with 500 µl of freshly prepared 15% trichloroacetic acid (TCA) and 1 ml of 1% thiobarbituric acid (TBA) (Appendix 1.1.4.3). The reaction mixture was further incubated in boiling water bath for 10 min. Once cooled to room temperature, the tubes were capped and centrifuged at 3500 rpm for 10 minutes. The supernatant layer was pippeted into a 96 well plate and absorbance reading at 532 nm was recorded. The percentage of inhibition was calculated using the following equation:

$$\text{Inhibition (\%)} = \left(\frac{A_0 - A_s}{A_0} \right) \times 100,$$

Where A_0 is the absorbance of the control whereas A_s is the absorbance of the sample.

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3.7.3 Cupric-Ion-Reducing Antioxidant Capacity (CUPRAC)

CUPRAC activity of the *S. commune* extracts were determined according to the method of Öztürk *et al.* (2007) with some modifications. This assay measured the orange-yellow colour of reduced Cu^{+}-Neocuproine at 450 nm at pH 7.

For the test, 1 ml of 10 mM of copper (II) and 1 ml of 7.5 mM Neocuproine was added into a test tube. Then, 1 ml of each 1 M ammonium acetate buffer (pH 7.0) and (100 µg/ml) of *S. commune* extracts were added to achieve a final volume of 4 ml. The mixture was vortexed, incubated for 30 min at room temperature and absorbance at 450 nm was recorded against a blank (Appendix 1.1.4.4).

3.7.4 Metal chelating activity

The metal chelating ability of the *S. commune* extracts on ferrous ion (Fe^{2+}) was measured as described by Decker and Welch (1990) with some modifications. For the test, 1 ml of 100 µg/ml extracts was added to 100 µl of 2 mM $FeCl_2$. After 1 min, 200 µl of 5 mM ferrozine and 3.7 ml of distilled water were added, the mixture was shaken vigorously and left standing at room temperature for 10 min (Appendix 1.1.4.5). After the mixture reach equilibrium, the absorbance was measured at 562 nm, and the results were given as percentage of inhibition as follows:

$$\text{Chelating activity (\%):} \quad 1- \left(\frac{A_s}{A_0} \right) \times 100,$$

Where A_0 is the absorbance of the control whereas A_s is the absorbance of the sample.

3.8 Angiotensin-I Converting Enzyme (ACE) inhibitory assay

ACE inhibitory activity of the samples was measured *in-vitro* using ACE Kit-WST (Dojindo Molecular Technologies, Inc.) based on the recommended protocols by

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the manufacturer. Captopril ($1x10^{-6}$ µg/ml) was used as positive control and the *S. commune* extracts were tested at a concentration of 100 mg/ml protein.

For the assay, 20 µl of *S. commune* extracts, 20 µl of substrate buffer and 20 µl of enzyme working solution were added into the 96-microplate well. For blank 1, 20 µl of deionized water and 20 µl of enzyme working solution added (Appendix 1.1.5.1). For blank 2, 40 µl of deionized water added instead of other solution. The microplate was then incubated at 37˚C for 60 minutes. Next, 200 µl of indicator working solution (Appendix 1.1.5.2) was added to each well and the plate was incubated at room temperature for 10 minutes. The absorbance readings were taken using Sunrise™ ELISA reader (Tecan, Switzerland) at 450 nm. Percentage of the inhibition was calculated based on the formula given in the protocol.

ACE inhibitory activity (%) =

$$\left[\frac{(\text{Absorbance blank 1} - \text{Absorbance sample})}{(\text{Absorbance blank 1} - \text{Absorbance blank 2})} \right] \times 100$$

3.9 Liquid Chromatography Mass Spectrometry (LC MS/MS) of selected extracts

Selected extracts with good antioxidant activity were analysed for compound identification via LCMS/MS. This step was employed to discover bioactive compounds such as phenolic acids, organic acids and amino acids that can be found in the extracts and contribute to the antioxidant activities. The analysis was performed using AB Sciex 3200 QTrap hybrid linear ion trap triple- quadruple mass spectrometer equipped with a turbo ion spray source; coupled with Perkin Elmer UHPLC Flexar FX15 ultra high-performance liquid chromatograph. Chromatographic separation was achieved on a Phenomenex Aqua C18 (5 mm, 50 mm62 mm) column where mobile

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phase A was composed of water with 0.1% (v/v) formic acid and 5 mM ammonium formate, whereas the mobile phase B consisted of acetonitrile containing 0.1% (v/v) formic acid and 5 mM ammonium formate. Elution was performed by means of a linear gradient from 10% to 90% B (0.01-8.0 min) held for 3 min, and back to 10% B in 0.1 min, and then re-equilibrated for 5 min. Full scan with MS/MS data collection analyses was performed in negative mode. Data analysis, processing, and interpretation were carried out using the ABSCIEX Analyst 1.5 and Advanced Chemistry Development, Inc., (ACD/Labs, Ontario, Canada) MS Processor software. Marker-View Software (AB SCIEX, Massachusetts, USA) was used for principal component analysis (PCA).

3.10 Partial Purification of selected extracts using ultracentrifugal filter device

S. commune extracts (WRE-SH, CMDE-ST, PE-ST and F90-ST) having potent ACE inhibition were separated using the Amicon® Ultra-0.5 centrifugal filter devices (Merck Millipore Ltd.) according to the recommended protocols by the manufacturer. The Amicon® Ultra 10K device with 10 000 Nomial Molecular Weight Limit (NMWL) was selected to separate the sample with initial concentration of 1 mg/ml.

First, 500 ul of sample was added into the Amicon® Ultra filter device assembled with microcentrifuge tube provided. The capped microcentrifuge tubes were centrifuged at 14 000 rpm for 15 mins. The filtrated sample collected by the microcentrifuge tube represent the <10 000 NMWL sample. The assembled devices were separated and the Amicon® Ultra filter device was placed upside down into a clean microcentrifuge tube, spun for 2 min at 1000 rpm to collect the concentrated or >10 000 NMWL sample. The collected filtrates and recovered concentrates were then freeze dried and stored at -20 ˚C for further analysis.

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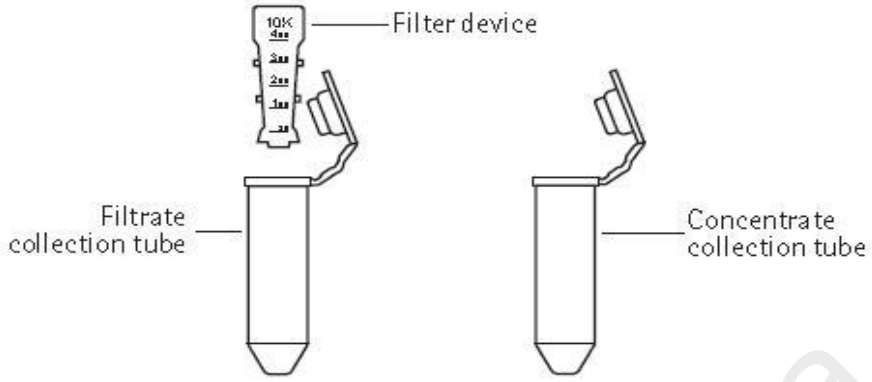

Figure 3.2: Amicon® centrifugal filter unit

3.11 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE)

SDS PAGE analysis was performed based on discontinuous buffering system according to the method of Laemmli (1970) with some modification. Fractionation on SDS PAGE is controlled by the molecular size and shape of the protein and dodecyl sulfate particle, its net charge, and the accessible spaces among the acrylamide fibers that comprise the gel matrix as determined by the total concentration of acrylamide and bis-acrylamide cross-linker. Larger particles become trapped within the gel meshwork and migrate slower than smaller species. Low-percentage gels are therefore typically used to resolve larger proteins, and high percentage gels for small proteins, in this study 18% polyacylamide was used as the target protein is <10 kDa.

F90-ST <10 kDa extract that showed high inhibition for *in vitro* ACE assay were subjected to SDS PAGE analysis. Separating gel solution of 18% polyacrylamide and stacking gel solution (4% polyacrylamide) were prepared and allowed to polymerize in appropriately assembled glass plates. An appropriate gel comb was inserted to the stacking gel layer. The sample buffer and protein extracts were mixed at a ratio of 1:3 (v/v) and boiled for 5 minutes. The sample and Spectra™

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multicolor broad range protein ladder (Thermo Scientific) were carefully loaded into the wells.

Electrophoresis was conducted at a constant current of 90V for stacking gel and followed by 100V for separating gel. The power supply was turned off once the blue dye front reached approximately 1 cm from the bottom of the gel. The gel was carefully removed from the gel cassette and the protein bands were then stained by Coomassie Brilliant Blue or silver nitrate. All solutions and reagents were prepared as in Appendix 1.1.6.

3.12 Identification of ACE inhibitor proteins by LCMS-QTOF

From the separated F90 extract, the F90 <10 kDa extract was further subjected to Liquid Chromatography Mass Spectrometry of Quadrupole Time of Flight (LCMS-QTOF) analysis. After the extract was separated by SDS PAGE and stained with Coomassie blue, the bands on the gel were cut accordingly using scalpel and placed into individual Eppendorf tubes. Each gel cuts were destained, reduced, alkylated and dehydrated. Next, the bands were digested with trypsin (6 ng/µl) at 37˚C overnight followed by alkylation, extraction and desalting using C18 Zip Tip (EMD Milipore Co., MA, USA) (Appendix 1.1.7).

Analysis was performed with peptide reconstituted in 7 µl 0.1% formic acid and separation was carried out using 1260 Infinity Nanoflow LC system (Agilent, Santa Clara, CA, USA) directly connected to Accurate-Mass Q-TOF 6550 with a nano electrospray ionization source for MS analysis and MS/MS data. The mobile phases used were highly purified water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B). Column used was HPLC Large-Capacity Chip Column (Zorbax 300SB-C18, 160 nL enrichment column, and 75 µm × 150 mm analytical column, and 5 µm particles (Agilent, Santa Clara, CA, USA). The flow rate capillary pump was set

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at 4.0 µl/min and the flow rate nano pump was set at 0.4 µl/min with 25 minutes total run time. The prepared samples were placed into the LCMS autosampler and the injection volume of sample was 2 µl. The samples were run at 5-70% linear gradient mobile phase B. Analysis was performed in positive ion mode.

Mass spectra were acquired using Mass Hunter acquisition software (Agilent, Santa Clara, CA, USA). Each mass spectra acquisition cycle (an acquisition rate of 8 spectra per second from 200 to 3000 m/z) was followed by collision-induced dissociation of the twenty most intensive ions. MS/MS data were acquired in the range of 50–3200 m/z (scan rate = 4 spectra/sec).

Spectrum Mill software (Agilent, Santa Clara, CA, USA) was used in MS/MS search and the database used was Swiss-Prot, updated on 22/7/2015, 168 628 entry sequences. Mass-tolerance of precursor was ±20 ppm and mass-tolerance of product ions was ±50 ppm. Iodoacetamide was used for alkylation during sample preparation, therefore carbamidomethylation was specified as a fixed modification and oxidized methionine as a variable modification. Precursor mass shift was set between -18 Da to 177 Da (to take into consideration of variable modifications such as presence of sodium and potassium adducts).

3.13 Statistical analysis

All results were expressed as mean ± standard deviation from triplicates data. Statistic significant differences were determined by Duncan test with p values <0.05 were considered as significant difference using Minitab statistical software (Minitab Incorporation, USA).

LCMS-QTOF analysis of proteins and peptides were validated using Spectrum Mill software, (Agilent, Santa Clara, CA, USA) based on the software default settings. Inclusion criteria: protein score > 20, peptide score > 10, Scored Peak Intensity

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(%SPI) > 70%. Proteins that shared at least one peptide were grouped together. Identified proteins were then filtered to achieve a false discovery rate (FDR) of <1% for the peptide-spectrum matches.

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CHAPTER 4:RESULTS & DISCUSSION

4.1 *Schizophyllum commune* mycelial growth

Schizophyllum commune demonstrated good mycelial growth. The mycelium grown on the malt extract agar (MEA) plate was observed as white-cottony and it took an average of seven days to fully colonize the 90 mm diameter Petri dish plate (Figure 4.1 (a)).

Figure 4.1 (b) showed the flask with mycelia plugs before incubation condition. After 14 days, mycelial biomass of *S. commune* obtained by liquid fermentation in GYMP media by shaking condition (SH) was observed to form clumps or disperse pellets. According to Fang *et al.* (2002), inoculation density or inoculation size affected cellular morphology and later can influence the production of active metabolites in the mycelia biomass. The mycelium pellets and clumps were white and yellowish in colour, while the media was pale yellow turning almost clear (Figure 4.1 (c)).

On the other hand, in the static flask condition (ST), the mycelia formed a single layer of mycelium on top of the media. The mycelium was white in colour while the media turned dark brown (Figure 4.1 (d)). The mycelia and broth of SH and ST flasks were collected respectively, freeze dried, ground to powder and then stored at 4 ˚C for analyses. The yield recorded of mycelial biomass was 5.0656±0.056 g/L and 5.8280±0.088 g/L for SH and ST flasks respectively. ST flask culture gave significantly higher yield than the yield of the SH flask culture.

In this study, two culture conditions were employed to determine which condition has high ACE inhibitory and antioxidant activity. As reported from other study, some secondary metabolites were not produced in active biomass at a high

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oxygen tension, but they were accumulated in producing biomass under a certain limited oxygen tension (Gehrig *et al.*, 1998). But, because of the favourable oxygen supply for cell growth, the mycelial yield was significantly higher in the shake culture. According to Yang and Liau (1998), yields and productivity of mushroom mycelium vary widely depending on the type of mushroom, substrate used and conditions applied. In shake flask culture, introduction of agitation was aimed to obtain optimal mycelial growth where it enhanced the secretion of extracellular substances as well as maximum oxygen transfer.

A study done by Lee *et al.* (2004a) on submerged culture conditions for the production of mycelial biomass and exopolysaccharides (EPS) by *Grifola frondosa* showed that the mycelial morphology was significantly altered by culture pH, aeration rate, and hydrodynamic behaviour, which subsequently affected the yield of EPS production. Another study by Madla *et al.* (2008) on culture conditions for production of hirsutellones by fungus *Hirsutella nivea* showed that shaking induced aeration and thus dramatically reduced the time required for production of hirsutellones and increased the effective growth of the compound.

On the other hand, Arora and Chandra (2010) observed that static culture give better antioxidant yield in comparison to shake flask cultures, which resulted in steady decline in the antioxidant activities with increase in revolutions per minute (rpm) of two *Aspergillus* species namely *Aspergillus* PR78 and *Aspergillus* PR66. Shih *et al.* (2007) reported static culture of *Cordyceps militaris* have the maximum mycelial production with 14.0 g/L obtained at day 30 of cultivation, compared to the static culture with 15.5 g/L obtained at day 36 of cultivation.

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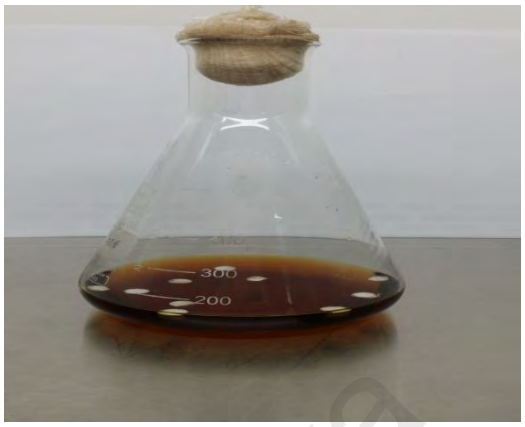
a



b

c

d

Figure 4.1: Submerged fermentation of (a) *Schizophyllum commune* 7-days old colony growing on MEA plate. (b) GYMP media flask with inoculated mycelial plugs at day 0. (c) 14-days old mycelium obtained by submerged fermentation under shaking condition. (d) 14-days old mycelium obtained by submerged fermentation under static condition.

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4.2 Preparation of Extracts

Solvent partitioning was employed in the preparation of extracts as it is one of the highly effective method (Otsuka, 2005). Secondary metabolites from organisms were isolated from its crude form into an extract or fraction suitable for chemical analysis, biological testing or chromatographic separation (Jones & Kinghorn, 2005). As solvent partitioning depends on solubility, two solvents that are not miscible with each other were chosen, which will formed two layers when mixed together separating compounds that dissolved in it respectively.

Methanol:DCM was used as the first solvent due to its polarity and its ability to extract compounds like flavonoids, phenolics and polar compounds. As methanolic extract consist of polar, moderately polar compounds and sometimes non polar compounds that soluble in methanol, fractionation was done to further partition the crude methanol extract to non-polar and polar components using different solvents.

Hexane was used to fractionate the non-polar compounds of the extract such as lipid, while DCM further partitioned lipid and fatty acids (Cequier-Sánchez *et al.*, 2008). Ethyl acetate which is more polar than hexane and DCM was used next, while water on the other hand, used to extract the polar compounds. In short, the non-polar solvents will extract non-polar compounds and polar solvents will extract polar compounds such as phenolic compounds and flavonoids (Houghton & Raman, 2012).

The extraction procedure yield a total of 22 extracts, 11 extracts from each of shaking and static submerged fermentation condition respectively. Five extracts were from solvent extraction (CMDE, HE, DCME, EAE and WRE), four extracts from water/protein extraction (CWE, F30, F60 and F90) and two extracts from hot water/polysaccharide extraction (HWE and PE). Table 4.1 showed the percentage yield of the sample extracts obtained from solvent, water/protein and hot water/polysaccharide extraction methods, from shake and static flasks culture.

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In this study, solvent extraction percentage yields were higher in SH compared to ST in all extracts except for HE, where HE-SH has 5.77% and HE-ST has 6.02%. Water extraction yields for CWE-SH was lower than CWE-ST with 63.18% and 70.72%, respectively. All protein fraction extracts of SH were higher than ST except for F60-SH with 25.81% compared to F60-ST with 26.09%. HWE-SH and HWE-ST percentage yields have no significant different; meanwhile, PE-SH has higher percentage yield with 10.76% and PE-ST has 9.89%. As mentioned before, different culture conditions can enhance or suppress the production of metabolites in submerged fermentation. Percentage yields of all extracts were tabulated in Table 4.1 below.

Table 4.1: Percentage yield (%) of samples extracts obtained from three different extraction methods and two culture conditions

Extraction methods	Shake flasks culture (SH)		Static flasks culture (ST)	
	Extract code	**% Yield**	**Extract code**	**% Yield**
Solvent	CMDE-SH	16.24	CMDE-ST	15.39
	HE-SH	5.77	HE-ST	6.02
	DCME-SH	9.21	DCME-ST	8.91
	EAE-SH	3.42	EAE-ST	3.22
	WRE-SH	15.65	WRE-ST	15.43
Water/Protein	CWE-SH	63.18	CWE-ST	70.72
	F30-SH	29.10	F30-ST	27.82
	F60-SH	25.81	F60-ST	26.09
	F90-SH	21.42	F90-ST	23.03
Hot Water/ Polysaccharide	HWE-SH	44.39	HWE-ST	44.19
	PE-SH	10.76	PE-ST	9.89

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4.3 Protein and carbohydrate content estimation

Protein and carbohydrate are the essential macronutrients which is beneficial to human health. The present of protein and carbohydrate in the sample extract may contribute to the synergism effect of the bioactive compounds in the extract.

4.3.1 Protein concentration estimation

All freeze dried extracts were assayed for protein concentration estimation using BCA protein assay kit. Standard curve of the bovine serum albumin (BSA) was plotted to estimate the protein concentration of each extract (Figure 4.2).

Protein concentration in 100 µg/ml of each extract was determined by plotting the absorbance on the standard curve generated. Table 4.2 showed the estimated protein concentration of the extracts. Among the SH extracts, four extracts showed high protein concentration with CMDE exhibited the highest protein concentration with 68.97±4.42 µg/ml followed by DCME and EAE both at 68.21±2.18 and 68.21±1.09 µg/ml, and also protein fraction F90 at 66.54±0.54 µg/ml.

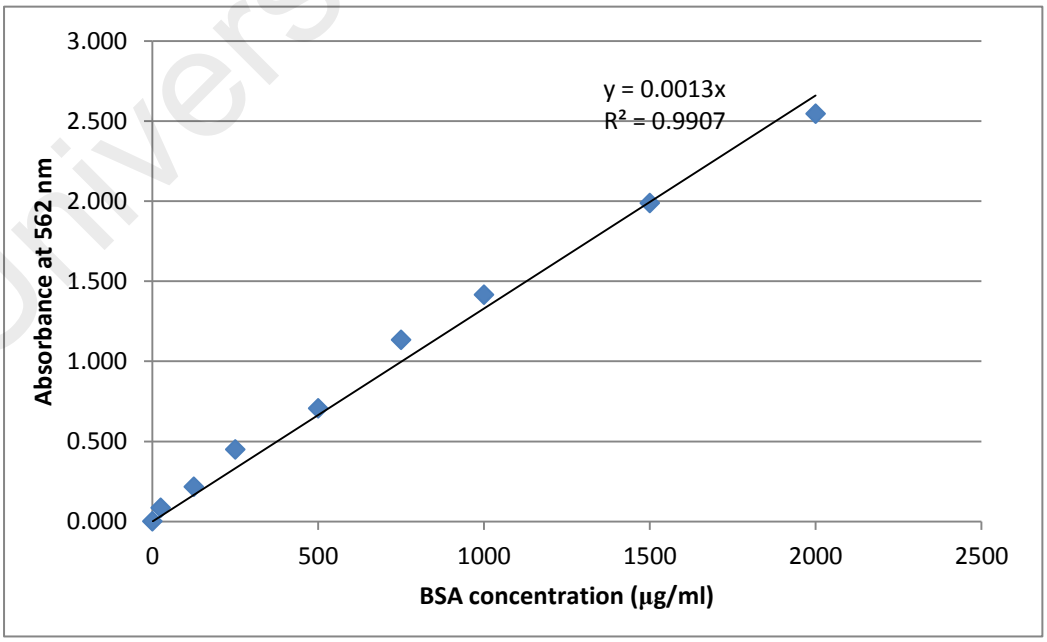

Figure 4.2: BSA standard curve

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On the other hand, the highest protein concentration in ST was protein fraction F90 at 178.97±1.63 µg/ml while the lowest protein concentration exhibited by DCME with 49.74±1.60 µg/ml. Comparing the SH and ST protein concentration, ST extracts contain more protein than the SH extracts.

4.3.2 Carbohydrate content estimation

A glucose standard curve was generated to serve as guide in estimating the carbohydrate content of the extracts. Figure 4.3 show the glucose standard curve of a known concentration of glucose.

The carbohydrate content of each extracts was determined by plotting the absorbance value on the standard curve and the result were tabulated in the following Table 4.2. Carbohydrate content of SH is in the range of 146.86±0.488 to 259.28±0.286 mg glucose/g extract, while in ST carbohydrate content is in the range of 147.38±0.210 to 201.70±0.165 mg glucose/g extract. In both SH and ST flask condition, the highest carbohydrate content was shown by the PE with 259.28±0.286 mg glucose/g extract and 201.70±0.165 mg glucose/g extract respectively.

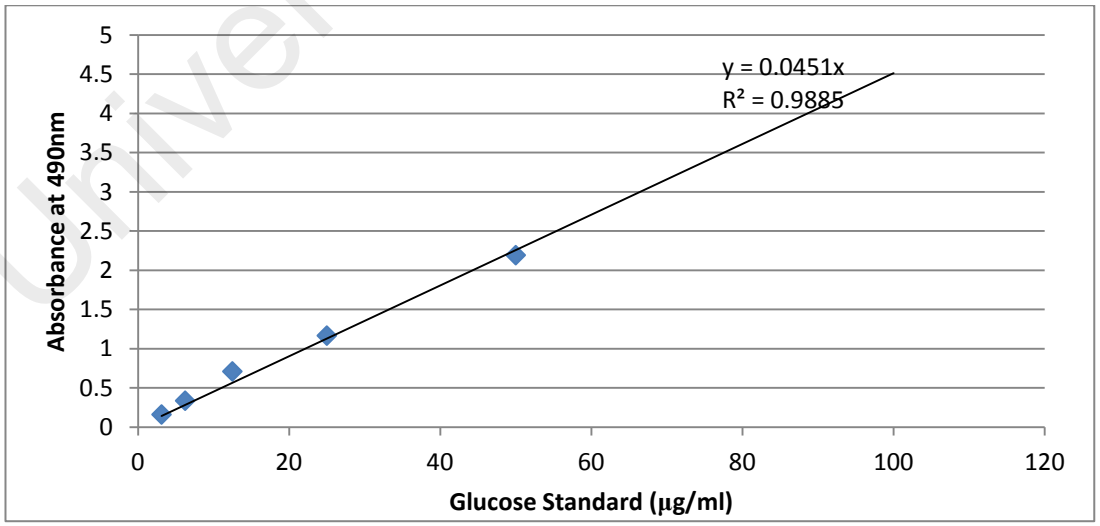

Figure 4.3: Glucose standard curve

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Table 4.2: Protein and carbohydrate concentration/content of SH and ST *S. commune* mycelial biomass

	Protein concentration (µg/ml)		**Carbohydrate content (mg glucose/g extract)**	
Extract	**Shake flasks (SH)**	**Static flasks (ST)**	**Shake flasks (SH)**	**Static flasks (ST)**
CMDE	$68.97\pm4.42_{a}$	$54.36\pm1.09_{e}$	$148.93\pm0.523_{e}$	$158.98\pm0.162_{d}$
HE	$46.67\pm3.26_{b}$	$50.26\pm2.18_{e}$	$146.86\pm0.488_{e}$	$149.15\pm0.178_{e}$
DCME	$68.21\pm2.18_{a}$	$49.74\pm1.60_{e}$	$150.18\pm0.023_{de}$	$149.15\pm0.184_{e}$
EAE	$68.21\pm1.09_{a}$	$84.62\pm0.54_{c}$	$149.15\pm0.132_{e}$	$147.38\pm0.210_{e}$
WRE	$51.79\pm1.09_{b}$	$73.33\pm2.18_{d}$	$149.52\pm0.447_{e}$	$149.08\pm0.262_{e}$
HWE	$48.21\pm0.44_{b}$	$81.79\pm3.55_{cd}$	$249.00\pm1.958_{a}$	$165.19\pm0.229_{c}$
PE	$44.36\pm0.54_{b}$	$56.92\pm1.33_{e}$	$259.28\pm0.286_{a}$	$201.70\pm0.165_{a}$
CWE	$64.62\pm2.31_{a}$	$83.59\pm1.63_{c}$	$172.51\pm0.124_{c}$	$154.77\pm0.130_{d}$
F30	$48.85\pm2.72_{b}$	$98.97\pm3.53_{b}$	$171.62\pm0.412_{cd}$	$157.06\pm0.094_{d}$
F60	$42.56\pm0.54_{b}$	$108.97\pm0.54_{b}$	$196.38\pm0.290_{b}$	$159.57\pm0.050_{d}$
F90	$66.54\pm0.54_{a}$	$178.97\pm1.63_{a}$	$168.00\pm0.191_{cde}$	$171.10\pm0.050_{b}$

Values are means of triplicate experiments. Means that do not share a letter are significantly different

Table 4.2 showed that protein concentration in F90-ST was the highest between the extracts tested and between culture conditions. This may because during the static flask culture condition, the lack of aeration and spatial homogeneity as well as the merging of growth phases in static cultures might have effects on the biosynthesis of secondary metabolites. Some metabolites are produced during suppressed condition such as ganoderic acid by *G. lucidum* in submerged cultures (Zhang & Zhong, 2013). Meanwhile, PE has the highest carbohydrate content compared to other extracts tested on both culture conditions. In PE, other debris or low molecular weight compounds in

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the extract were removed beforehand giving the extract in its semi-purify forms, this may contribute to its high carbohydrate content.

4.4 Antioxidant activities of *S. commune* extracts

Antioxidant activities need to be assessed using different methods in order to understand the mechanism of the extracts as antioxidant since there is no single testing method which is sufficient to estimate antioxidant activity of test samples (Huang *et al.,* 2005). In this study, four assays were conducted in estimating the antioxidant activities of the extracts.

Mechanism of antioxidant can be based on hydrogen atom transfer (HAT) or an electron transfer (ET). The HAT-based assays quantify hydrogen atom donating capacity, while the ET-based assays measure an antioxidant's reducing capacity. Folin-Ciocalteu, DPPH, CUPRAC and metal-chelating assays are ET-based mechanisms with different type of oxidant and redox reagents, on the other hand, inhibition of lipid peroxidation is a representative of HAT-based mechanism.

4.4.1 Phenolic content estimation

Phenolics have been suggested to be the main compounds responsible for the radical scavenging activity as the antioxidant activity is well correlated with phenolic content (Cheung *et al.*, 2003). Phenolic compounds may have one or more aromatic rings, with one or more hydroxyl groups, they can be found as simple molecules such as phenolic acids, phenylpropanoids and flavonoids, or as highly polymerized compounds such as lignin, melanin and tannins (Zárate-Chaves *et al.*, 2013). Polyphenols may act as antioxidants, with its ability to chelate metals, inhibit lipoxygenase and scavenge free radicals, the risk of atherosclerosis and coronary heart disease can be reduce (Tribble & Committee, 1999).

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For each extract, phenolic content was estimated by Folin-Ciocalteu assay, this assay measures the total reducing capacity of a sample. The Folin-Ciocalteu assay is a colorimetric method based on electron transfer reactions between the Folin-Ciocalteu reagent and any reducing substance such as phenolic compounds, some nitrogen-containing compounds, thiols, many vitamins, the nucleotide base guanine, the trioses glyceraldehyde and dihydroxyacetone, and some inorganic ions.

During the assay, the reaction between phenolic compounds and the Folin-Ciocalteu reagent takes place at pH 10, which is reached by sodium carbonate addition. Under the basic conditions, dissociation of a phenolic proton leads to the formation of a phenolate ion, which is capable of reducing the Folin-Ciocalteu reagent (Singleton & Rossi, 1965). It was reported that phenolic compounds were associated with antioxidant activity and play an important role in stabilizing lipid peroxidation (Keleş *et al.*, 2011).

This assay is convenient, simple and reproducible assay and it is a routine assay that is used in studying phenolic antioxidants (Huang *et al.*, 2005). A gallic acid standard curve as shown in Figure 4.4 was generated to serve as guide in estimating the phenolic acid content in the extracts and the result was expressed as mg of gallic acid equivalence (GAE) per gram of sample.

Based on the result in Figure 4.5, the highest phenolic content of SH was observed in CWE with 7.803±0.254 mg GAE/g extract and the lowest phenolic content observed in the protein fraction F60 with 1.637±0.095 mg GAE/g extract. On the other hand, the highest phenolic content for ST was protein fraction F90 with 15.037±0.392 mg GAE/g extract and the lowest was DCME extract with 3.034±0.185 mg GAE/g extract. All extracts were tested at 100 µg/ml concentration while Quercetin used as positive control was tested at 10 µg/ml which showed phenolic content of 5.404±0.032 mg GAE/g extract.

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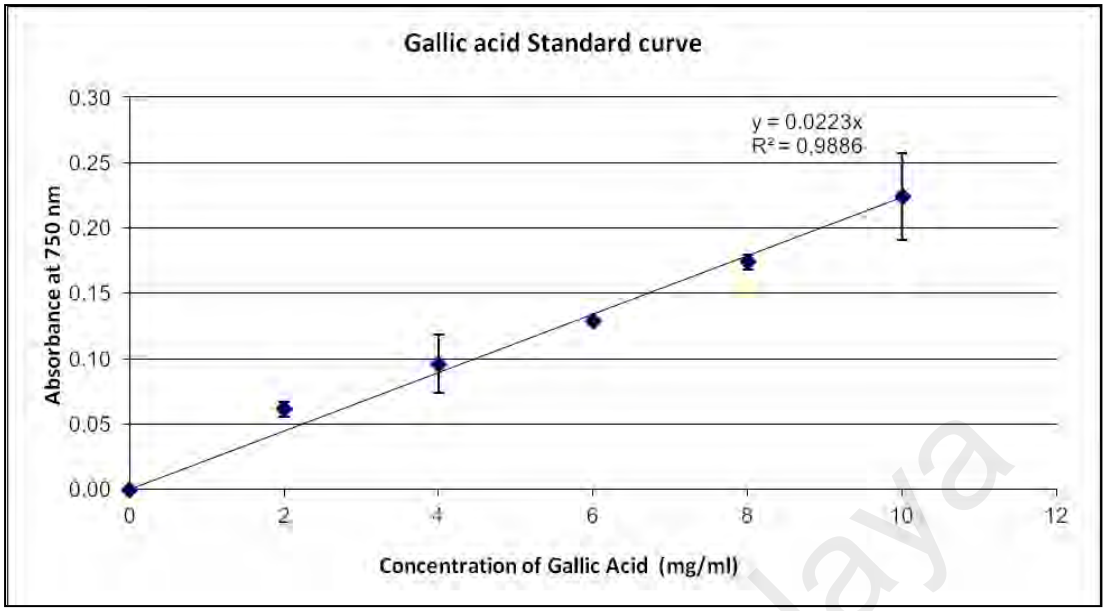

Figure 4.4: Gallic acid standard curve

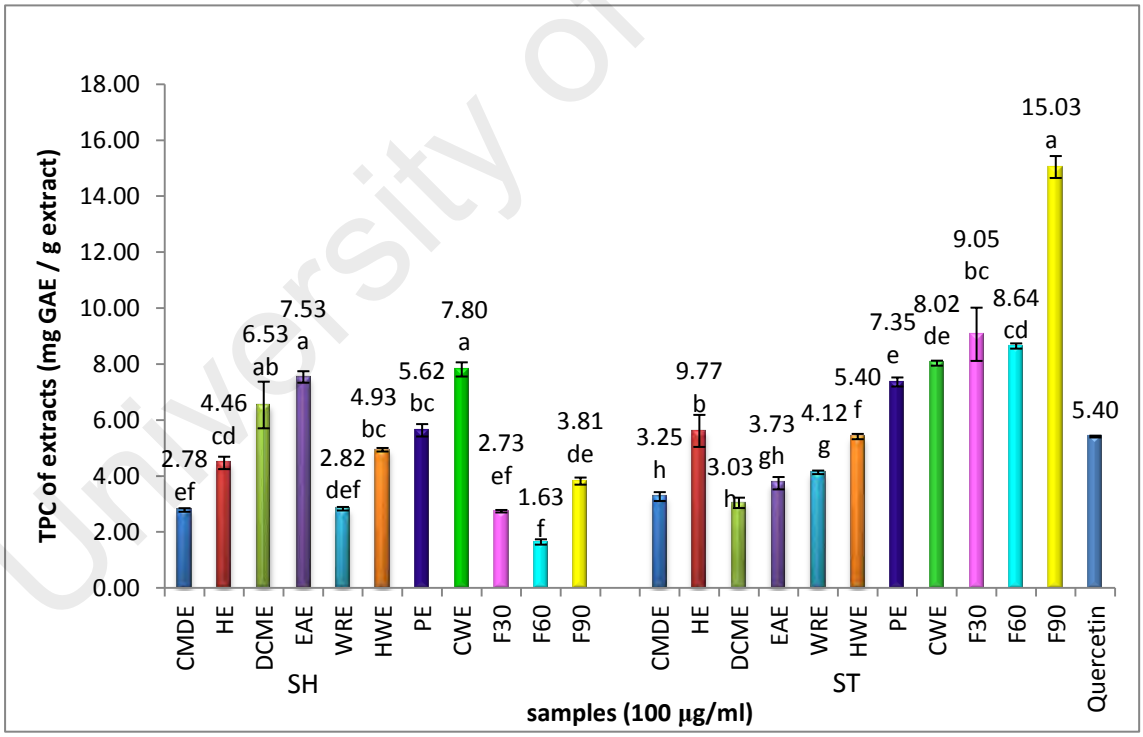

Figure 4.5: Folin-Ciocalteu assay of *S. commune* extracts at 100 µg/ml and Quercetin at 10 µg/ml. Means that do not share a letter in the same culture condition are significantly different.

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Tripathi and Tiwary (2013) reported that a mycelial ethanolic extract of the wild strain of *S. commune* from a region in India has total phenolic compounds of 0.573±0.06 Catechol equivalent μg/g fresh weight. While Rangkhawong (2014) reported that the total phenolic compound of *S. commune* ethanolic mycelium extract from a shake flask culture was 8.54±0.0 mg GAE/g extract, which is high compared to the highest phenolic content of the shake flasks extracts in this study which is CWE with 7.803±0.254 mg GAE/g extract.

On the other hand, *S. commune* mycelium grown in coconut water as media for static flask recorded the highest phenolic content of 25.52 mg ascorbic acid equivalent/g sample (Dulay *et al.*, 2016). In a study by Badalyan (2003) seven out of 14 mycelial samples of mushroom cultures including *S. commune* inhibit the reaction of free-radical peroxide oxidation of lipids in rat brain homogenate by more than 20% antioxidant activity. Meanwhile, *S. commune* fruit body hot water extract contained 16.47±0.42 mg GAE/g extract of phenolic was reported by Abdullah *et al.* (2012).

In another studies performed by Salahuddin (2008) and Wong and Chye (2009), methanol extract from *S. commune* fruit bodies reported to contained total phenolic content of 1.70±0.00 and 25.03±2.53 mg GAE/g extract respectively. On the other hand, highest phenolic content was found in DCM extract of *S. commune* fruiting body with 86.51±6.70 mg GAE/g extract by Mayakrishnan *et al.* (2013).

The antioxidant activities of mushrooms seemed to rely highly on the polarity of the solvent used in extraction and extracts using polar solvents such as methanol and water had tendency to have higher total phenolic content (Cheung *et al.*, 2003). Phenolics compounds reported to typically found in methanolic extract of several mushroom species indicating that most of the phenolic components in mushrooms were more polar and had lower molecular weight (Wong & Chye, 2009); contrasting

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with the finding of this study where phenolic content of CMDE for both SH and ST were low at 2.780±0.063 and 3.259±0.159 mg GAE/g extract, respectively.

CWE-SH had the highest phenolic when compared to other extracts in the same culture condition with 7.803±0.254 mg GAE/g extract, since water was used as extractant, the extract was expected to contain polar compounds which contributed to the high reading in Folin-Ciocalteu assay. Yeh *et al.* (2011) reported cold water extract of *G. fondosa* strain T1 and T2 both contained higher total phenols compared to the ethanolic extract with 39.78 ± 1.86 and 38.96 ± 1.14 mg GAE/g extract respectively. Moreover, Lee *et al.* (2007) reported that cold water extract of *Hypsizigus marmoreus* possessed 30.8 ± 0.45 mg GAE/g extract total phenols compared to the ethanolic and hot water extracts.

Protein also reported to possessed antioxidant activities such as in food proteins including milk proteins's lactoferrin and casein, potato patatin, maize zein and mushroom proteins (Arcan & Yemenicioğlu, 2007). As phenolic compounds have various subgroups distinguishing by a large structural diversity particularly phenolic acids, among frequently detected are *p*-hydroxybenzoic, protocatechuic, gallic, gentisic, vanilic acids, *p*-coumaric, caffeic and ferulic acids; which most of the acids are mostly bound in various complex structure (Kalač, 2009). This may explain the reason why protein fraction of F90-ST having highest phenolic content among the extracts tested.

4.4.2 Scavenging Effect on 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Radicals

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) is a stable free radical having an unpaired valence electron at one atom of nitrogen bridge and scavenging of this radical is the basis of the DPPH antioxidant assay (Sharma & Bhat, 2009). DPPH

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assay is a sensitive, easy to perform and offers a rapid way to screen radical scavenging activity of the isolated natural components, crude plant extracts and foods.

During the assay, the DPPH free radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors from antioxidants. Frequently used technique is the discoloration assay, where purple colour turned to yellow with respect to amount of DPPH present in the reaction mixture which evaluates the absorbance decrease at 515–528 nm (Szabo *et al.*, 2007).

In this study, both the SH and ST extracts showed DPPH anion scavenging power, as referring to Figure 4.6, at 100 μg/ml, highest radical scavenging activity for both culture conditions were shown by CWE with 20.94±1.93% and 16.93±2.65% respectively. Quercetin (10 μg/ml) as the positive control had the radical scavenging activity at 48.11±0.88%. Furthermore, IC_{50} for the SH and ST CWE were 38.46 mg/ml and 17.24 mg/ml, respectively compared to IC_{50} of the quercetin at 0.258 mg/ml.

Abdullah *et al.* (2012) reported that hot water extract of *S. commune* fruit body have IC_{50} of 35.659±0.055 mg/ml. Meanwhile, at 10 mg/ml of hot water extract and hot water polysaccharide extract from fruiting body of *S. commune*, 79.5±0.6% and 79.5±0.3% of DPPH radical scavenging activity were recorded respectively by Klaus *et al.* (2011). On the contrary, HWE of both culture conditions in this study showed DDPH scavenging activity at 7.30±0.83% and 5.77±0.72% respectively.

Previous study by Mayakrishnan *et al.* (2013) reported that ethyl acetate fraction of *S. commune* fruiting body showed the highest DPPH activity with 70.52±2.17 mg/ml. On the other hand, IC_{50} of *S. commune* mycelial ethanolic extract was documented at 50.95±1.45 ug/ml by Tripathi and Tiwary (2013).

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Reviewing DPPH inhibition percentage of other edible and medicinal mushrooms mycelia extract, *Omphalotus olearius* ethanolic extract showed radical scavenging effect (60.25%) at 1 mg/ml, meanwhile water extracts of *Chroogomphus rutilus* and *Rhizopogon roseolus* have 40.84% and 35.38% inhibition respectively (Kalyoncu *et al.*, 2010). According to Mau *et al.* (2004) the scavenging effects of *Termitomyces albuminosus*, *Grifola frondosa* and *Morchella esculenta* mycelia at 10 mg/ml were 78.8%, 79.4% and 94.1%, respectively. On the other hand, (Tseng & Mau, 2007) reported, at 1 mg/mL, the scavenging abilities of *Ganoderma tsugae* mycelia cold water extracts on DPPH radicals was 20.2%.

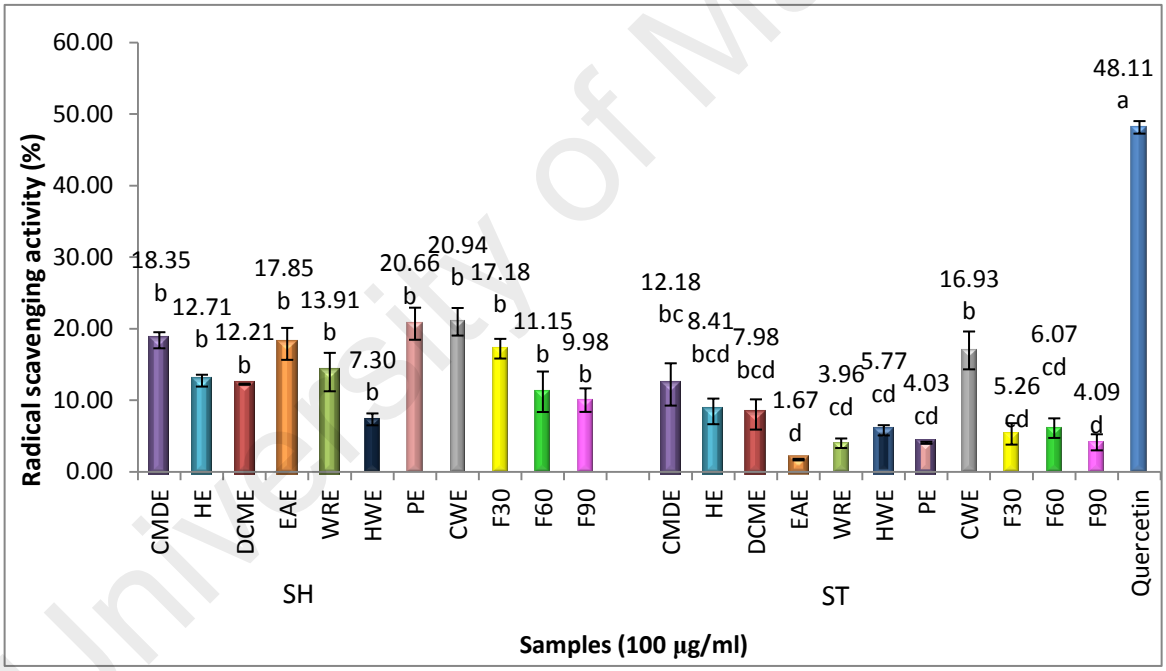

Figure 4.6: DPPH Radical Scavenging Activity (%). All extracts were tested at 100 µg/ml while Quercetin at 10 µg/ml. Means that do not share a letter in the same culture condition are significantly different.

4.4.3 Cupric-Ion-Reducing Antioxidant Capacity (CUPRAC)

The CUPRAC method is a simple and versatile antioxidant capacity assay because its reagent is stable, easily accessible, low-cost and selective besides capable

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to react to all types of antioxidants. The assay method is based on the reduction of a cupric neocuproine complex (Dimitrijevic *et al.*, 2015). A rise in the absorbance reading indicates higher cupric ion reducing power in an extract correspond with the the increase of orange-yellow colour formation.

CUPRAC measures chromophore absorbance, Cu(I)-neocuproine (Nc) chelate, formed as a result of the redox reaction of antioxidants with the CUPRAC reagent, copper(II)-neocuproine cation Cu(II)-Nc, where absorbance is recorded at the maximal light absorption wavelength of 450 nm (Özyürek *et al.*, 2011). CUPRAC assay is an electron-transfer based assays and it was proved to be efficient for glutathione and thiol-type antioxidants. Furthermore, this assay measures the antioxidant capacity at near pH7 compared to other antioxidant assays, it is better in stimulating the physiological action of the antioxidants (Özyürek *et al.*, 2011).

In this study, protein fraction F90 showed highest CUPRAC absorbance at 450 nm for SHFM and STFM with 0.064±0.00 and 0.420±0.00 respectively (Table 4.3). Meanwhile, the lowest CUPRAC absorbances were recorded by HWE-SH at 0.009±0.00 and HE-ST at 0.016±0.004 respectively. Quercetin (10 µg/ml) as the positive control gave absorbance value of 0.174±0.014 at 450 nm. Comparing the overall result for SHFM and STFM, STFM extracts showed higher absorbance compared to SHFM.

CUPRAC assay conducted by Mayakrishnan *et al.* (2013) on *S. commune* fruiting body showed that ethyl acetate extract have the highest absorbance at 0.38±0.03 at 1 mg/ml while EAE-ST in this study has 0.137±0.003 at 100 ug/ml extract. CUPRAC of *S. commune* fruit body hot water extract reported by Abdullah *et al.* (2012) showed that at 1 mg/ml and 10 mg/ml were 0.114±0.009 and 2.060±0.031, respectively. The ethyl acetate extract of *Tricholoma fracticum* was found to have 1.89± 0.05 absorbance at 450 nm when tested at 800 µg/ml (Tel *et al.*, 2012). Smith

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(2014) reported methanol extract at concentration of 10 mg/ml of *G. frondosa* mycelia have significant CUPRAC activity (2.343±0.048) as the positive controls α-tocopherol and BHT at 1 mg/ml (2.412±0.022 and 2.396±0.002 respectively).

CUPRAC method was able to demonstrate antioxidant capacity on both hydrophilic and lipophilic antioxidant, and this method is highly recommended in evaluating antioxidant capacity of natural extract rich in phenolic compounds (Apak *et al.*, 2007). Although proteins are not considered as true antioxidants but they are accepted to protect antioxidants from oxidation in various antioxidant activity assays. Çekiç *et al.* (2009) studied the contribution of proteins, especially thiol-containing proteins, where it may either respond to total antioxidant capacity directly *via* their free sulfhydryl or thiol (–SH) groups, or indirectly after protein denaturation through their exposed thiol groups.

Table 4.3: Cupric-Ion-Reducing Antioxidant Capacity (CUPRAC) of *S. commune* extracts.

Samples	CMDE	HE	DCME	EAE	WRE	HWE	PE	CWE	F30	F60	F90
SH	0.061±0.002 *a*	0.037±0.002 a*b*	0.011±0.001 *b*	0.023±0.001 *ab*	0.016±0.002 *ab*	0.009±0.000 *b*	0.025±0.006 *ab*	0.026±0.003 *ab*	0.053±0.001 *ab*	0.04±0.002 a*b*	0.064±0.000 *a*
ST	0.026±0.002 *ef*	0.016±0.004 *f*	0.044±0.001 *de*	0.137±0.003 *bc*	0.055±0.003 *de*	0.127±0.000 *c*	0.033±0.002 *def*	0.161±0.010 *b*	0.038±0.001 *def*	0.056±0.004 *d*	0.420±0.02 *a*

Values are the mean of three replication and the values that do not share a letter in the same row are significantly different $p<0.05$.

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The constituent of amino acids in the proteins are responsible for their antioxidant activity. The amino acids have the ability to donate protons to free radicals such as tyrosine, phenylalanine, tryptophan and cysteine; other than that, amino acids such as lysine, arginine, aspartate and glutamate can chelate metal ions as their antioxidant ability (Arcan & Yemenicioğlu, 2007). Moreover, amino acids positioning in the protein sequence can also determine the antioxidative properties. According to Chen *et al.* (1996), peptides having proline at the N-terminus more effectively prevent oxidation of linoleic acid than do peptides having proline at the C-terminus.

4.4.4 Metal chelating activity

Iron play an important role in cell functions, such as oxygen transport, cellular respiration, and is a co-factor for a number of iron metallic enzymes. Iron can be found either as ferrous (Fe^{2+}) or ferric ion (Fe^{3+}), the Fe^{2+} was found to cause the production of ROS. Therefore, chelation of ferrous ion is important antioxidant effects via retarding metal catalysed oxidation. Oxy-radical generation and its consequence oxidative damage can be prevented by the metal chelating capacity of an antioxidant compound (Srivastava *et al.*, 2006).

Iron can stimulate lipid peroxidation by the Fenton reaction, and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can abstract hydrogen and perpetuate the chain reaction of lipid peroxidation (Halliwell, 1991). Hence, Fe^{2+} chelators are important as they have the ability to protect against oxidative damage by preventing the production of radical species from the Fenton reactions as described below.

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + \cdot OH$$

In metal chelating activity assay, ferrozine was added which will form complexes with ferrous ion (Fe^{2+}). Fe^{2+}-Ferrozine complex can be disrupted by the

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presence of chelating agents resulting in decreased red colour of the complex. Measurement of colour reduction therefore allows estimation of the chelating activity of the coexisting chelator (Gülçin *et al.*, 2005). The lower the absorbance indicates higher metal chelating activity.

From the result in Figure 4.7, it is shown that the highest metal chelating activity for SH was CWE with 67.51±0.77% while the lowest was shown by EAE with 7.82±0.45%. For ST, the highest metal chelating activity was observed in PE with 30.02±1.23%, while the lowest activity was observed in CWE with 1.32±0.38%. Ethylenediaminetetra-aceticacid **(**EDTA) served as standard, as it is a strong metal chelator thus gave the highest metal chelating activity compared to all extracts (16.61±1.53%) at 10 μg/ml.

Wong and Chye (2009) reported methanolic extract of *S. commune* fruiting body having 50.0% metal chelating effect at 1 mg/ml. The chelating effect of the PE-ST in this study is considered higher with 30.02±1.23% at 100 μg/ml, when compared to HWP extract and PE extract from *S. commune* fruiting body reported by Klaus *et al.* (2011) and Wong and Chye (2009) with 72.9±0.3% and 60.0%, both at 20 mg/ml respectively. On the other hand, Mayakrishnan *et al.* (2013) found that ethyl acetate extract of *S. commune* fruit body have the highest metal chelating activity with 81.29±4.19% at 1 mg/ml concentration. Mau *et al.* (2005) reported that *Ganoderma tsugae* mycelia hot water extract chelated 4.9% of ferrous ions at 20 mg/ml, while its methanolic extract has chelating ability of 85.9% at 10 mg/ml.

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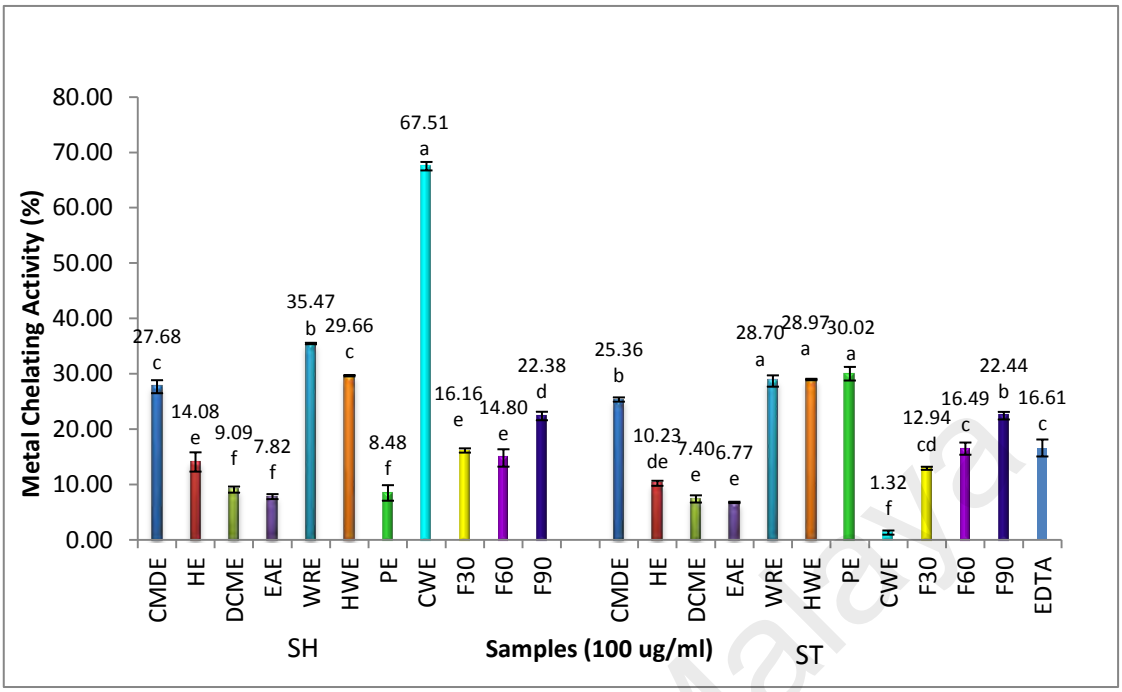

Figure 4.7: Metal chelating activity of *S. commune* extracts. Means that do not share a letter in the same culture condition are significantly different $p<0.05$.

Chelation activity of an extract has close relationship with its compounds structure, where chelating agents possess "ligand" binding atoms that form either two covalent linkages or one covalent and one co-ordinate or two co-ordinate linkages and mainly atoms like S, N and O function as ligand atoms in the form of chemical groups such as the hydroxyl (-OH), thiol (-SH), carboxyl (-COOH), carbonyl (C=O), phosphonic ($–PO_3H_2$) and amino ($–NR_2$) in a favourable structure-function configuration will more likely to have chelation activity (Flora & Pachauri, 2010; Yuan *et al.*, 2005).

Chelating agents may serve as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of metal ions. It is reported that iron chelators isolated from fungi have been hydroxamic acid derivatives and the screening of several white and brown rot fungi showed that Fe^{3+} chelating compounds were produced extracellularly in fungi grown in liquid, or on solid plating medium

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(Goodell *et al.*, 1997). Based on the finding of the metal chelating activity in this study, it is propose that the moderate ferrous ion chelating effects of the *S. commune* mycelia extracts could protect against oxidative damage.

4.4.5 Inhibition of Lipid Peroxidation

Lipid peroxidation occurs when free radicals oxidize polyunsaturated fatty acids in biological systems. This reaction starts a free radical chain reaction that produces highly reactive secondary products. The secondary products of the reaction can cause harm to biological components such as lysosomal destabilization, apoptosis and cellular changes, change in membrane structure and cellular necrosis and death (Ayala *et al.*, 2014) which can further engage in cellular disorders such as neurodegeneration, cardiovascular disease, and cancer (McIntyre & Hazen, 2010).

First-chain initiation of a peroxidation sequence in a membrane or polyunsaturated fatty acid results from the attack by any species with sufficient reactivity to abstract a hydrogen atom (H) from a methylene (-CH2-) group. Because a hydrogen atom contains only one electron, abstraction leaves behind an unpaired electron on the carbon, -CH-. The presence of a double bond in the fatty acid weakens the C-H bonds on the carbon atom adjacent to the double bond therefore the hydrogen in more susceptible to abstraction. The unpaired electron is stabilized by a molecular rearrangement of the double bonds to form a conjugated diene which then combines with oxygen to form a peroxyl radical. The peroxyl radical is itself capable of abstracting a hydrogen atom from another polyunsaturated fatty acid and starting a chain reaction (Halliwell & Gutteridge, 1984; McIntyre & Hazen, 2010; Repetto *et al.*, 2012).

Lipid peroxidation assay was done using egg yolk homogenates as lipid-rich media. Egg yolk lipids undergo rapid non-enzymatic peroxidation when incubated in

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the presence of ferrous sulphate. Malondialdehyde (MDA), a secondary end product of the oxidation of polyunsaturated fatty acids, reacts with two molecules of thiobarbituric acid (TBA) yielding a pinkish red chromogen with an absorbance maximum at 532 nm. This assay is one of the important assays as it shows the first line of antioxidant defence of the test sample, in which preventative antioxidants stops the formation of free radicals. Hence, the damage of fatty acids can be inhibited and prevent various diseases (Vimala *et al.*, 2003).

From Figure 4.8, both HWE of SH and ST showed highest inhibition of lipid peroxidation among the extracts tested with 26.40±0.57% and 20.07±0.78%. For SH culture condition, the lipid peroxidation inhibitory potency decreased in the order: HWE > HE > WRE > DCME > PE > EAE > CMDE > F90 > F30 > F60 > CWE. For ST culture condition, the lipid peroxidation inhibitory potency decreased in the order: HWE > PE > F30 > F90 > CWE > HE > EAE > F60 > CMDE > CWE > DCME. Quercetin as the positive control inhibit the lipid peroxidation with 7.67±0.19% at 10 µg/ml concentration compared to other extracts which were tested at 100 µg/ml extract concentration.

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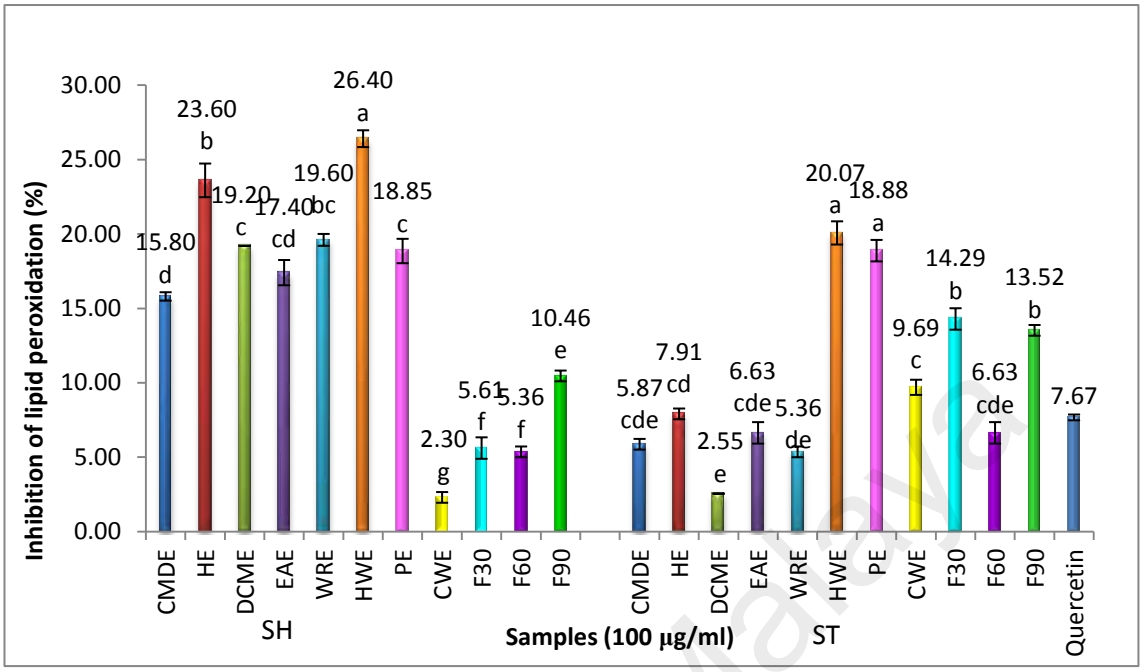

Figure 4.8: Inhibition of lipid peroxidation (%) of *S. commune* extracts. Means that do not share a letter in the same culture condition are significantly different.

Abdullah *et al.* (2012) reported the hot water extract of *G. lucidum* showed the highest inhibition of lipid peroxidation with 57.18%, whereas *S. commune* fruit body have an inhibition of lipid peroxidation of 36.24±9.41% at 10 mg/ml, in the same test. An ethyl acetate fraction of *S. commune* fruiting body exhibited the highest inhibition of lipid peroxidation (73.38±1.39%) compared to the positive control quercetin (59.91±1.19%) (Mayakrishnan *et al.*, 2013).

Methanolic extract of *Antrodia camphorate* and *Agaricus blazei* were reported to have 5.88-7.59% and 6.09% of lipid peroxidation inhibition at 5 mg/ml, respectively (Huang, 2000). On the other hand, Mau *et al.* (2001) studied the lipid peroxidation of several ear mushrooms and found that all studied ear mushrooms inhibit lipid peroxidation in the range of 38.6-74.6% at 1.0 to 5.0 mg/ml. *Ganoderma lucidum*, *G. lucidum* antler and *G. tsugae* methanolic extract were found to inhibit lipid peroxidation at 6.41%, 2.62% and 2.30% at 0.6 mg/ml respectively (Lin, 1999).

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According to Liu (2003), additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their potent bioactive properties and the benefit of a diet rich in fruits and vegetables is attributed to the complex mixture of phytochemicals present in whole foods. This explains why no single antioxidant can replace the combination of natural phytochemicals to achieve health benefits. The antioxidative activities could be ascribed to the different mechanisms exerted by different phenolic compounds and due to the synergistic effects of different compounds (Chang *et al.*, 2007).

4.5 Angiotensin-I Converting Enzyme (ACE) inhibitory activity

As one of the key elements in vasopressure action, ACE inhibitors work by preventing a chemical in the blood, angiotensin-I, from being converted into a substance that increases water and salt retention in the body, the angiotensin-II. Conventionally, ACE activity was determined by the UV reading of the hippuric acid produced from a synthetic substrate, Hyppuryl-His-Leu. Due to the complicated procedure and requirement of organic solvent, a more safe and simple modified method is more favourable. In this study, an enzymatic-based assay of ACE kit (Dojindo, Japan) was used where 3-Hydroxybutyryl glycylglycylglycine (3HB-GGG) served as a substrate for ACE where the amount of fragmented 3-hydroxybutric acid (3HB) cleaved from the substrate was calculated as the percentage of inhibition.

ACE inhibitor reported to originate from proteins or peptides, since the first ACE inhibitory peptides were discovered from snake venom. Since then, ACE inhibitory peptides have been isolated from numerous sources including dairy products such as milk, yogurt and cheese (FitzGerald *et al.*, 2004; López-Fandiño *et al.*, 2006), meat by-products (Jang & Lee, 2005), fish protein/ peptides (Fujita & Yoshikawa, 1999; Lee *et al.*, 2010), vegetables such as broccoli (Lee *et al.*, 2006),

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cereals (Shamloo *et al.*, 2015), soybean (Rho *et al.*, 2009), and mushrooms such as *Tricholoma giganteum* (Lee *et al.*, 2004b).

Figure 4.9 showed that at 100 μg/ml concentration of *S. commune* mycelial biomass extracts, the highest ACE inhibitor activity of SHFM was in WRE-SH with 58.20±1.81%, while the lowest ACE inhibitor activity was shown by EAE-SH with 13.19±1.74%. Meanwhile, the highest ACE inhibitory activity in STFM was in PE-ST with 56.67±1.79%, followed by CMDE-ST with 50.89±0.85% then by protein fraction F90 with 49.30±2.28%. The lowest inhibition was in protein fraction F30 with 7.51±0.85%. On the other hand, Captopril ($1x10^{-6}$ μg/ml) served as positive control, inhibited ACE with 97.34±0.38%.

IC_{50} values of the extracts with highest ACE inhibitory activity of SHFM and STFM were determined at the concentration of 0, 10, 20, 50, 100, 150 and 200 μg/ml. The result in Figure 4.10 showed that WRE-SH have an IC_{50} value of 70 μg/ml while PE-ST have an IC_{50} value of 82 μg/ml. IC_{50} of positive control, captopril was recorded at $1x10^{-9}$ μg/ml. From the inhibitory curve, the inhibition percentage was increased with the increased extracts concentrations. On the other hand, hot water extract from *S. commune* fruiting body was reported to show ACE inhibitory activity with IC_{50} of 0.320±0.070 mg/ml (Abdullah *et al.*, 2012).

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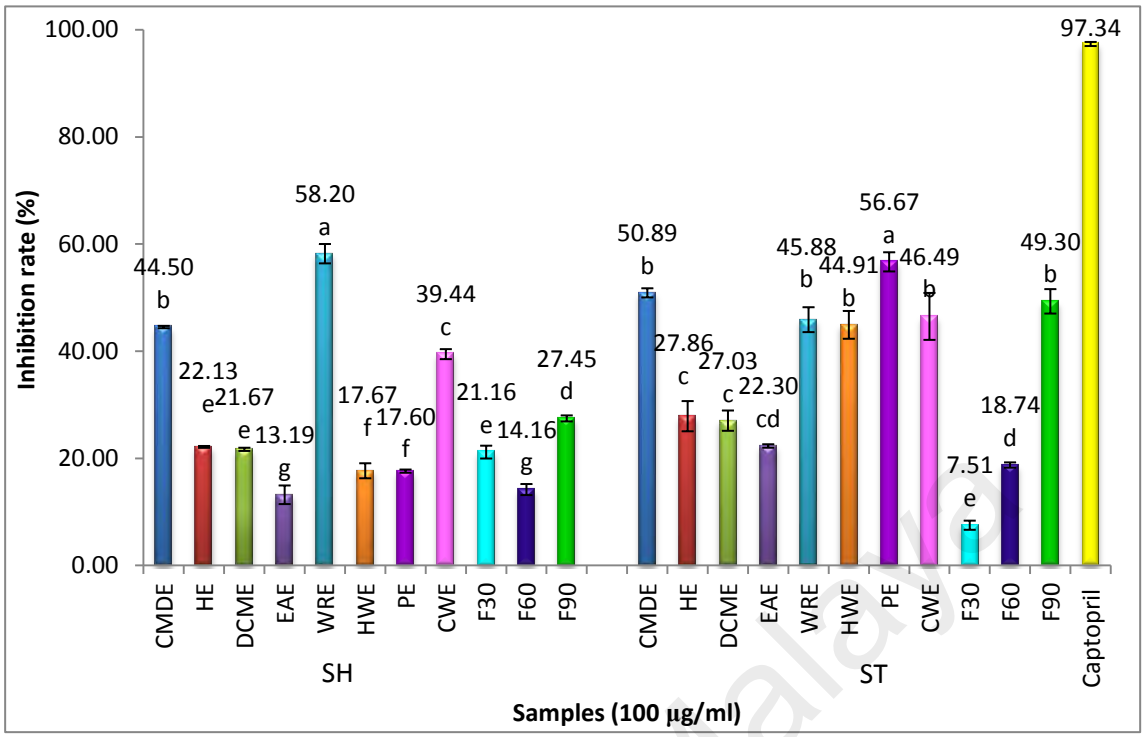

Figure 4.9: ACE Inhibitory activity. Means that do not share a letter in the same culture condition are significantly different.

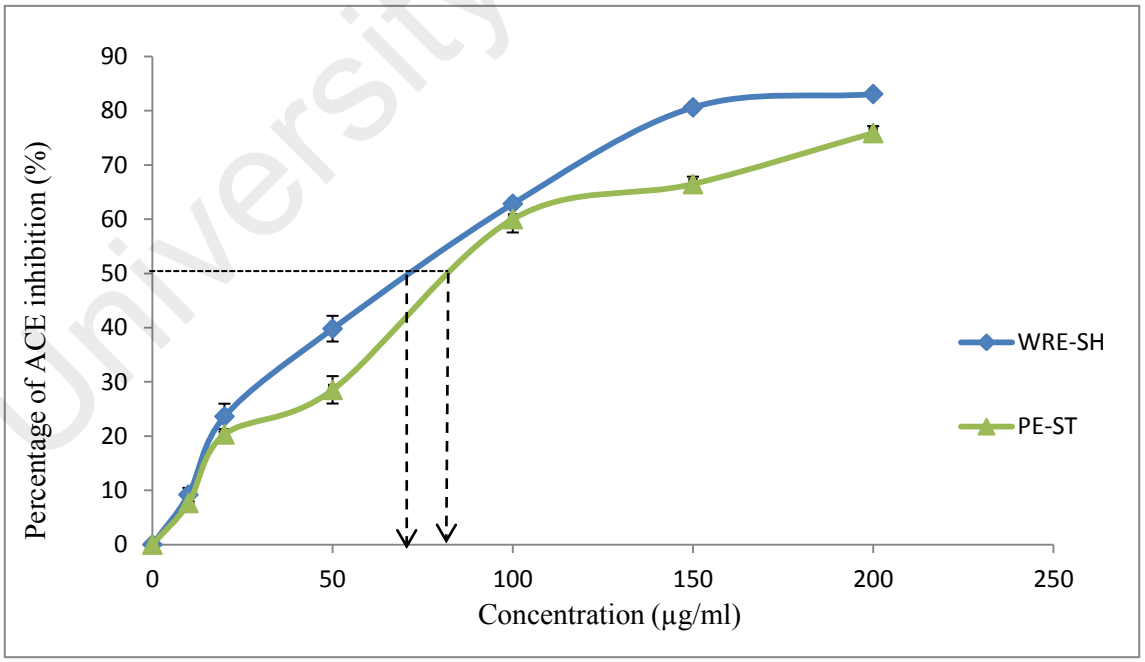

Figure 4.10: IC_{50} of ACE inhibition activity of WRE-SH and PE-ST extracts

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Mushrooms were extensively being studied as a source of hypotensive agents. Many mushroom extracts have been investigated for antihypertensive effect and the responsible bioactive compounds can come from several chemical groups such as polysaccharides, triterpenoids and proteins (Lindequist *et al.*, 2005). In addition, phenolic compounds from mushroom extracts can also play a role in ACE inhibitory activity as reported by Tran *et al.* (2014).

Morigawa *et al.* (1986) reported *G. lucidium* possessed antihypertensive property due to the presence of triterpenes and ganoderic acids. While Hagiwara *et al.* (2005) found that sugar alcohol from *Pleurotus cornucopiae* was able to inhibit ACE. Izawa and Aoyagi (2006) on the other hand reported that nicotinamine from *Rhodophyllus clypeatus* have the ACE inhibitory activity. The result from this study showed all extract gave high inhibition towards ACE, in lines with reports mentioned.

4.6 Identification of active compounds by LCMS

4.6.1 Identification of antioxidant compounds by LCMS

Liquid chromatography coupled with mass spectrometry (LC-MS) has been recognized as a powerful analytical tool with its high sensitivity, short run time and less use of toxic organic solvents as mobile phase compared to reversed phase stand alone high performance liquid chromatography (HPLC) coupled with Diode-Array Detector (Hossain *et al.*, 2010). LCMS also enables the analysis of very complex samples with a high degree of confidence with the presence of mass spectrometry (MS) where the mass-to-charge ratio (m/z) of charged particles measured.

From the result of the antioxidant assays conducted, potent extracts were selected and subjected to LCMS analysis. Table 4.4 show the extracts that have highest activity in each assay conducted. The mass fragmentations of the compounds were based on journal references and Advance Chemistry Development (ACD)/Labs

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advanced chemometrics mass fragmentations predictive software. For further confirmation of the compounds presence, reference standards or complimentary data from various analytical instrumentations can be use.

Initial investigation of LCMS/MS analysis showed that all sample extracts were found to contain rich amount of phenolics based compounds. *Follin-ciocalteu*, DPPH and metal chelating activity assays share common potent extract which is the CWE-SHF. The LCMS analysis of CWE-SHF showed that compounds such as gluconic acid, tryptophan and phenolic acid were identified. Figure 1.2 (a) and (b) in Appendix 1.2.1 shows the total ion current (TIC) chromatogram of CWE-SH CWE-ST, HWE-SH and HWE-ST, while the major peaks observed have been assigned in Table 4.5, 4.6, 4.7 and 4.8 respectively.

Table 4.4: Extracts exhibiting highest potency in each antioxidant assays

Antioxidant assay	Culture conditions	
	SHFM	**STFM**
Folin-Ciocalteu	CWE	F90
DPPH	CWE	CWE
CUPRAC	F90	F90
Metal Chelating Activity	CWE	PE
Inhibition of Lipid Peroxidation	HWE	HWE

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Table 4.5: Compounds identification in CWE-SH based on LCMS/MS

R_T(min)	m/z	Mass fragments, MS/MS	Suggested identification	Reference
0.52	195	177, 159, 129, 97, 87, 85, 75, 73	Gluconic acid	Felipe *et al.* (2014), Göger *et al.* (2015)
0.92	203	186, 159, 142, 130, 116, 104, 74, 59	Tryptophan	Lau *et al.* (2014), Ying *et al.* (2013)

In the LCMS analysis of CWE-SH of *S. commune* mycelia using the negative ion mode, the first peak at retention time of 0.52 minute was proposed as gluconic acid, based on the $[M-H]^-$ ion at m/z 195 and fragment ions at 177, 159, 129, 97, 87, 85, 75 and 73. Gluconic acid and its derivatives are produced by glucose oxidase from glucose in the presence of oxygen, and naturally found in fruit, honey and wine (Ahmad *et al.*, 2009). Gluconic acid is one of the antioxidant that can react with transition metals to form complexes and thus avoid the catalytic effect of the metals in oxidation process (Decker *et al.*, 2010).

At retention time of 0.92 minute, an amino acid with hydrophobic side chain, tryptophan, was identified from CWE-SH. Tryptophan exhibited a deprotonated molecular ion $[M-H]^-$ at m/z 203; a mass fragment at m/z 186 possibly corresponding to a loss of hydroxy group (OH) and further loss of carboxyl group (CO_2) gave a fragment at m/z 159. Tryptophan and several peptides were found to have higher radical scavenging capacity compared to butylated hydroxyanisole; tryptophan acted as antioxidant by serving as a hydrogen donor (Elias *et al.*, 2005).

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Table 4.6: Compounds identification in CWE-ST based on LCMS/MS.

R_T(min)	m/z	Mass fragments, MS/MS	Suggested identification	Reference
0.52	195	177, 159, 129, 101, 99, 87, 75, 73, 71	Gluconic acid	Felipe *et al.* (2014), Göger *et al.* (2015)
1.20	241	197, 169, 154, 141, 130, 112, 98, 84, 82, 66	2-(2-amino-3-imidazol-5-yl-propanoylamino)-3-hydroxypropanoic acid (Histidylserine)	Lau *et al.* (2014), Horai *et al.* (2010)
3.58	367	349, 323, 305, 261, 245, 235, 219, 195, 135, 123, 87,85, 69, 57	Quinic acid derivatives	NA
4.23	349	305, 287, 247, 221, 191, 137, 84, 83, 67, 57, 53	Phenolic acid	NA
5.55	311	239, 211, 197, 183, 170, 119	2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzene propanoic acid	Jamaludin (2013)

NA: not available

2-(2-amino-3-imidazol-5-yl-propanoylamino)-3-hydroxypropanoic acid or known as Histidylserine (HisSer) is a histidine-containing peptide which contains two N-atoms in the aromatic imidazole ring of the histidine moiety where one of it can be protonated in the biologically relevant pH range 5 – 7 (Reddy *et al.*, 2005). The antioxidant activity shown by histidine and its derivatives is due to the imidazole moiety of the molecule, which Kohen *et al.* (1988) concluded from their study that the hydrogen on the nitrogen ring and on the methylene carbon next to the imidazole ring are likely donors and the imidazolones themselves have antioxidant activity.

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Quinic acid or its conjugates was reported to possess DNA repair and/or immune enhancing properties, Pero *et al.* (2009) in their study showed that serum protein thiols can be increased in humans supplemented with quinic acid and that these effects mirrored DNA repair enhancement. Quinic acid also has been shown to have an anti-inflammatory mode of action, contributes to its antioxidant properties (Akesson *et al.*, 2005). Besides that, quinic acid is one of coffee's total acid content along with citric and malic acids, where the cleavage of chlorogenic acid moiety results the quinic acid formation.

Table 4.7: Compounds identification in HWE-SH based on LCMS/MS.

R_T(min)	m/z	Mass fragments, MS/MS	Suggested identification	Reference
0.52	195	177, 159, 129, 99, 97, 87, 75, 74, 73, 71, 59	Gluconic acid	Felipe *et al.* (2014), Göger *et al.* (2015)
0.658	151	136, 108, 95, 82, 66, 65, 62	Phenolic acid	NA

NA: not available.

Table 4.8: Compounds identification in HWE-ST based on LCMS/MS.

R_T(min)	m/z	Mass fragments, MS/MS	Suggested identification	Reference
0.52	195	177, 129, 99, 97, 87, 79, 75, 73, 59	Gluconic acid	Felipe *et al.* (2014), Göger *et al.* (2015)
1.19	225	207, 181, 179, 165, 153, 137, 112, 106, 82	Phenolic acid	NA
1.32	153	109, 108, 95, 91, 67, 65	Protocatechuic acid	Ju *et al.* (2010)

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Table 4.8, continued,

3.58	367	323, 261, 245, 87, 69, 59, 57	Quinic acid conjugate	NA
5.41	311	239, 225, 197, 183, 170, 119	2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzene propanoic acid	NA
11.05	175	147, 145, 133,118, 116, 103, 91	Phenylvaleric acid	NA

NA: not available

Protocatechuic acid is a dihydroxybenzoic acid phenolic compound found in many edible and medicinal plants. It is a major metabolite of antioxidant polyphenols found in plant such as green tea. Phenolic acids can occur in conjugated or esterified form in providing an efficient antioxidant activity. Protochatechuic acid is a form of hydroxylated phenolic acid with the introduction of hydroxyl group in the *ortho* or *para* position which is *o*-diphenol and is more powerful than its respective monophenol, *p*-hydroxybenzoic acid (Shahidi, 1997).

At retention time of 11.05 min, phenylvaleric acid was identified, it exhibited a deprotonated molecular ion $[M-H]^-$ at m/z 175. Phenylvaleric acid is a monophenolic acid that originated from degradation of polymers or scission of monomeric flavonoids forming low molecular weight aromatic acids such as phenylvaleric acid (Kasote *et al.*, 2015).

4.6.2 Identification of ACE inhibitory compounds by LCMS

WRE-SH had showed the highest ACE inhibition among all other extracts, therefore it was subjected for LCMS analysis. Based on the chromatogram obtained

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(Figure 1.2 (c) and 1.2 (d), Appendix 1.2.1), several compounds were detected and tabulated in Table 4.9.

Table 4.9: Compounds identification in WRE-SH based on LCMS/MS

R_T(min)	m/z	Mass fragments, MS/MS	Suggested identification	Reference
1.207	164	147, 119, 103, 72	Hydroxylated cinnamic acid	Mikhaeil *et al.* (2004)
1.652	203	186, 159, 142, 130, 116, 74	Tryptophan	Lau *et al.* (2014), Ying *et al.* (2013)
1.763	130	128	Leucine	Barnett *et al.* (1999)
2.99	997	954, 910, 872, 828, 746, 728, 524, 299, 282, 207, 168, 150	Dicarboxylic acid derivatives	Not Available
10.18	1034	1017, 998, 776, 692, 665, 452, 339, 321, 240, 221	Polysaccharide	Tissot *et al.* (2007)
4.107	265	249, 247, 177, 162, 150, 148, 143, 134, 109	Thiamine	Horai *et al.* (2010)

The compound detected at retention time of 1.207 min in the negative ion mode was proposed as hydroxylated cinnamic acid. The molecular ion peak present at m/z 164 correspond to the molecular formula $C_9H_8O_3$ while the peak at m/z 146 resulted from the dehydration of the parent ion [M-H_2O^+]. Decarboxylation of the parent ion had resulted in the peak at m/z 119 (Mikhaeil *et al.*, 2004). Moreover, cinnamic acid derivatives were pharmacologically evaluated for its potent antioxidant, antihyperglycemic and peroxynitrite scavenging effects; furthermore, hypertensive rats treated with cinnamic acid derivatives demonstrated attenuated hypertension and improved cardiovascular function (Silambarasan *et al.*, 2014).

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At 1.763 min, a peak with molecular ion $[M-H]^-$ at m/z 130 was suspected to be amino acid leucine. Leucine is a branched-chain essential amino acid and has an indirect effect on lowering blood pressure through its influence on a number of metabolic processes including insulin signalling. One of several metabolic alterations and downstream effects that increase blood pressure was insulin resistance, where leucine increases protein synthesis in skeletal muscle and improves insulin resistance by modulating hepatic gluconeogenesis by contributing to the de novo synthesis of glucose (Vasdev & Stuckless, 2010).

Tryptophan was proposed as the compound representing peak at retention time of 1.652 min. It exhibited a deprotonated molecular ion $[M-H]^-$ at m/z 203; a mass fragment at m/z 186 possibly corresponding to a loss of hydroxy group (OH) and further loss of carboxyl group (CO_2) gave a fragment at m/z 159. Tryptophan was reported by several studies to lower blood pressure in rat models with hypertension. Fregly *et al.* (1989) pointed the important fact regarding the antihypertensive effect of tryptophan where it can prevented or attenuated all major types of experimentally induced hypertension in rats.

At retention time of 10.18 min, compound with m/z 1034 was suggested to be a polysaccharide. Thiamine was detected in the positive ion mode with m/z 265 at retention time of 4.107 min. Thiamine or vitamin B1 was reported to improves vasodilation in patients with high blood sugar or diabetes, which can enhance the vasodilatory effect of adrenergic blocking agents and lower blood pressure (Hechtman, 2012).

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4.7 Partial Purification of selected extracts using ultracentrifugal filter device and ACE inhibitory activity

To further study the antihypertensive activity of *S. commune* mycelial biomass extracts, the extracts with high inhibition activity from the preliminary ACE test were chosen to be separated using Amicon® Ultra-0.5 centrifugal filter devices (Merck Millipore Ltd.). WRE-SH, CMDE-ST, PE-ST and F90-ST were separated by the filter device with the nomial molecular weight cut off 10 kDa.

Basically, the filter unit consist of two microcentrifuge tubes, during operation, one tube is used to collect filtrate; the other one to recover the concentrated sample (Figure 3.2). The membranes used in Amicon® Ultra devices are characterized by a nominal molecular weight limit (NMWL); that is, their ability to retain molecules above a specified molecular weight. At the end, >10 kDa and <10 kDa fraction of each sample extracts were obtained and labelled as in Table 4.10 below.

ACE inhibition assay of the extracts was then determined as shown in Figure 4.11. All proteins showed positive inhibition towards ACE with the highest inhibition by F90-ST <10 kDa extract with 32.62±0.70% at 50 µg/ml. The IC_{50} value of the F90-ST <10 kDa was further determined at 0, 5, 10, 25, 50, 100, 150 and 200 µg/ml and the result in Figure 4.12 showed the IC_{50} was at 120 µg/ml.

Table 4.10: Sample extracts obtrained after separation by Amicon® Ultra-0.5 centrifugal filter unit with nomial molecular cut off 10 kDa

Sample obtained with 10 kDa cut off	
<10 kDa	**>10 kDa**
WRE < 10 kDa	WRE > 10 kDa
CMDE < 10 kDa	CMDE > 10 kDa
PE < 10 kDa	PE > 10 kDa
F90 < 10 kDa	F90 > 10 kDa

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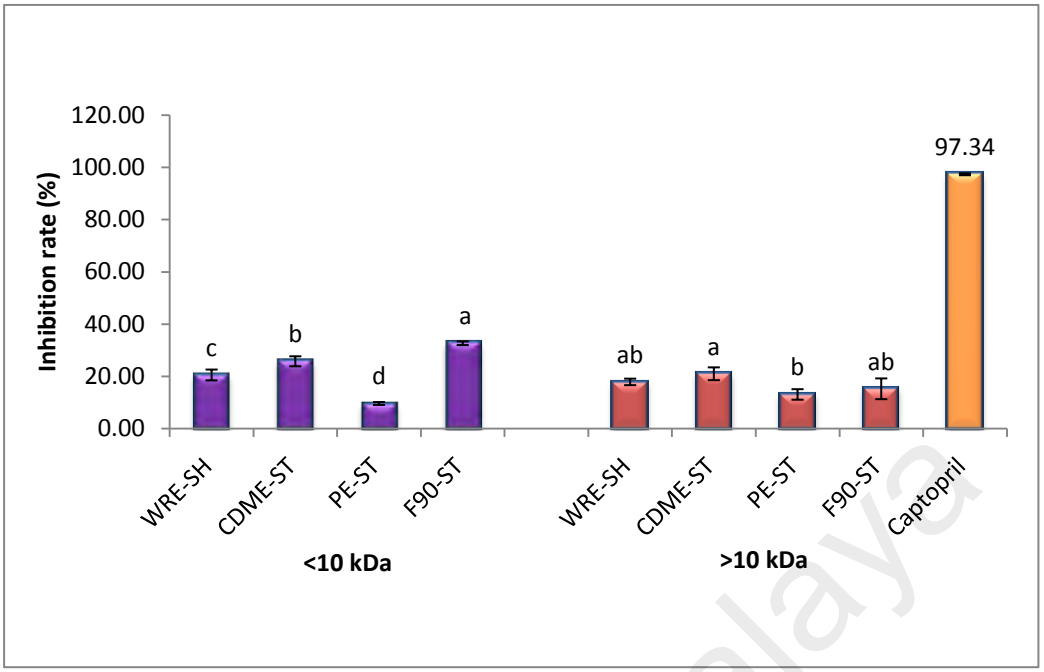

Figure 4.11: ACE inhibition activity of separated extracts (%) at 50 µg/ml, Captopril as positive control at 10 µg/ml. Means that do not share a letter are significantly different ($p<0.05$).

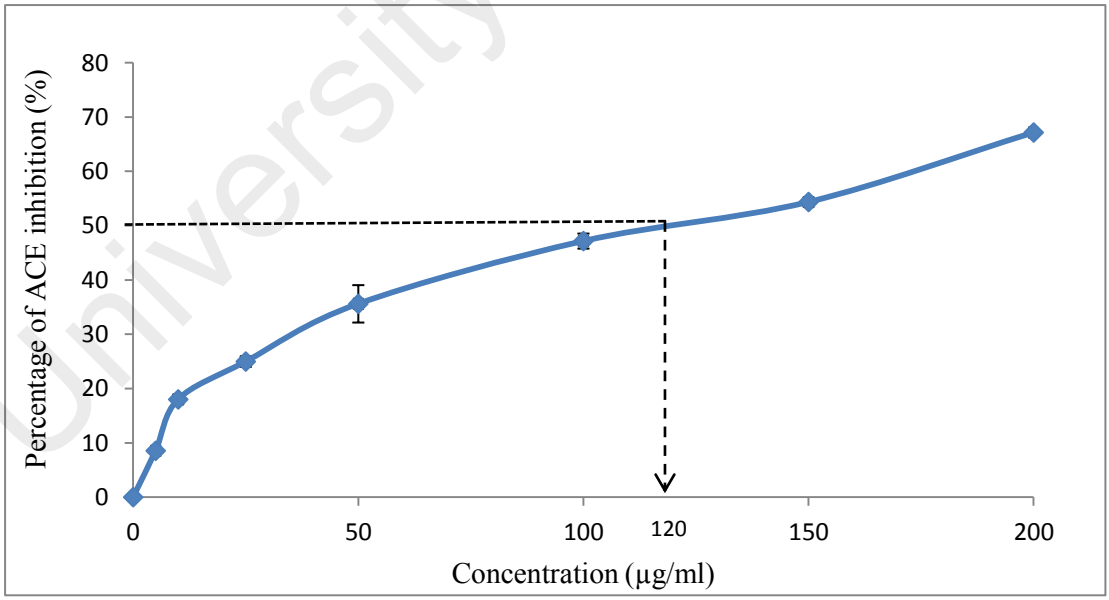

Figure 4.12: IC_{50} value of ACE inhibition activity of F90 <10 kDa extract

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ACE inhibitors from other natural sources have been reported to be in the low molecular weight fraction as reported by previous studies (Kinoshita *et al.*, 1993{Rho, 2009 #480)}. Pihlanto *et al.* (2012) reported that ACE inhibition of fermented rapeseed and flaxseed meals was found in low molecular weight in the range of 300 to 500 Da. Furthermore, ACE inhibitor from plants, foods, microbial sources, marine organisms and other natural sources have been found mainly in the form of bioactive peptides therefore, it is interesting to discover that F90 <10 kDa extract also exhibited high ACE inhibition among the extracts assayed.

Extracts separation using ultracentrifugal unit were executed to observe the inhibition of ACE from high molecular weight extracts (>10 kDa) and also the low molecular weight extracts (<10 kDa). From the result, protein fraction F90 <10 kDa showed highest inhibition of ACE suggested that separating extract performed better inhibition compared to the non-separated extract, 30.03±2.96% at 50 μg/ml and 49.30±2.28% at 100 μg/ml, respectively.

A study conducted by Jeon *et al.* (1999) showed that separation of cod frame protein hydrolysate by ultrafiltration membrane had high antioxidant activity (10 kDa) and was excellent in ACE inhibitory activity (3 kDa). This is mainly due to the reason that active components should possess high activity at low concentrations when it is present in the isolated form. Di Bernardini *et al.* (2012) also reported that 3-kDa filtrates of bovine brisket sarcoplasmic proteins using ultrafiltration had higher ACE-I inhibitory activity (40.64 ± 7.05%**)** compared to the non-ultra filtered hydrolysate at 20 mg/ml.

In order to obtain a peptide fraction with both a desired molecular size and a functional property, ultrafiltration membrane system has the major advantage, where the molecular weight distribution of the desired peptide can be controlled by adoption of an appropriate ultrafiltration membrane molecular cut-off. ACE inhibitory activities

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were reported to increase with the lowering of molecular weight as shown in a study by Jeon *et al.* (1999), the IC_{50} value of the 3-K hydrolysate was recorded at 0.08 mg protein/ml and was approximately threefold lower compared to the original hydrolysate of cod frame protein.

ACE inhibitory peptides can exhibit the ACE activity either by competing with substrate where it can bind to the active site to block it or to the inhibitor binding site that is remote from the active site to alter the enzyme conformation such that the substrate no longer binds to the active sites. Example of these peptides includes tryptophan, tyrosine, proline or phenylalanine at the C-terminal and branched-chain aliphatic amino acids at the N-terminal of the peptide sequence (Cushman & Cheung, 1971).

Besides that, the ACE inhibitory peptide can combine with an enzyme molecule to produce a dead-end complex by blocking the active site. The hydrophobicity of the N-terminus, which is one of the common features of these peptides, may contribute to the inhibitory activity (Rho *et al.*, 2009). ACE inhibitory peptides are generally short chain peptides, and binding to ACE is strongly influenced by the C-terminal tripeptide sequence of the substrate, and it is suggested that peptides, which contain hydrophobic amino acids at these positions, are potent inhibitors (Wijesekara & Kim, 2010).

4.8 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE)

SDS PAGE is most widely used analytical method to resolve separate components of a protein mixture. SDS PAGE simultaneously exploits differences in molecular size to resolve proteins differing by as little as 1% in their electrophoretic mobility through the gel matrix. The technique is also a powerful tool for estimating the molecular weights of proteins.

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Since F90-ST <10 kDa extract had the highest ACE inhibition activity, the protein profiling by SDS PAGE was executed. From the SDS-PAGE result, F90-ST <10 kDa sample was resolved into several protein bands by the 18% polyacrylamide gel. First, the gel was stained with Coomassie blue where 16 protein bands appeared and later when stained with silver nitrate, 20 protein bands were exhibited on the gel (Figure 4.13).

Several protein bands appeared on the gel suggested that different peptides may be working together creating synergistic effect on ACE inhibition activity. Previous study on ACE inhibition activity also showed that synergistic effect of peptides/ proteins in a sample (Choi *et al.*, 2001). Afterwards, protein bands were subjected for LCMS QTOF analysis for protein identification.

4.9 Protein identification by LCMS-QTOF

Determination of the protein profile in a sample can be accomplished using the mass spectrometry (MS). Among the MS platforms, LCMS-QTOF mass spectrometry offers high sample throughput and capability of identifying proteins from one- and two-dimensional SDS-PAGE gels and also in solution sample. Protein can be identified by the masses of constituent peptides, the list of observed peptides masses was matched with a calculated list of all the expected peptide masses for each entry in a database (Karpievitch *et al.*, 2010).

Protein bands from the SDS PAGE were cut and digested individually; and upon zip tip the bands were pooled into five groups, based on their appearance on the gel. Group A represent protein bands of above 50 kDa, group B represent protein bands 6, 7, 8, 9 and 10 which were around 15 kDa to 50 kDa, protein bands 11, 12 and 13 were grouped together denoted as group C, meanwhile, group D corresponded to bold protein bands 14, 15, 16 and 17 that can be seen clearly on the gel, lastly, group

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E consisted of protein bands 18, 19, 20 and the rest of protein bands towards the end of the gel which may not appeared clearly (Figure 4.13(b)).

The five groups of protein bands (A, B, C, D and E) were subjected to LCMS-QTOF for peptide identification. Table 4.11 displayed the proteins/peptides that were identified from Swissprot database search. Table 4.12 on the other hand lists the proteins/peptides that potentially have antihypertensive effects.

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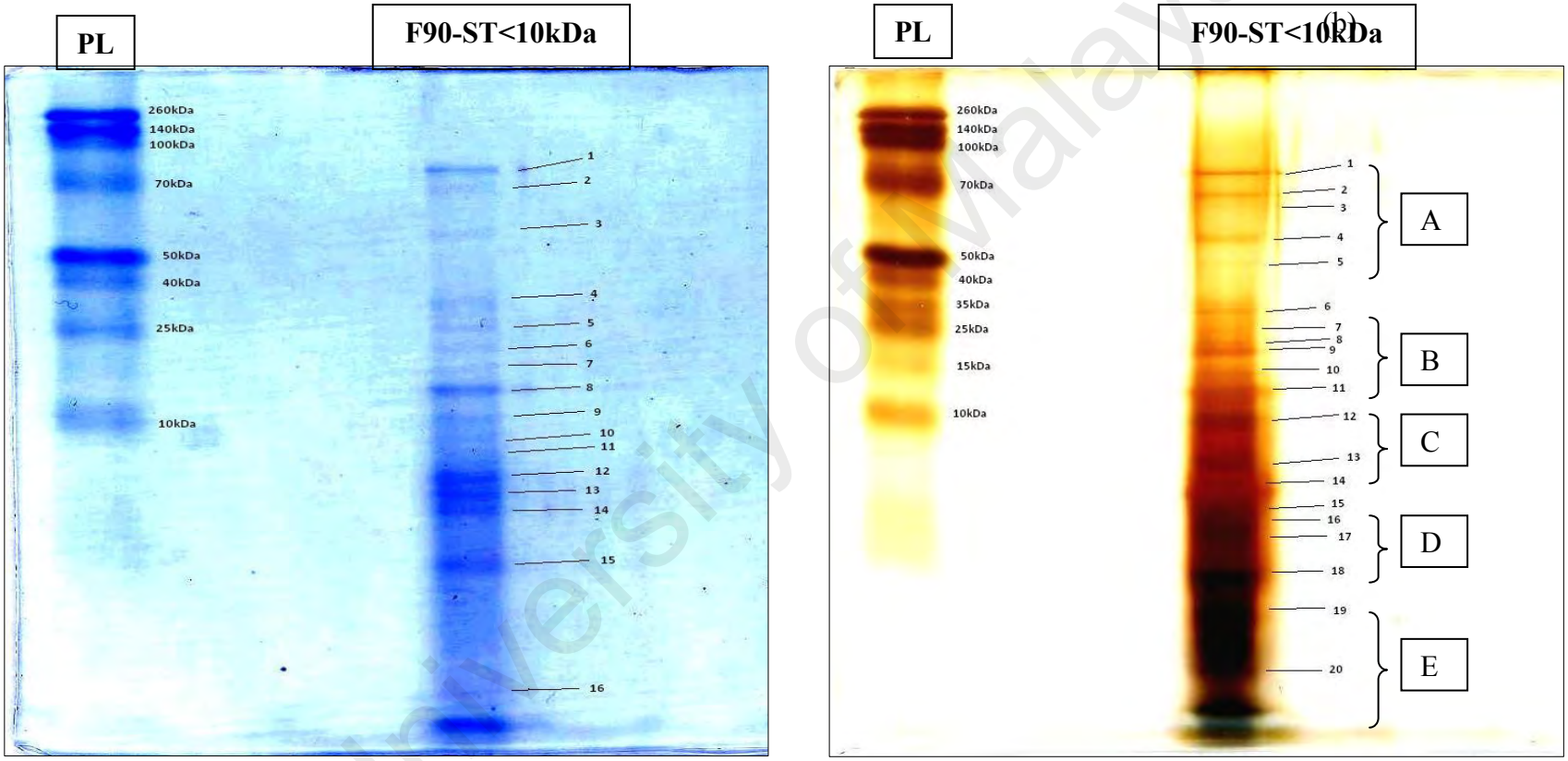

Figure 4.13: SDS PAGE profile of F90-ST <10 kDa extract stained with (a) Coomassie blue and (b) silver nitrate. Lane PL: protein ladder and lane F90-ST<10kDa: 100 µg/ml F90-ST<10 kDa extract in sample buffer with DTT. A: Protein size >50 kDa, B: Protein size 15-50 kDa, C: Protein size 8-10 kDa, D: Protein size 5-8 kDa, E: Protein size <5 kDa

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Table 4.11: Protein identified by LCMS/MS QTOF

Group	Protein candidates	Database Accession No.	Distinct summed MS/MS search score	% Amino acid coverage	MW (Da)	Protein PI	Distinct peptides No.
1	Glycoside hydrolase family 93 protein	D8QFW8	214.66	45.6	39315.4	4.57	11
2	Glycoside hydrolase family 31 protein	D8PUK9	172.19	15.5	100563.4	4.63	10
	Uncharacterized protein	A0A067TQG8	33.44	2.2	98756.5	5.75	2
	Uncharacterized protein	J4GCB0	33.44	2.2	98189.2	4.66	2
3	Putative uncharacterized protein	D8PQZ6	152.57	23.5	56299.5	4.57	8
4	Glycoside hydrolase family 3 protein	D8PU51	126.84	10.6	98151.1	4.49	7
5	Putative uncharacterized protein	D8PRI9	125.44	14.1	67095.9	4.52	6
6	Alpha-1,2-Mannosidase	D8QH88	123.32	15.9	59878.3	4.31	6
7	Glucoamylase ARB_02327-1	D8Q9M3	117.11	18.2	61225.0	4.46	6

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Table 4.11, continued,

8	Alpha-galactosidase	D8PQ92	112.41	13.5	55260.9	4.69	7
9	Carbohydrate-binding module family 13 protein (fragment)	D8Q7S3	104.69	25.1	33006.6	6.24	5
10	Endo-1,4-beta-xylanase	D8PN69	104.26	39.4	24223.8	5.20	5
	Endo-1,4-beta-xylanase A	P35809	104.26	45.6	21092.2	5.16	5
11	Putative uncharacterized protein	D8Q9N4	100.04	14.3	57832.3	4.31	5
	Alpha/beta-hydrolase	R7SVB2	22.29	3.1	5650-3.3	4.57	1
	Carboxypeptidase	V2XLJ0	22.29	3.2	56142.0	4.64	1
12	Putative uncharacterized protein	D8QFR9	98.38	15	53844.7	4.35	5
13	Putative uncharacterized protein	D8PSG6	86.70	13.6	61585.2	4.64	5
14	Putative uncharacterized protein	D8PZU4	83.86	20.1	27779.2	4.14	4
15	Putative uncharacterized protein	D8QLX5	82.67	22.5	38067.3	4.12	5

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Table 4.11, continued,

16	Putative uncharacterized protein	D8Q1P7	81.82	58.4	15070.6	6.03	4
17	Putative uncharacterized protein	D8Q7W8	79.43	34.7	27750.1	4.32	5
	Related to pathogenesis-related protein PR5K (Thaumatin family)	G4TJ49	32.57	8.8	28545.4	4.93	2
	Pathogenesis-related protein PR5K (Thaumatin family)	X8JDR4	21.07	5	27885.6	7.82	1
	Thaumatin-like protein	S7PTS7	21.07	5	27835.2	4.64	1
	Pathogenesis-related protein 5	A0A0B7F6I4	21.07	5.1	27704.4	6.57	1
	Uncharacterized protein	A0A0C3PQB4	21.07	5.1	27351.0	5.71	1
18	Expressed protein	D8PX57	75.90	40.7	19048.1	5.19	4
19	Putative uncharacterized protein	D8PRJ2	75.76	13.7	59879.1	4.58	4
20	Carbohydrate-binding module family 13 protein	D8PNF6	75.45	45.3	17182.9	4.31	4

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Table 4.11, continued,

21	Glucanase	D8QGE7	73.11	10.9	41099.6	4.37	4
22	Glycoside hydrolase family 18 protein	D8QLJ3	72.68	13.8	49837.2	5.03	4
23	Glycoside hydrolase family 114 protein	D8PVE3	72.17	28.6	28493.6	4.21	5
24	Putative uncharacterized protein	D8PJY8	70.65	8.4	56730.3	4.50	4
25	Glycoside hydrolase family 43 and carbohydrate-binding module family 35 protein	D8PL55	66.65	10.5	48132.9	4.59	4
26	Putative uncharacterized protein (Fragment)	D8QDQ6	64.25	26.4	19878.5	8.02	3
27	Glucanase	D8PLS5	63.18	13.8	50263.7	4.23	4
28	Glycoside hydrolase family 16 protein	D8Q6U1	60.33	15.1	33979.8	4.43	3
29	Putative uncharacterized protein	D8PU43	54.33	16.8	37497.3	4.84	3
30	Putative uncharacterized protein	D8QI38	53.81	6.4	63728.3	4.49	3

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Table 4.11, continued,

31	Glycoside hydrolase family 3 protein	D8Q6V5	53.32	5.9	81254.7	4.09	3
32	Expressed protein	D8Q1J2	51.68	18.4	25443.0	9.02	3
33	Putative uncharacterized protein	D8PXE8	51.27	6	60810.2	4.55	2
	Putative uncharacterized protein	D8PXE9	24.30	2.5	61832.0	4.69	1
34	Putative uncharacterized protein (Fragment)	D8PQS8	50.91	24.4	15586.3	4.42	3
35	Putative uncharacterized protein	D8PUZ1	41.43	12	13447.9	4.05	2
36	Glycoside hydrolase family 18 protein	D8QCH6	41.35	14.5	36358.5	4.26	2
37	Putative uncharacterized protein	D8QDL8	40.87	6.8	35736.9	4.53	2
38	Putative uncharacterized protein	D8PK56	40.11	16.7	19541.0	5.10	2
39	Putative uncharacterized protein	D8QLT4	39.29	9.9	22242.7	5.01	2
40	Putative uncharacterized protein	D8Q666	38.62	11.4	36976.6	4.78	2

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Table 4.11, continued,

41	Putative uncharacterized protein	D8Q3J7	37.41	22.5	16737.2	6.26	2
42	Putative uncharacterized protein (Fragment)	D8PUH8	35.72	34.1	9134.1	5.62	2
43	Glycoside hydrolase family 76 protein (Fragment)	D8QAB5	35.61	10	39722.1	4.46	2
44	Glycoside hydrolase family 16 protein (Fragment)	D8Q9E0	35.26	14.8	23396.1	4.27	2
45	Putative uncharacterized protein	D8Q6D5	35.08	8	43514.0	4.10	2
46	Putative uncharacterized protein (Fragment)	D8PN77	34.71	9.6	34457.4	4.22	2
47	Non-Catalytic module family EXPN protein (Fragment)	D8PUF8	34.54	28.4	13794.2	6.82	2
48	Cytolysin	D8PSZ1	34.36	6.1	31973.8	5.25	2

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Table 4.11, continued,

49	Putative uncharacterized protein	D8QJC3	33.29	11.1	38712.7	4.34	2
50	Putative uncharacterized protein	D8PRQ8	33.06	6.5	47983.1	4.40	2
51	Alpha-amylase	D8Q8W0	30.27	5.1	53073.1	4.79	2
52	Putative uncharacterized protein	D8QHI9	29.61	7.4	32248.8	4.07	2
53	Glycoside hydrolase family 45 protein	D8QEK9	29.16	7.6	24269.2	4.48	2
54	Uncharacterized protein	W9CPR3	29.01	5.2	65652.2	5.49	2
55	Glycoside hydrolase family 18 protein	D8PS75	26.91	4.8	44695.4	4.10	1
56	Defence-related protein SCP domain-containing protein (Fragment)	D8Q6B5	25.69	9.6	19173.6	5.17	1
57	Pectinesterase	D8PYT0	24.20	4.6	35028.6	4.27	1

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Table 4.11, continued,

58	Putative uncharacterized protein (Fragment)	D8PK60	22.79	8.8	16806.1	4.65	1
59	Putative uncharacterized protein (Fragment)	D8QLX4	22.51	10.9	20425.0	4.50	1
60	Putative uncharacterized protein	D8PK53	21.71	14.4	17976.1	4.41	1
61	Carbohydrate esterase family 12 protein (Fragment)	D8Q5H3	21.33	9.2	16350.7	4.24	1
62	Fruiting body protein SC7	D8Q6B1	20.16	9.3	22529.6	6.40	1

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Table 4.12: Putative antihypertensive proteins

Group	Protein name	Organism Species	Gene Ontology (GO) function	Database Accession No.	Distinct summed MS/MS search score	Protein MW (Da)	Protein sequence
3	Putative uncharacterized protein	*Schizophyllum commune*	serine-type peptidase activity (Source: InterPro)	D8PQZ6	152.57	56299.5	MVYGMSLARLALAGALAVGVAQA
5	Putative uncharacterized protein	*Schizophyllum commune*	serine-type endopeptidase activity (Source: InterPro)	D8PRI9	125.44	67095.9	MNGLTWRALLSLVLAAAAVA
11	Putative uncharacterized protein	*Schizophyllum commune*	serine-type carboxypeptidase activity (Source: InterPro)	D8Q9N4	100.04	57832.3	MQLLQVLSLALLASVASA
11	Alpha/beta-hydrolase	*Dichomitus squalens*	serine-type carboxypeptidase activity (Source: InterPro)	R7SVB2	22.29	56276	MEGMETAETI QREQLDRLRD
11	Carboxypeptidase	*Moniliophthora roreri*	serine-type carboxypeptidase activity (Source: InterPro)	V2XLJ0	22.29	55915	MQGMEDAMEI QREQMQRMRD

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Table 4.12, continued,

19	Putative uncharacterized protein	*Schizophyllum commune*	serine-type peptidase activity (Source: InterPro)	D8PRJ2	75.76	59481	MMPHPQVPKV PMESVGPVVS
33	Putative uncharacterized protein	*Schizophyllum commune*	serine-type peptidase activity (Source: InterPro)	D8PXE8	51.27	60355	MLPPLLSALGLTIAVAAAGIADA
33	Putative uncharacterized protein	*Schizophyllum commune*	serine-type peptidase activity (Source: InterPro)	D8PXE9	24.30	61434	MGVPLALLTLAVLSGVTA

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The Spectrum Mill listed 62 groups of proteins/peptides from the LCMS-QTOF analysis by Spectrum Mill software and Swissprot database. Table 4.12 revealed that 8 proteins were identified that may have anti-hypertensive related function based on their gene onthology in InterPro database (Finn *et al.*, 2017). Out of the 8 proteins, Carboxypeptidase and Alpha/beta-hydrolase were the antihypertensive related proteins based on literature search, while the other proteins were putative uncharacterized proteins.

4.10 Anti-hypertensive related proteins

4.10.1 Carboxypeptidase/ Serine-type carboxypeptidase/ Serine-type peptidase

Endothelial dysfunction (EC) play important physiological functions in regulation of the vascular homeostasis. Vascular imbalance is responsible for various cardiovascular diseases including hypertension. Endothelial cells produce a number of vasoactive substances, including nitric oxide (NO) and endothelin (ET-1). NO the key vasodilator, and ET-1 a potent vasoconstrictor, are vital mediators of endothelial functions. An imbalance between these two factors is a feature of endothelial dysfunction.

Vasoconstrictory properties of circulating endothelins have three isoforms (ET-1, ET-2, and ET-3, where ET-1 is the dominant form and actively modulates vascular functions (Barton & Yanagisawa, 2008). ET-1 is synthesized predominantly in endothelial cells and vascular smooth muscle cells**,** other than in airway epithelial cells, macrophages, fibroblasts, cardiac myocytes, brain neurons, and pancreatic islets (Ortmann *et al.*, 2005). The ET-1 gene encodes a 212-amino acid precursor, functionally inactive peptide, preproET-1. Removal of the 17-amino acid signal by a signal peptidase generates proET-1, which in turn is cleaved at both the C and N

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terminals by furin enzymes, which remove 35 and 122 amino acids, respectively, called Big-ET-1 (bET-1) (Davenport *et al.*, 2016).

Figure 4.14 shows under normal physiological conditions, endothelin-converting enzyme (ECE) converts bET-1 to ET-1, whereas current evidence suggests ET-1 can also be produced from bET-1 through several other proteolytic digestions involving matrix metalloproteinases (MMPs), and neutral endopeptidase (NEP). ET-1 exerts its functions by binding to G protein-coupled ET receptors, endothelin receptor A (ETA) and endothelin receptor B (ETB) (Bourque *et al.*, 2011). ETA receptors are located within the vascular smooth muscle cells (VSMC), whereas ETB receptors are located both on vascular endothelium, as well as, on VSMC. Binding with ETA and ETB receptors in vascular smooth muscle ET-1 exerts vasoconstriction. Alternatively, ET-1 binding to ETB receptors in the endothelium results in vasodilation through increased of NO production and prostacyclin synthesis (Barton & Yanagisawa, 2008).

The bioavailability and potency of ET-1 can be regulated through many factors such as alteration of its receptor density and affinity, up- and down-regulation of peptide synthesis or release, enzymatic activation such as ECE and MMP-2, or degradation NEP for ET-1 (Pan *et al.*, 2014). The elevated levels of ET-1 have been previously documented in patients with the acute peripheral ischemia and myocardial infarction and in patients with such chronic conditions as congestive heart failure, atherosclerosis, hypercholesterolemia, and systemic and pulmonary hypertension (Goraca, 2002).

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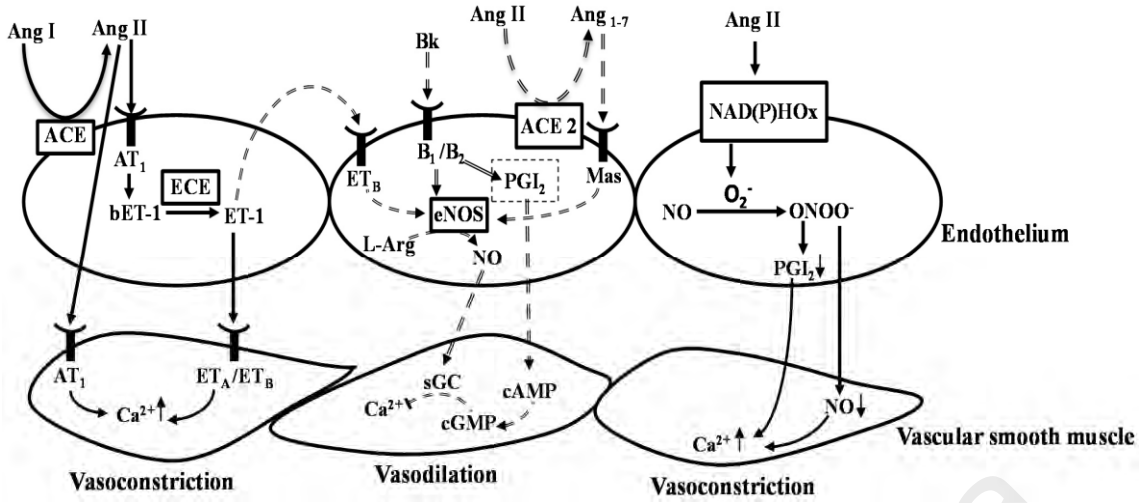

Figure 4.14: Endothelial dysfunction and blood pressure regulation. Angiotensin converting enzyme (ACE) converts angiotensin I (Ang I) to angiotensin II (Ang II), Ang II binds with angiotensin receptor 1 (AT1) on endothelium cells as well as vascular smooth muscle cells, then AT1 receptor increases calcium ion ($Ca2^{+}$) concentration in vascular smooth muscle cells (VSMC) and exerts vasoconstriction. In endothelium cells activation of AT1 receptor increases the production of bET-1 (big endothelin-1). Endothelin-Converting Enzyme (ECE) converts bET-1 to endothelin-1 (ET-1) and exerts vasoconstriction by activating endothelin A/B receptors (ETA/B) in the VSMC. In contrast, activation of ETB receptor in endothelium cells mediates vasodilatory effects via release of nitric oxide (NO) by activating endothelial nitric oxide synthase (eNOS). ACE also converts Bradykinin (Bk) into inactive peptides. Bk binds with bradykinin receptor (B1/2) and activates eNOS, which converts L-Arginine to L-Citrulline and produces NO. NO exerts vasodilation by activating cyclic guanosine monophosphate (cGMP) by inhibiting the concentration of $Ca2^{+}$ in VSM. In endothelium cells Ang II produces superoxide ($O2^{-}$) which scavenges NO and produces peroxynitrite ($ONOO^{-}$), exerts vasoconstriction effect by limiting the supply of NO to the VSM. Signaling pathways illustrated with solid line arrows are representing vasoconstriction and with compound line arrows are representation vasodilation network. Adapted from (Sudano *et al.*, 2006).

Previous study showed that a lysosomal serine carboxypeptidase, cathepsin A, and lysosomal serine carboxypeptidase 1 rapidly inactivates ET-1 thus regulate vascular tone and hemodynamics (Pan *et al.*, 2014). A lysosomal carboxypeptidase or serine carboxypeptidase, are widely distributed in fungi, higher plants, and animal tissues. Generally, serine carboxypeptidases are glycoproteins with subunit molecular weights of 40,000 - 75,000 Da and carboxypeptidase from fungi apparently contain only a single peptide chain (Breddam, 1986). Serine carboxypeptidase has a cleavable

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signal peptide, N-linked glycans and the Ser-Asp-His catalytic triad (Deng *et al.*, 1994).

Serine carboxypeptidase rapidly inactivates ET-1 by converting it into biologically inactive des-Trp21-endothelin-1 and hydrolaze the last residue of Angiotensin I to angiotensin 1–9 which later converted by ACE to Angiotensin II but at much slower rate (Pan *et al.*, 2014). Formation of des-Trp21-endothelin-1 will cause endothelin receptors (ETA) in the vascular smooth muscle cells not being activated thus the vasoconstrictor effect will not take place.

4.10.2 Alpha/Beta Hydrolase

Alpha/beta hydrolases function as hydrolases, lyases, transferases, hormone precursors or transporters, chaperones or routers of other proteins (Lenfant *et al.*, 2013). This group of protein have diverse catalytic functions which include acetylcholinesterase, lipase and others. This protein superfamily can be classified into its similar function by dividing them according to sequence and structural homologies.

Currently, there is no report on direct relationship between alpha/beta hydrolase protein and antihypertensive activity, however, a few studies show indirect contribution of alpha/beta hydrolase in the mechanism of hypertension. A study showed that soluble epoxide hydrolase (sEH) inhibitor have antihypertensive effect and ability to protect the brain, heart and kidney from damage (Imig & Hammock, 2009). It is through the role in blood pressure regulation and renal function of arachidonic acid metabolism in which epoxyeieosatrienoic acids (EETs) were formed.

EET various region-isomers can antagonize renal tubular sodium reabsorption and may have both vasoconstrictive and vasodilatory effects on renal vasculature. sEH have an important role inregulating EET levels and thus an important mediator of cardiorenal mechanisms and blood pressure regulation (Fornage *et al.*, 2002). sEH

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metabolizes EETs to its corresponding diols which is lacking in renal vasodilatory effects, thus inhibiting the sEH results in modest blood pressure reduction (Sinal *et al.*, 2000).

Mohamad Ansor *et al.* (2013) discovered an alpha/beta hydrolase-like protein from mycelia of *G. lucidum.* In their study, the mechanism of ACE inhibition by alpha/beta hydrolase-like protein was speculated to act as alpha/beta hydrolase inhibitor in restoring blood pressure level. As there is no report on alpha/beta hydrolase having specific role in inhibition of ACE, synergism effect of different proteins may contribute in the ACE inhibition mechanism. Further study on the alpha/beta hydrolase protein will give better understanding on how this protein works especially as an anti-hypertensive agent.

4.10.3 Putative uncharacterized proteins

Apart from the carboxypeptidase and alpha/beta hydrolase proteins, the putative uncharacterized proteins from Table 4.12 were identified. Putative uncharacterized proteins refer to protein identified by computer software/s, and the function is still unknown. The listed putative uncharacterized proteins may possibly have anti hypertensive properties based on the gene onthology function. These proteins have serine carboxypeptidase function as described earlier besides several other characteristics of antihypertensive protein.

Amino acid position on the peptide chain plays a significant role on ACE inhibition. This is because some important amino acid residues are required on a peptide chain to allow selection of specific enzymes that will release peptides with desirable amino acid sequences. Furthermore, structure-activity relationship between different ACE's peptide inhibitors suggests that binding to ACE is strongly influenced by the C-terminal tripeptide sequence of the substrate (FitzGerald & Meisel, 2000).

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ACE appears to prefer competitive inhibitors containing hydrophobic amino acid residues at each of three C-terminal positions, and many naturally occurring peptide inhibitors contain amino acid proline (P) at the C-terminal (Mine & Shahidi, 2005). The major di- and tripeptide inhibitors reported to have amino acid tyrosine (Y), phenylalanine (F), tryptophan (W) and proline (P) residue at the C-terminus and branched aliphatic amino acids at the N-terminus (Ni *et al.*, 2012).

In this study, putative uncharacteristic proteins from group 3, 5 and 11 (Table 4.12) have high percentage of hydrophobic proteins especially alanine (A), leucine (L) and valine (V) in the sequences. Lau *et al.* (2013) identified peptide containing alanine (A) in their ACE inhibitory peptide from *Pleurotus cystidiosus* which is also similar with ACE inhibitor from sea squirt (AHIII). Branched-chain amino acids such as valine and leucine play crucial roles in the interaction of the transmembrane domains of ACE with phospholipid bilayers as the amino acids were at the N-terminus.

Putative uncharacteristic proteins from group 19 and 33 contained many hydrophobic proteins such as methionine (M), proline (P), leucine (L), alanine (A) and valine (V) in the sequence which may contributes to the ACE inhibitory activity. At the same time, most proline is positioned close to the N-terminal position of the sequence. Hydrophobic amino acids, especially those with aliphatic chains such as glycine, isoleucine, leucine, and valine are characteristic for the N-terminal of a peptide (Iwaniak *et al.*, 2014). This is consistent with the conclusion made by (Wu & Aluko, 2007) who emphasized the impact of physicochemical attributes of amino acids such as hydrophobicity, bulkiness, and electronic properties on the bioactivity of peptides.

Putative uncharacterized protein is not homologous to any well characterised proteins and thus its function is unknown. One of the most reliable ways to characterize proteins with unknown activity is by conducting experimental

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determination of protein function. However, this method is difficult, as a protein have many possible functions it can perform. Besides that, computer approaches such as web databases and services have been developed for prediction of protein function. Sequence and structure homology can also contribute in giving hypotheses to guide functional experiments on these proteins. Further investigation on these proteins may give an insight on the role they may impose in anti hypertension mechanism.

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CHAPTER 5:CONCLUSION AND RECOMMENDATIONS

Mushrooms as the natural sources of various secondary metabolites have became one of the alternatives to diseases treatment and prevention. Extracts from *S. commune* mycelial biomass in this study showed fairly good antioxidant activities.

In Folin-Ciocalteu assay, CWE showed highest phenolic content with 7.80±0.25 mg GAE/g extract, while for STFM protein fraction F90 gave highest phenolic content with 15.04±0.39 mg GAE/g extract. In DPPH scavenging activity, CWE from both SHFM and STFM conditions gave highest scavenging activity with 20.94±1.93% and 16.93±2.65%, and IC_{50} of 38.46 mg/ml and 17.24 mg/ml respectively. In Cupric ion reduction antioxidant capacity (CUPRAC), protein fraction F90 of both SHFM and STFM conditions gave highest absorbance value of 0.420±0.00 and 0.064±0.00 at 450 nm respectively. CWE of SHFM condition gave highest percentage of metal chelating activity with 67.51±0.77% while PE of STFM scored 30.02±1.23%. For inhibition of lipid peroxidation assay, HWE from both culture conditions showed highest inhibition percentage with 26.40±0.57% and 20.07±0.78%.

The LCMS/MS analysis showed that, compounds such as tryptophan, gluconic acid and phenolic acid, while in CWE-ST compounds such as 2(3,4-dihydroxyphenyl)-7-hydroxy-5-benzene propanoic acid, gluconic acid and quinic acid conjugate were present in CWE-SH. In addition, compounds such as phenolic acid was found in HWE-SH, while HWE-ST contained compounds such as propanoic acid, gluconic acid, quinic acid conjugate, phenylvaleric acids and protocatechuic acid. LCMS analysis of WRE-SH exhibited the presence of compounds such as hydroxylated cinnamic acid, tryptophan, leucine and thiamine.

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ACE inhibitory assay and result showed the highest ACE inhibition at 50 μg/ml was F90-ST <10 kDa with 30.0% inhibition with IC50 of 120 μg/ml. Protein identifications by LCMS-QTOF have revealed two potential antihypertensive proteins: carboxypeptidase and alpha/beta hydrolase protein.

In this study, only one-dimensional chromatography was used for protein separation which is the SDS PAGE. Hence, sample F90-ST <10 kDa may still contain mixture of proteins. In SDS-PAGE, a single protein band might also contain proteins with similar molecular weight. The impurity of sample, together with the limitation of databases may affect protein scores and cause imprecision of protein identification**.** More comprehensive proteomic approaches such as 2D electrophoresis, HPLC and FPLC may be beneficial in future works.

Evaluation of the potential antihypertensive proteins *in vivo* may offer better understanding of its effect on hypertension mechanisms. Furthermore, elucidation and purification of the proteins can be considered in the future works. Nevertheless, this study has discovered potential antihypertensive proteins thus offered better understanding of the *S. commune* medicinal properties in antioxidant and hypertension.

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REFERENCES

Abdullah, N., Ismail, S. M., Aminudin, N., Shuib, A. S., & Lau, B. F. (2012). Evaluation of selected culinary-medicinal mushrooms for antioxidant and ace inhibitory activities. *Evidence Based Complementary Alternative Medicine, 2012*, 464238.

Ahmad, I., Zahin, M., Aqil, F., Khan, M. S. A., & Ahmad, S. (2009). Novel approaches to combat drug-resistant bacteria. *New strategies combating bacterial infection* (pp. 47-70). Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA.

Akesson, C., Lindgren, H., Pero, R. W., Leanderson, T., & Ivars, F. (2005). Quinic acid is a biologically active component of the *Uncaria tomentosa* extract C-Med 100. *International Immunopharmacology, 5*(1), 219-229.

American Heart Association. (2015). *Step 5: Prevention and Treatment, types of blood pressure medications.* Retrieved from http://www.heart.org/HEARTORG/Conditions/HighBloodPressure/PreventionTreatmentofHighBloodPressure/Ty

Apak, R., Gorinstein, S., Böhm, V., Schaich, K. M., Özyürek, M., & Güçlü, K. (2013). Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report). *Pure and Applied Chemistry, 85*(5), 957-998.

Apak, R., Güçlü, K., Demirata, B., Özyürek, M., Çelik, S. E., Bektaşoğlu, B., . . . Özyurt, D. (2007). Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. *Molecules, 12*(7), 1496-1547.

Arcan, I., & Yemenicioğlu, A. (2007). Antioxidant activity of protein extracts from heat-treated or thermally processed chickpeas and white beans. *Food Chemistry, 103*(2), 301-312.

Arora, D. S., & Chandra, P. (2010). Assay of antioxidant potential of two *Aspergillus* isolates by different methods under various physio-chemical conditions. *Brazilian Journal of Microbiology, 41*, 765-777.

Aruoma, O. (1998). Free radicals, oxidative stress, and antioxidants in human health and disease. *Journal of the American Oil Chemists' Society, 75*(2), 199-212.

University of Malaya

Atlas, S. A. (2007). The renin-angiotensin aldosterone system: Pathophysiological role and pharmacologic inhibition. *Journal of Managed Care Pharmacy, 13*(8 Suppl B), 9-20.

Ayala, A., Muñoz, M. F., & Argüelles, S. (2014). Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxidative Medicine and Cellular Longevity, 2014*, 31.

Badalyan, S. M. (2003). Edible and medicinal higher basidiomycetes mushrooms as a source of natural antioxidants. *International Journal of Medicinal Mushrooms, 5*(2), 10.

Balasuriya, B. N., & Rupasinghe, H. V. (2011). Plant flavonoids as angiotensin converting enzyme inhibitors in regulation of hypertension. *Functional Foods in Health and Disease, 1*(5), 172-188.

Barnett, D. A., Ells, B., Guevremont, R., & Purves, R. W. (1999). Separation of leucine and isoleucine by electrospray ionization–high field asymmetric waveform ion mobility spectrometry–mass spectrometry. *Journal of the American Society for Mass Spectrometry, 10*(12), 1279-1284.

Barton, M., & Yanagisawa, M. (2008). Endothelin: 20 years from discovery to therapy. *Canadian Journal of Physiology and Pharmacology, 86*(8), 485-498.

Bath, J., Bohin, G., Jones, C., & Scarle, E. (2009). *Cardiac rehabilitation: A workbook for use with group programmes*. West Sussex, UK: John Wiley & Sons Ltd.

Bernstein, K. E., Ong, F. S., Blackwell, W.-L. B., Shah, K. H., Giani, J. F., Gonzalez-Villalobos, R. A., . . . Fuchs, S. (2013). A modern understanding of the traditional and nontraditional biological functions of angiotensin-converting enzyme. *Pharmacological Reviews, 65*(1), 1-46.

Bisen, P. S., Baghel, R. K., Sanodiya, B. S., Thakur, G. S., & Prasad, G. B. (2010). *Lentinus edodes*: A macrofungus with pharmacological activities. *Current Medinal Chemistry, 17*(22), 2419-2430.

Bourque, S. L., Davidge, S. T., & Adams, M. A. (2011). The interaction between endothelin-1 and nitric oxide in the vasculature: new perspectives. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, 300*(6), R1288.

Breddam, K. (1986). Serine carboxypeptidases. A review. *Carlsberg Research Communications, 51*(2), 83.

University of Malaya

Breene, W. M. (1990). Nutritional and medicinal value of specialty mushrooms. *Journal of Food Protection, 53*(10), 883-899.

Brown, N. J., & Vaughan, D. E. (1998). Angiotensin-converting enzyme inhibitors. *Circulation, 97*(14), 1411-1420.

Bryan, J. (2009). From snake venom to ACE inhibitor — the discovery and rise of captopril. *The Pharmaceutical Journal, 282*, 455.

Carretero, O. A., & Oparil, S. (2000). Essential hypertension: part I: Definition and etiology. *Circulation, 101*(3), 329-335.

Çekiç, S. D., Başkan, K. S., Tütem, E., & Apak, R. (2009). Modified cupric reducing antioxidant capacity (CUPRAC) assay for measuring the antioxidant capacities of thiol-containing proteins in admixture with polyphenols. *Talanta, 79*(2), 344-351.

Cequier-Sánchez, E., Rodríguez, C., Ravelo, Á. G., & Zárate, R. (2008). Dichloromethane as a solvent for lipid extraction and assessment of lipid classes and fatty acids from samples of different natures. *Journal of Agricultural and Food Chemistry, 56*(12), 4297-4303.

Challem, J. (2003). *User's guide to nutritional supplements*. North Bergen, New Jersey: Basic Health Publications, Inc.

Chang, H.-Y., Ho, Y.-L., Sheu, M.-J., Lin, Y.-H., Tseng, M.-C., Wu, S.-H., . . . Chang, Y.-S. (2007). Antioxidant and free radical scavenging activities of *Phellinus merrillii* extracts. *Botanical Studies, 48*(4), 407-417.

Chang, S. T., & Buswell, J. A. (1996). Mushroom nutriceuticals. *World Journal of Microbiology and Biotechnology, 12*(5), 473-476.

Chatatikun, M. C., A. (2013). Phytochemical screening and free radical scavenging activities of orange baby carrot and carrot (*Daucus carota* Linn.) root crude extracts. *Journal of Chemical and Pharmaceutical Research, 5*(4), 97-102.

Chen, H.-M., Muramoto, K., Yamauchi, F., & Nokihara, K. (1996). Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein. *Journal of Agricultural and Food Chemistry 44*(9), 2619-2623.

Cheung, L. M., Cheung, P. C. K., & Ooi, V. E. C. (2003). Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chemistry, 81*(2), 249-255.

University of Malaya

Cheung, P. C. K. (1996). The hypocholesterolemic effect of two edible mushrooms: *Auricularia auricula* (tree-ear) and *Tremella fuciformis* (white jelly-leaf) in hypercholesterolemic rats. *Nutrition Research, 16*(10), 1721-1725.

Chobanian, A. V., Bakris, G. L., Black, H. R., Cushman, W. C., Green, L. A., Izzo, J. L., Jr., . . . Roccella, E. J. (2003). The seventh report of the joint national committee on prevention, detection, evaluation, and treatment of high blood pressure: The JNC 7 report. *Journal of the American Medical Association, 289*(19), 2560-2572.

Chockalingam, A., Campbell, N. R., & Fodor, J. G. (2006). Worldwide epidemic of hypertension. *The Canadian Journal of Cardiology, 22*(7), 553-555.

Choi, H. S., Cho, H. Y., Yang, H. C., Ra, K. S., & Suh, H. J. (2001). Angiotensin I-converting enzyme inhibitor from *Grifola frondosa*. *Food Research International, 34*(2–3), 177-182.

Curzen, N. P., & Fox, K. M. (1997). Do ACE inhibitors modulate atherosclerosis? *European Heart Journal, 18*(10), 1530-1535.

Cushman, D. W., & Cheung, H. S. (1971). Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochemical Pharmacology, 20*(7), 1637-1648.

Davenport, A. P., Hyndman, K. A., Dhaun, N., Southan, C., Kohan, D. E., Pollock, J. S., . . . Maguire, J. J. (2016). Endothelin. *Pharmacological Reviews, 68*(2), 357-418.

De Leo, F., Panarese, S., Gallerani, R., & Ceci, L. R. (2009). Angiotensin converting enzyme (ACE) inhibitory peptides: production and implementation of functional food. *Current Pharmaceutical Design, 15*(31), 3622-3643.

Decker, E. A., Elias, R. J., & McClements, D. J. (2010). *Oxidation in foods and beverages and antioxidant applications: management in different industry sectors*. Cambridge, UK: Woodhead Publishing Limited.

Decker, E. A., & Welch, B. (1990). Role of ferritin as a lipid oxidation catalyst in muscle food. *Journal of Agricultural and Food Chemistry, 38*(3), 674-677.

Deng, A. Y., Martin, L. L., Balwierczak, J. L., & Jeng, A. Y. (1994). Purification and characterization of an endothelin degradation enzyme from rat kidney. *The Journal of Biochemistry, 115*(1), 120-125.

University of Malaya

Di Bernardini, R., Mullen, A. M., Bolton, D., Kerry, J., O'Neill, E., & Hayes, M. (2012). Assessment of the angiotensin-I-converting enzyme (ACE-I) inhibitory and antioxidant activities of hydrolysates of bovine brisket sarcoplasmic proteins produced by papain and characterisation of associated bioactive peptidic fractions. *Meat Science, 90*(1), 226-235.

Dimitrijevic, M., Jovanovic, V. S., Cvetkovic, J., Mihajilov-Krstev, T., Stojanovic, G., & Mitic, V. (2015). Screening of antioxidant, antimicrobial and antiradical activities of twelve selected Serbian wild mushrooms. *Analytical Methods, 7*(10), 4181-4191.

Drexler, H. (1997). Endothelial dysfunction: Clinical implications. *Progress in Cardiovascular Diseases, 39*(4), 287-324.

DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry, 28*(3), 350-356.

Dulay, R., Vicente, J., Dela Cruz, A., Gagarin, J., Fernando, W., Kalaw, S., & Reyes, R. (2016). Antioxidant activity and total phenolic content of *Volvariella volvacea* and *Schizophyllum commune* mycelia cultured in indigenous liquid media. *Mycosphere, 7*(2), 131-138.

Elias, R. J., McClements, D. J., & Decker, E. A. (2005). Antioxidant activity of cysteine, tryptophan, and methionine residues in continuous phase *β*-lactoglobulin in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 53*(26), 10248-10253.

Elisashvili, V. (2012). Submerged cultivation of medicinal mushrooms: bioprocesses and products (review). *International Journal of Medicinal Mushrooms, 14*(3), 211-239.

Endringer, D., Oliveira, O., & Braga, F. (2014). *In vitro* and in silico inhibition of angiotensin-converting enzyme by carbohydrates and cyclitols. *Chemical Papers, 68*(1), 37-45.

Erjavec, J., Kos, J., Ravnikar, M., Dreo, T., & Sabotič, J. (2012). Proteins of higher fungi – from forest to application. *Trends in Biotechnology, 30*(5), 259-273.

Esther Jr, C. R., Marino, E. M., & Bernstein, K. E. (1997). The role of angiotensin-converting enzyme in blood pressure control, renal function, and male fertility. *Trends in Endocrinology & Metabolism, 8*(5), 181-186.

University of Malaya

Ewart, H. S., Dennis, D., Potvin, M., Tiller, C., Fang, L.-h., Zhang, R., . . . Barrow, C. (2009). Development of a salmon protein hydrolysate that lowers blood pressure. *European Food Research and Technology, 229*(4), 561-569.

Fang, Q.-H., Tang, Y.-J., & Zhong, J.-J. (2002). Significance of inoculation density control in production of polysaccharide and ganoderic acid by submerged culture of *Ganoderma lucidum*. *Process Biochemistry, 37*(12), 1375-1379.

Felipe, D. F., Brambilla, L. Z., Porto, C., Pilau, E. J., & Cortez, D. A. (2014). Phytochemical analysis of *Pfaffia glomerata* inflorescences by LC-ESI-MS/MS. *Molecules, 19*(10), 15720-15734.

Ferreira, I. C., Vaz, J. A., Vasconcelos, M. H., & Martins, A. (2010). Compounds from wild mushrooms with antitumor potential. *Anticancer Agents in Medicinal Chemistry, 10*(5), 424-436.

Finn, R. D., Attwood, T. K., Babbitt, P. C., Bateman, A., Bork, P., Bridge, A. J., . . . Mitchell, A. L. (2017). InterPro in 2017—beyond protein family and domain annotations. *Nucleic Acids Research, 45*(D1), D190-D199.

FitzGerald, R. J., & Meisel, H. (2000). Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. *British Journal of Nutrition, 84*(S1), 33-37.

FitzGerald, R. J., Murray, B. A., & Walsh, D. J. (2004). Hypotensive peptides from milk proteins. *The Journal of Nutrition, 134*(4), 980s-988s.

Fleming, I. (2006). Signaling by the angiotensin-converting enzyme. *Circulation Research, 98*(7), 887-896.

Flora, S. J. S., & Pachauri, V. (2010). Chelation in metal intoxication. *International Journal of Environmental Research and Public Health, 7*(7), 2745-2788.

Fornage, M., Hinojos, C. A., Nurowska, B. W., Boerwinkle, E., Hammock, B. D., Morisseau, C. H., & Doris, P. A. (2002). Polymorphism in soluble epoxide hydrolase and blood pressure in spontaneously hypertensive rats. *Hypertension, 40*(4), 485-490.

Fregly, M. J., Sumners, C., & Cade, J. R. (1989). Effect of chronic dietary treatment with L-tryptophan on the maintenance of hypertension in spontaneously hypertensive rats. *Canadian Journal of Physiology & Pharmacology, 67*(6), 656-662.

Friel, M., & McLoughlin, A. (2000). Production of a liquid inoculum/spawn of *Agaricus bisporus*. *Biotechnology Letters, 22*(5), 351-354.

University of Malaya

Fujita, H., & Yoshikawa, M. (1999). LKPNM: a prodrug-type ACE-inhibitory peptide derived from fish protein. *Immunopharmacology, 44*(1–2), 123-127.

Gehrig, I., Bart, H. J., Anke, T., & Germerdonk, R. (1998). Influence of morphology and rheology on the production characteristics of the basidiomycete *Cyathus striatus*. *Biotechnology & Bioengineering, 59*(5), 525-533.

Göger, F., Köse, Y. B., Göger, G., & Demirci, F. (2015). Phytochemical characterization of phenolics by LC-MS/MS and biological evaluation of *Ajuga orientalis* from Turkey. *Bangladesh Journal of Pharmacology, 10*(3), 6.

Goldman, M. B., & Hatch, M. (2000). *Women and health*. London, UK: Academic Press.

Goodell, B., Jellison, J., Liu, J., Daniel, G., Paszczynski, A., Fekete, F., . . . Xu, G. (1997). Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood. *Journal of Biotechnology, 53*(2–3), 133-162.

Goraca, A. (2002). New views on the role of endothelin (minireview). *Endocrine Regulations, 36*(4), 161-167.

Griendling, K. K., Murphy, T. J., & Alexander, R. W. (1993). Molecular biology of the renin-angiotensin system. *Circulation, 87*(6), 1816-1828.

Griensven, L. J. L. D. (2000). Science and Cultivation of Edible Fungi. *Proceedings of the 15th International Congress on the Science and Cultivation of Edible Fungi, Maastricht, Netherlands*. Rotterdam, Netherlands: A.A. Balkema.

Guang, C., Phillips, R. D., Jiang, B., & Milani, F. (2012). Three key proteases – angiotensin-I-converting enzyme (ACE), ACE2 and renin – within and beyond the renin-angiotensin system. *Archives of Cardiovascular Diseases, 105*(6–7), 373-385.

Gülçin, İ., Alici, H. A., & Cesur, M. (2005). Determination of *in vitro* antioxidant and radical scavenging activities of propofol. *Chemical and Pharmaceutical Bulletin, 53*(3), 281-285.

Hagiwara, S.-y., Takahashi, M., Shen, Y., Kaihou, S., Tomiyama, T., Yazawa, M., . . . Terazawa, M. (2005). A phytochemical in the edible Tamogi-take mushroom (*Pleurotus cornucopiae*), D-mannitol, inhibits ACE activity and lowers the blood pressure of spontaneously hypertensive rats. *Bioscience Biotechnology and Biochemistry, 69*(8), 1603-1605.

University of Malaya

Halliwell, B. (1991). Reactive oxygen species in living systems: source, biochemistry, and role in human disease. *American Journal of Medicine, 91*.

Halliwell, B. (2000). Lipid peroxidation, antioxidants and cardiovascular disease: How should we move forward? *Cardiovascular Research, 47*(3), 410-418.

Halliwell, B., & Gutteridge, J. M. (1984). Lipid peroxidation, oxygen radicals, cell damage, and antioxidant therapy. *Lancet, 1*(8391), 1396-1397.

Hasler, C. M. (1998). Functional foods: their role in disease prevention and health promotion. *Food Technology, 52*, 63-147.

Hechtman, L. (2012). *Clinical naturopathic medicine*. Chatswood, New South Wales: Elsevier Australia.

Heitzer, T., Schlinzig, T., Krohn, K., Meinertz, T., & Münzel, T. (2001). Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. *Circulation, 104*(22), 2673-2678.

Hernandez, A. F., & Harrington, R. A. (2008). Comparative effectiveness of angiotensin-converting-enzyme inhibitors: Is an ACE always an ace? *Canadian Medical Association Journal, 178*(10), 1316-1319.

Higashi, Y., Noma, K., Yoshizumi, M., & Kihara, Y. (2009). Endothelial function and oxidative stress in cardiovascular diseases. *Circulation Journal : Official Journal of the Japanese Circulation Society, 73*(3), 411-418.

Hong, F., Ming, L., Yi, S., Zhanxia, L., Yongquan, W., & Chi, L. (2008). The antihypertensive effect of peptides: A novel alternative to drugs? *Peptides, 29*(6), 1062-1071.

Horai, H., Arita, M., Kanaya, S., Nihei, Y., Ikeda, T., Suwa, K., . . . Nishioka, T. (2010). MassBank: A public repository for sharing mass spectral data for life sciences. *Journal of Mass Spectrometry, 45*(7), 703-714.

Hornig, B., Kohler, C., & Drexler, H. (1997). Role of bradykinin in mediating vascular effects of angiotensin-converting enzyme inhibitors in humans. *Circulation, 95*(5), 1115-1118.

Hossain, M. B., Rai, D. K., Brunton, N. P., Martin-Diana, A. B., & Barry-Ryan, C. (2010). Characterization of phenolic composition in *Lamiaceae* spices by LC-ESI-MS/MS. *Journal of Agricultural and Food Chemistry, 58*(19), 10576-10581.

University of Malaya

Houghton, P., & Raman, A. (2012). *Laboratory handbook for the fractionation of natural extracts*. London, UK: Springer

Huang, D., Ou, B., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry, 53*(6), 1841-1856.

Huang, L. C. (2000). *Antioxidant properties and polysaccharide composition analysis of Antrodia camphorata and Agaricus blazei.* (Unpublished master's thesis). National Chung-Hsing University, Taichung, Taiwan.

Humfeld, H. (1954). *United States* Patent No. US2693665 A. Berkeley, California: S. o. Agriculture.

Imig, J. D., & Hammock, B. D. (2009). Soluble epoxide hydrolase as a therapeutic target for cardiovascular diseases. *Nature Reviews Drug Discovery, 8*(10), 794-805.

Israili, Z. H., & Hall, W. D. (1992). Cough and angioneurotic edema associated with angiotensin-converting enzyme inhibitor therapy. A review of the literature and pathophysiology. *Annals of Internal Medicine, 117*(3), 234-242.

Ito, N., Fukushima, S., & Tsuda, H. (1985). Carcinogenicity and modification of the carcinogenic response by BHA, BHT, and other antioxidants. *Critical Reviews in Toxicology, 15*(2), 109-150.

Iwaniak, A., Minkiewicz, P., & Darewicz, M. (2014). Food-originating ACE inhibitors, including antihypertensive peptides, as preventive food components in blood pressure reduction. *Comprehensive Reviews in Food Science and Food Safety, 13*(2), 114-134.

Izawa, H., & Aoyagi, Y. (2006). Inhibition of angiotensin converting enzyme by mushroom. *Journal of the Japanese Society for Food Science and Technology, 53*.

Jahanbin, K., Gohari, A. R., Moini, S., Emam-Djomeh, Z., & Masi, P. (2011). Isolation, structural characterization and antioxidant activity of a new water-soluble polysaccharide from *Acanthophyllum bracteatum* roots. *International Journal of Biological Macromolecules, 49*(4), 567-572.

Jamaludin, F. L. (2013). *Isolation and characterization of angiotensin converting enzyme (ACE) inhibitory compounds and antioxidant activities of Tacca integrifolia.* (Unpublished master's thesis). University of Malaya.

University of Malaya

Jang, A., & Lee, M. (2005). Purification and identification of angiotensin converting enzyme inhibitory peptides from beef hydrolysates. *Meat Science, 69*(4), 653-661.

Japanese Society of Hypertension. (2014). Chapter 5. Treatment with antihypertensive drugs. *Hypertension Research, 37*(4), 291-300.

Jeon, Y.-J., Byun, H.-G., & Kim, S.-K. (1999). Improvement of functional properties of cod frame protein hydrolysates using ultrafiltration membranes. *Process Biochemistry, 35*(5), 471-478.

Jonathan, S. G., & Fasidi, I. O. (2003). Antimicrobial activities of two Nigerian edible macro-fungi *Lycoperdon pusilum* (Bat. Ex) and *Lycoperdon giganteum* (Pers.). *African Journal of Biomedical Research, 6*(2), 85-90.

Jones, W. P., & Kinghorn, A. D. (2005). Extraction of plant secondary metabolites. In S. D. Sarker, Z. Latif, & A. I. Gray (Eds.), *Natural products isolation* (pp. 323-351). Totowa, New Jersey: Humana Press.

Ju, H. K., Chung, H. W., Hong, S.-S., Park, J. H., Lee, J., & Kwon, S. W. (2010). Effect of steam treatment on soluble phenolic content and antioxidant activity of the Chaga mushroom (*Inonotus obliquus*). *Food Chemistry, 119*(2), 619-625.

Kakumu, S., Fujisawa, K., Suzuki, H., Ichida, F., Yamamoto, S., Hirayama, C., & Ogawa, N. (1994). Effect of the polysaccharide Sizofiran, an immunotherapeutic agent, on HBeAg-positive chronic hepatitis. In K. Nishioka, H. Suzuki, S. Mishiro, & T. Oda (Eds.), *Viral hepatitis and liver disease: Proceedings of the International Symposium on Viral Hepatitis and Liver Disease: Molecules Today, More Cures Tomorrow, Tokyo, (1993 ISVHLD)* (pp. 650-653). Tokyo: Springer Japan.

Kalač, P. (2009). Chemical composition and nutritional value of European species of wild growing mushrooms: A review. *Food Chemistry, 113*(1), 9-16.

Kalyoncu, F., Oskay, M., & Kayalar, H. (2010). Antioxidant activity of the mycelium of 21 wild mushroom species. *Mycology, 1*(3), 195-199.

Karpievitch, Y. V., Polpitiya, A. D., Anderson, G. A., Smith, R. D., & Dabney, A. R. (2010). Liquid chromatography mass spectrometry-based proteomics: biological and technological aspects. *The Annals of Applied Statistics, 4*(4), 1797-1823.

University of Malaya

Kasote, D. M., Katyare, S. S., Hegde, M. V., & Bae, H. (2015). Significance of antioxidant potential of plants and its relevance to therapeutic applications. *International Journal of Biological Sciences, 11*(8), 982-991.

Kearney, P. M., Whelton, M., Reynolds, K., Muntner, P., Whelton, P. K., & He, J. (2005). Global burden of hypertension: analysis of worldwide data. *Lancet, 365*(9455), 217-223.

Keleş, A., Koca, İ., & Gençcelep, H. (2011). Antioxidant Properties of Wild Edible Mushrooms. *Journal of Food Processing Technology, 2*(6), 1-6.

Khan, M. A., Tania, M., Liu, R., & Rahman, M. M. (2013). *Hericium erinaceus*: an edible mushroom with medicinal values. *Journal of Complementary and Integrative Medicine, 10*.

Kikumoto, S., Miyajima, T., Yoshizumi, S., Fujimoto, S., & Kimura, K. (1970). Polysaccharide produced by *Schizophyllum commune* Part I: Formation and some properties of an extracellular polysaccharide. *Nippon Nōgeikagaku Kaishi, 44*(8), 337-342.

Kimura, Y., Tojima, H., Fukase, S., & Takeda, K. (1994). Clinical evaluation of sizofilan as assistant immunotherapy in treatment of head and neck cancer. *Acta Oto-Laryngologica, 114*(sup511), 192-195.

Kinoshita, E., Yamakoshi, J., & Kikuchi, M. (1993). Purification and identification of an angiotensin I-converting enzyme inhibitor from soy sauce. *Bioscience, Biotechnology, and Biochemistry 57*(7), 1107-1110.

Klabunde, R. E. (2011, 24 April 2014). Renin-Angiotensin-Aldosterone System. *Cardiovascular physiology concepts.* 2nd Edition. Retrieved from http://cvphysiology.com/Blood%20Pressure/BP015.htm

Klaus, A., Kozarski, M., Niksic, M., Jakovljevic, D., Todorovic, N., & Van Griensven, L. J. L. D. (2011). Antioxidative activities and chemical characterization of polysaccharides extracted from the basidiomycete *Schizophyllum commune*. *LWT - Food Science and Technology, 44*(10), 2005-2011.

Knight, J. (2000). Review: Free radicals, antioxidants, and the immune system. *Annals of Clinical & Laboratory Science, 30*(2), 145-158.

Kohen, R., Yamamoto, Y., Cundy, K. C., & Ames, B. N. (1988). Antioxidant activity of carnosine, homocarnosine, and anserine present in muscle and brain. *Proceedings of the National Academy of Sciences, 85*(9), 3175-3179.

University of Malaya

Kohn, F. M., Muller, C., Drescher, D., Neukamm, C., el Mulla, K. F., Henkel, R., . . . Schill, W. B. (1998). Effect of angiotensin converting enzyme (ACE) and angiotensins on human sperm functions. *Andrologia, 30*(4-5), 207-215.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680-685.

Lau, B. F., Abdullah, N., Aminudin, N., Lee, H. B., Yap, K. C., & Sabaratnam, V. (2014). The potential of mycelium and culture broth of *Lignosus rhinocerotis* as substitutes for the naturally occurring sclerotium with regard to antioxidant capacity, cytotoxic effect, and low-molecular-weight chemical constituents. *PLOS ONE, 9*(7), e102509.

Lau, C.-C., Abdullah, N., Shuib, A. S., & Aminudin, N. (2012). Proteomic analysis of antihypertensive proteins in edible mushrooms. *Journal of Agricultural and Food Chemistry, 60*(50), 12341-12348.

Lau, C. C., Abdullah, N., & Shuib, A. S. (2013). Novel angiotensin I-converting enzyme inhibitory peptides derived from an edible mushroom, *Pleurotus cystidiosus* O.K. Miller identified by LC-MS/MS. *BMC Complementary and Alternative Medicine, 13*, 313-313.

Lee, B. C., Bae, J. T., Pyo, H. B., Choe, T. B., Kim, S. W., Hwang, H. J., & Yun, J. W. (2004a). Submerged culture conditions for the production of mycelial biomass and exopolysaccharides by the edible Basidiomycete *Grifola frondosa*. *Enzyme and Microbial Technology, 35*(5), 369-376.

Lee, D. H., Ho Kim, J., Sik Park, J., Jun Choi, Y., & Soo Lee, J. (2004b). Isolation and characterization of a novel angiotensin I-converting enzyme inhibitory peptide derived from the edible mushroom *Tricholoma giganteum*. *Peptides, 25*(4), 621-627.

Lee, J.-E., Bae, I. Y., Lee, H. G., & Yang, C.-B. (2006). Tyr-Pro-Lys, an angiotensin I-converting enzyme inhibitory peptide derived from broccoli (*Brassica oleracea* Italica). *Food Chemistry, 99*(1), 143-148.

Lee, S.-H., Qian, Z.-J., & Kim, S.-K. (2010). A novel angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. *Food Chemistry, 118*(1), 96-102.

Lee, S.-S., Lee, P.-L., Chen, C.-F., Wang, S.-Y., & Chen, K.-Y. (2003). Antitumor effects of polysaccharides of *Ganoderma lucidum* (Curt.:Fr.) P. Karst. (Ling Zhi, Reishi Mushroom) (Aphyllophoromycetideae). *International Journal of Medicinal Mushrooms, 5*(1), 16.

University of Malaya

Lee, Y.-L., Yen, M.-T., & Mau, J.-L. (2007). Antioxidant properties of various extracts from *Hypsizigus marmoreus*. *Food Chemistry, 104*(1), 1-9.

Lenfant, N., Hotelier, T., Bourne, Y., Marchot, P., & Chatonnet, A. (2013). Proteins with an alpha/beta hydrolase fold: Relationships between subfamilies in an ever-growing superfamily. *Chemico-Biological Interactions, 203*(1), 266-268.

Lewis, E. J., Hunsicker, L. G., Bain, R. P., & Rohde, R. D. (1993). The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy. *New England Journal of Medicine, 329*(20), 1456-1462.

Li, Y. R. (2015). *Cardiovascular Diseases: From molecular pharmacology to evidence-based therapeutics*. Hoboken, New Jersey: John Wiley and Sons.

Lin, H. C. (1999). *Evaluation of taste quality and antioxidant properties of edible and medicinal mushrooms.* (Unpublished master's thesis). National Chung-Hsing University, Taichung, Taiwan.

Lindequist, U., Niedermeyer, T. H., & Julich, W. D. (2005). The pharmacological potential of mushrooms. *Evidence Based Complementary and Alternative Medicine, 2*(3), 285-299.

Liu, R. H. (2003). Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *The American Journal of Clinical Nutrition, 78*(3), 517S-520S.

Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy Reviews, 4*(8), 118-126.

Longvah, T., & Deosthale, Y. G. (1998). Compositional and nutritional studies on edible wild mushroom from northeast India. *Food Chemistry, 63*(3), 331-334.

López-Fandiño, R., Otte, J., & van Camp, J. (2006). Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity. *International Dairy Journal, 16*(11), 1277-1293.

Machlin, L. J., & Bendich, A. (1987). Free radical tissue damage: protective role of antioxidant nutrients. *The Federation of American Societies for Experimental Biology Journal, 1*(6), 441-445.

Madhur, M. S. (2014, 30 Sep 2014). Hypertension treatment & management. *Drugs & diseases.* Retrieved from http://emedicine.medscape.com/article/241381-treatment

University of Malaya

Madla, S., Isaka, M., & Wongsa, P. (2008). Modification of culture conditions for production of the anti-tubercular hirsutellones by the insect pathogenic fungus *Hirsutella nivea* BCC 2594. *Letters in Applied Microbiology, 47*(2), 5.

Mau, J.-L., Chang, C.-N., Huang, S.-J., & Chen, C.-C. (2004). Antioxidant properties of methanolic extracts from *Grifola frondosa*, *Morchella esculenta* and *Termitomyces albuminosus* mycelia. *Food Chemistry, 87*(1), 111-118.

Mau, J.-L., Chao, G.-R., & Wu, K.-T. (2001). Antioxidant properties of methanolic extracts from several ear mushrooms. *Journal of Agricultural and Food Chemistry, 49*(11), 5461-5467.

Mau, J.-L., Tsai, S.-Y., Tseng, Y.-H., & Huang, S.-J. (2005). Antioxidant properties of hot water extracts from *Ganoderma tsugae* Murrill. *LWT - Food Science and Technology, 38*(6), 589-597.

Mayakrishnan, V., Abdullah, N., Abidin, M. H. Z., Fadzil, N. H. M., Johari, N. M. K., Aminudin, N., & Abidin, N. Z. (2013). Investigation of the antioxidative potential of various solvent fractions from fruiting bodies of *Schizophyllum commune* (Fr.) mushrooms and characterization of phytoconstituents. *Journal of Agricultural Science, 5*(6).

McCusker, M. E., Yoon, P. W., Gwinn, M., Malarcher, A. M., Neff, L., & Khoury, M. J. (2004). Family history of heart disease and cardiovascular disease risk-reducing behaviors. *Genetics in Medicine, 6*(3), 153-158.

McIntyre, T. M., & Hazen, S. L. (2010). Lipid oxidation and cardiovascular disease: Introduction to a review series. *Circulation Research, 107*(10), 1167-1169.

Mhd Omar, N. A., Abdullah, N., Kuppusamy, U. R., Abdulla, M. A., & Sabaratnam, V. (2011). Nutritional composition, antioxidant activities, and antiulcer potential of *Lentinus squarrosulus* (Mont.) mycelia extract. *Evidence Based Complementary and Alternative Medicine, 2011*.

Mikhaeil, B. R., Badria, F. A., Maatooq, G. T., & Amer, M. (2004). Antioxidant and immunomodulatory constituents of henna leaves. *Zeitschrift für Naturforschung C, 59*(7-8), 468-476.

Miles, P. G., & Chang, S.-T. (2004). *Mushrooms: Cultivation, nutritional value, medicinal effect, and environmental impact*. Boca Raton, Florida: CRC press.

Mimić-Oka, J., Simić, D. V., & Simić, T. P. (1999). Free radicals in cardiovascular diseases. *Facta Universitatis, 6*, 11-22.

University of Malaya

Mine, Y., & Shahidi, F. (2005). *Nutraceutical proteins and peptides in health and disease*. Florida: CRC Press.

Mirfat, A., Noorlidah, A., & Vikineswary, S. (2010). Scavenging activity of *Schizophyllum commune* extracts and its correlation to total phenolic content. *Journal of Tropical Agriculture and Food Science, 38*(2), 231-238.

Mittal, B. V., & Singh, A. K. (2010). World kidney forum, hypertension in the developing world: Challenges and opportunities. *American Journal of Kidney Diseases : The Official Journal of the National Kidney Foundation, 55*(3), 590-598.

Mohamad Ansor, N., Abdullah, N., & Aminudin, N. (2013). Anti-angiotensin converting enzyme (ACE) proteins from mycelia of *Ganoderma lucidum* (Curtis) P. Karst. *BMC Complementary and Alternative Medicine, 13*, 256.

Morigawa, A., Kitabatake, A., Fijimoto, Y., & Ikekawa, N. (1986). Angiotensin converting enzyme inhibitory triterpenes from *G. lucidium*. *Chemical & Pharmaceutical Bulletin, 34*, 3025-3028.

Mukamal, K. J. (2006). The effects of smoking and drinking on cardiovascular disease and risk factors. *Alcohol Research and Health, 29*(3), 199.

Mullally, M. M., Meisel, H., & FitzGerald, R. J. (1997). Angiotensin-I-converting enzyme inhibitory activities of gastric and pancreatic proteinase digests of whey proteins. *International Dairy Journal, 7*(5), 299-303.

Ni, H., Li, L., Liu, G., & Hu, S.-Q. (2012). Inhibition mechanism and model of an angiotensin i-converting enzyme (ACE)-inhibitory hexapeptide from yeast (*Saccharomyces cerevisiae*). *PLOS ONE, 7*(5), e37077.

Nicholls, S. J., Tuzcu, E. M., Kalidindi, S., Wolski, K., Moon, K. W., Sipahi, I., . . . Nissen, S. E. (2008). Effect of diabetes on progression of coronary atherosclerosis and arterial remodeling: a pooled analysis of 5 intravascular ultrasound trials. *Journal of the American College of Cardiology, 52*(4), 255-262.

Ohm, R. A., de Jong, J. F., Lugones, L. G., Aerts, A., Kothe, E., Stajich, J. E., . . . Wosten, H. A. B. (2010). Genome sequence of the model mushroom *Schizophyllum commune*. *Nature Biotechnology, 28*(9), 957-963.

Okamura, K., Hamazaki, Y., Yajima, A., & Noda, K. (1989). Adjuvant immunotherapy: Two randomized controlled studies of patients with cervical cancer. *Biomedicine & Pharmacotherapy, 43*(3), 177-181.

University of Malaya

Okwulehie, I., Nwosu, C., & Okoroafor, C. (2007). Pharmaceutical and nutritional prospects of two wild macro-fungi found in Nigeria. *Research Journal of Applied Sciences, 2*(6), 715-720.

Ortmann, J., Nett, P. C., Celeiro, J., Traupe, T., Tornillo, L., Hofmann-Lehmann, R., . . . Barton, M. (2005). Endothelin inhibition delays onset of hyperglycemia and associated vascular injury in type I diabetes: Evidence for endothelin release by pancreatic islet *β*-cells. *Biochemical and Biophysical Research Communications, 334*(2), 689-695.

Otsuka, H. (2005). Purification by solvent extraction using partition coefficient. In S. D. Sarker, Z. Latif, & A. I. Gray (Eds.), *Natural products isolation* (pp. 269-273). Totowa, New Jersey: Humana Press.

Öztürk, M., Aydoğmuş-Öztürk, F., Duru, M. E., & Topçu, G. (2007). Antioxidant activity of stem and root extracts of Rhubarb (*Rheum ribes*): An edible medicinal plant. *Food Chemistry, 103*(2), 623-630.

Özyürek, M., Güçlü, K., & Apak, R. (2011). The main and modified CUPRAC methods of antioxidant measurement. *Trends in Analytical Chemistry, 30*(4), 652-664.

Pan, X., Grigoryeva, L., Seyrantepe, V., Peng, J., Kollmann, K., Tremblay, J., . . . Pshezhetsky, A. V. (2014). Serine carboxypeptidase SCPEP1 and Cathepsin A play complementary roles in regulation of vasoconstriction *via* inactivation of endothelin-1. *PLOS Genetics, 10*(2), e1004146.

Pedroche, J., Yust, M. M., Megias, C., Lqari, H., Alaiz, M., Giron-Calle, J., . . . Vioque, J. (2004). Utilisation of rapeseed protein isolates for production of peptides with angiotensin I-converting enzyme (ACE)-inhibitory activity. *Grasas Y Aceites, 55*(4), 354-358.

Peplow, P. V., & Adams Jr, J. D. (2015). The relevance of biomarkers, risk factors and gene-environment interactions in disease: Scientific developments and therapeutic approaches. In *Cardiovascular and metabolic disease: Scientific discoveries and new therapies* (pp. 1-13). The Royal Society of Chemistry.

Pero, R. W., Lund, H., & Leanderson, T. (2009). Antioxidant metabolism induced by quinic acid. Increased urinary excretion of tryptophan and nicotinamide. *Phytotherapy Research, 23*(3), 335-346.

Pham-Huy, L. A., He, H., & Pham-Huy, C. (2008). Free radicals, antioxidants in disease and health. *International Journal of Biomedical Science : IJBS, 4*(2), 89-96.

University of Malaya

Pihlanto-Leppälä, A. (2000). Bioactive peptides derived from bovine whey proteins: Opioid and ACE-inhibitory peptides. *Trends in Food Science & Technology, 11*(9–10), 347-356.

Pihlanto, A., Johansson, T., & Mäkinen, S. (2012). Inhibition of angiotensin I-converting enzyme and lipid peroxidation by fermented rapeseed and flaxseed meal. *Engineering in Life Sciences, 12*(4), 450-456.

Ramli, A. S., & Taher, S. W. (2008). Managing chronic diseases in the Malaysian primary health care - A need for change. *Malaysian Family Physician, 3*(1), 7.

Rampal, L., Rampal, S., Azhar, M. Z., & Rahman, A. R. (2008). Prevalence, awareness, treatment and control of hypertension in Malaysia: A national study of 16,440 subjects. *Public Health, 122*(1), 11-18.

Rang, H. P., Dale, M. M., Ritter, J. M., Flower, R. J., & Henderson, G. (2011). *Rang & Dale's pharmacology*. London, UK: Elsevier Health Sciences UK.

Rang, H. P., Ritter, J. M., Flower, R. J., & Henderson, G. (2014). *Rang & Dale's Pharmacology: with STUDENT CONSULT Online Access*: Elsevier Health Sciences UK.

Rangkhawong, P. (2014). Mycelial fermentation in submerged culture of *Schizophyllum commune* and its properties. *Journal of Science and Technology Mahasarakham University, 33*(5), 420-427.

Rao, S., Sun, J., Liu, Y., Zeng, H., Su, Y., & Yang, Y. (2012). ACE inhibitory peptides and antioxidant peptides derived from *in vitro* digestion hydrolysate of hen egg white lysozyme. *Food Chemistry, 135*(3), 1245-1252.

Reddy, P. R., Manjula, P., & Mohan, S. K. (2005). Novel peptide-based copper(II) complexes for total hydrolytic cleavage of DNA. *Chemistry & Biodiversity, 2*(10), 1338-1350.

Repetto, M., Boveris, A., & Semprine, J. (2012). *Lipid peroxidation: chemical mechanism, biological implications and analytical determination*. Rijeka, Croatia: INTECH Open Access Publisher.

Rho, S. J., Lee, J.-S., Chung, Y. I., Kim, Y.-W., & Lee, H. G. (2009). Purification and identification of an angiotensin I-converting enzyme inhibitory peptide from fermented soybean extract. *Process Biochemistry, 44*(4), 490-493.

Riley, P. A. (1994). Free radicals in biology: oxidative stress and the effects of ionizing radiation. *International Journal of Radiation Biology, 65*(1), 27-33.

University of Malaya

Salahuddin, M. A. H. (2008). *Biological activities of Schizophyllum commune Fr.* (Unpublished master's thesis), University of Malaya, Kuala Lumpur, Malaysia.

Sarmadi, B. H., & Ismail, A. (2010). Antioxidative peptides from food proteins: A review. *Peptides, 31*(10), 1949-1956.

Shahidi, F. (1997). *Natural Antioxidants: Chemistry, Health Effects, and Applications*. Champaign, Illinois: AOCS Press.

Shamloo, M., Eck, P., & Beta, T. (2015). Angiotensin converting enzyme inhibitory peptides derived from cereals. *Journal of Human Nutrition & Food Science, 3*(1), 1057.

Sharma, O. P., & Bhat, T. K. (2009). DPPH antioxidant assay revisited. *Food Chemistry, 113*(4), 1202-1205.

Shih, I.-L., Tsai, K.-L., & Hsieh, C. (2007). Effects of culture conditions on the mycelial growth and bioactive metabolite production in submerged culture of *Cordyceps militaris*. *Biochemical Engineering Journal, 33*(3), 193-201.

Silambarasan, T., Manivannan, J., Krishna Priya, M., Suganya, N., Chatterjee, S., & Raja, B. (2014). Sinapic acid prevents hypertension and cardiovascular remodeling in pharmacological model of nitric oxide inhibited rats. *PLOS ONE, 9*(12), e115682.

Sinal, C. J., Miyata, M., Tohkin, M., Nagata, K., Bend, J. R., & Gonzalez, F. J. (2000). Targeted disruption of soluble epoxide hydrolase reveals a role in blood pressure regulation. *The Journal of Biological Chemistry, 275*(51), 40504-40510.

Singleton, V., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16*(3), 144-158.

Skeggs, L. T., Marsh, W. H., Kahn, J. R., & Shumway, N. P. (1954). The existence of two forms of hypertensin. *The Journal of Experimental Medicine, 99*(3), 275-282.

Smith, H. A. (2014). *Production of antimicrobials and antioxidants from filamentous fungi.* National University of Ireland Maynooth.

Smith, J. E., Rowan, N. J., & Sullivan, R. (2002). Medicinal mushrooms: a rapidly developing area of biotechnology for cancer therapy and other bioactivities. *Biotechnology letters, 24*(22), 1839-1845.

University of Malaya

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., . . . Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry, 150*(1), 76-85.

Soccol, M. C. H., & Oetterer, M. (2003). Seafood as functional food. *Brazilian Archives of Biology and Technology, 46*, 443-454.

Srivastava, A., Harish, R., & Shivanandappa, T. (2006). Novel antioxidant compounds from the aqueous extract of the roots of *Decalepis hamiltonii* (Wight and Arn.) and their inhibitory effect on low-density lipoprotein oxidation. *Journal of Agricultural and Food Chemistry 54*(3), 790-795.

Streatfeild-James, R. M. A., Williamson, D., Pike, R. N., Tewksbury, D., Carrell, R. W., & Coughlin, P. B. (1998). Angiotensinogen cleavage by renin: importance of a structurally constrained N-terminus. *The Federation of European Biochemical Societies Letters, 436*(2), 267-270.

Strube, G., & Strube, G. (1992). The ACE inhibitor drugs: history and pharmacology. *ACE Inhibitors in Hypertension* (pp. 55-67). West Sussex, UK: Springer Netherlands.

Sudano, I., Spieker, L. E., Hermann, F., Flammer, A., Corti, R., Noll, G., & Luscher, T. F. (2006). Protection of endothelial function: targets for nutritional and pharmacological interventions. *Journal of Cardiovascular Pharmacology, 47(Suppl 2)*, 136-150.

Swales, J. D. (1994). The renin-angiotension system as a target for therapeutic intervention. *Journal of Cardiovascular Pharmacology, 24*(2), S1-S5.

Szabo, M. R., Idiţoiu, C., Chambre, D., & Lupea, A. X. (2007). Improved DPPH determination for antioxidant activity spectrophotometric assay. *Chemical Papers, 61*(3), 214-216.

Tel, G., Apaydın, M., Duru, M. E., & Öztürk, M. (2012). Antioxidant and cholinesterase inhibition activities of three *Tricholoma* Species with total phenolic and flavonoid contents: The edible mushrooms from Anatolia. *Food Analytical Methods, 5*(3), 495-504.

Tissot, B., Gasiunas, N., Powell, A. K., Ahmed, Y., Zhi, Z.-l., Haslam, S. M., . . . Dell, A. (2007). Towards GAG glycomics: Analysis of highly sulfated heparins by MALDI-TOF mass spectrometry. *Glycobiology, 17*(9), 972-982.

Tran, H. B., Suhara, H., Doi, K., Ishikawa, H., Fukami, K., Parajuli, G. P., . . . Shimizu, K. (2014). Wild mushrooms in Nepal: Some potential candidates as

University of Malaya

antioxidant and ACE-inhibition sources. *Evidence-Based Complementary and Alternative Medicine, 2014*, 11.

Tribble, D. L., & Committee, F. t. N. (1999). Antioxidant consumption and risk of coronary heart disease: Emphasis on vitamin C, vitamin E, and *β*-Carotene: A statement for healthcare professionals from the American Heart Association. *Circulation, 99*(4), 591-595.

Tripathi, A., & Tiwary, B. (2013). Biochemical constituents of a wild strain of *Schizophyllum commune* isolated from Achanakmar-Amarkantak Biosphere Reserve (ABR), India. *World Journal of Microbiology and Biotechnology, 29*(8), 1431-1442.

Tseng, Y.-H., & Mau, J.-L. (2007). Antioxidant properties of cold water extracts from *Ganoderma tsugae*. *Fungal Science, 22*, 15-25.

Valavanidis, A., Vlachogianni, T., & Fiotakis, K. (2009). Tobacco smoke: Involvement of reactive oxygen species and stable free radicals in mechanisms of oxidative damage, carcinogenesis and synergistic effects with other respirable particles. *International Journal of Environmental Research and Public Health, 6*(2), 445-462.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology, 39*(1), 44-84.

Valverde, M. E., Hernandez-Perez, T., & Paredes-Lopez, O. (2015). Edible mushrooms: improving human health and promoting quality life. *International Journal of Microbiology, 2015*, 14.

Vamanu, E. (2014). Antioxidant properties of mushroom mycelia obtained by batch cultivation and tocopherol content affected by extraction procedures. *BioMed Research International, 2014*, 8.

Vasdev, S., & Stuckless, J. (2010). Antihypertensive effects of dietary protein and its mechanism. *The International Journal of Angiology : Official Publication of the International College of Angiology, Inc, 19*(1), e7-e20.

Vellinga, E. (2013). Spilt Gill - *Schizophyllum commune*. *Mycena News, 64*(09). http://www.mssf.org/mycena-news/pdf/1305mn.pdf

University of Malaya

Vimala, S., Adenan, M. I., & Ahmad, A. R. (2003). *Nature's choice to wellness : Antioxidant vegetables/ulam*. Kepong, Kuala Lumpur (Malaysia): Forest Research Institute Malaysia (FRIM).

Walden, R., & Tomlinson, B. (2011). Cardiovascular Disease. In Benzie, I. F. F & Wachtel, G. S (Eds.), *Herbal Medicine: Biomolecular and Clinical Aspects* (2nd ed.). Boca Raton, Florida: CRC Press.

Wang, T. J., & Vasan, R. S. (2005). Epidemiology of uncontrolled hypertension in the United States. *Circulation, 112*(11), 1651-1662.

Wang, W., Dia, V. P., Vasconez, M., de Mejia, E. G., & Nelson, R. L. (2008). Analysis of soybean protein-derived peptides and the effect of cultivar, environmental conditions, and processing on lunasin concentration in soybean and soy products. *Journal of Association of Official Agricultural Chemists International, 91*(4), 936-946.

Wijesekara, I., & Kim, S.-K. (2010). Angiotensin-I-Converting Enzyme (ACE) inhibitors from marine resources: Prospects in the pharmaceutical industry. *Marine Drugs, 8*(4), 1080-1093.

Wong, J. Y., & Chye, F. Y. (2009). Antioxidant properties of selected tropical wild edible mushrooms. *Journal of Food Composition and Analysis, 22*(4), 269-277.

World Health Organization. (2009). *Global health risks: mortality and burden of disease attributable to selected major risks*: World Health Organization.

World Health Organization. (2011). *Noncommunicable diseases in the South-East Asia region: Situation and response 2011*. Retrieved from http://www.searo.who.int/topics/cardiovascular_diseases/en/

World Health Organization. (2014). *World Health Organization - Noncommunicable diseases (NCD) country profiles , 2014*. Retrieved from http://www.who.int/nmh/countries/mys_en.pdf?ua=1

World Health Organization. (2017). Fact sheet No 317: *Cardiovascular diseases* (CVDs). Retrieved from http://www.who.int/mediacentre/factsheets/fs317/en/

Wu, J., & Aluko, R. E. (2007). Quantitative structure-activity relationship study of bitter di- and tri-peptides including relationship with angiotensin I-converting enzyme inhibitory activity. *Journal of Peptide Science, 13*(1), 63-69.

University of Malaya

Yang, F. C., & Liau, C. B. (1998). Effects of cultivating conditions on the mycelial growth of *Ganoderma lucidum* in submerged flask cultures. *Bioprocess Engineering, 19*(3), 233-236.

Yeh, J., Hsieh, L., Wu, K., & Tsai, C. (2011). Antioxidant properties and antioxidant compounds of various extracts from the edible basidiomycete *Grifola frondosa* (Maitake). *Molecules, 16*(4), 3197-3211.

Ying, X., Ma, J., Liang, Q., Wang, Y., Bai, G., & Luo, G. (2013). Identification and analysis of the constituents in an aqueous extract of *Tricholoma matsutake* by HPLC coupled with diode array detection/electrospray ionization mass spectrometry. *Journal of Food Sciences, 78*(8), C1173-1182.

Young, I. S., & Woodside, J. V. (2001). Antioxidants in health and disease. *Journal of Clinical Pathology, 54*(3), 176-186.

Yuan, Y. V., Bone, D. E., & Carrington, M. F. (2005). Antioxidant activity of dulse (*Palmaria palmata*) extract evaluated *in vitro*. *Food Chemistry, 91*(3), 485-494.

Zárate-Chaves, C. A., Romero-Rodríguez, M. C., Niño-Arias, F. C., Robles-Camargo, J., Linares-Linares, M., Rodríguez-Bocanegra, M. X., & Gutiérrez-Rojas, I. (2013). Optimizing a culture medium for biomass and phenolic compounds production using *Ganoderma lucidum*. *Brazilian Journal of Microbiology, 44*(1), 215-223.

Zhang, W.-X., & Zhong, J.-J. (2013). Oxygen limitation improves ganoderic acid biosynthesis in submerged cultivation of *Ganoderma lucidum*. *Biotechnology and Bioprocess Engineering, 18*(5), 972-980.

Zhang, Y., Geng, W., Shen, Y., Wang, Y., & Dai, Y.-C. (2014). Edible mushroom cultivation for food security and rural development in China: Bio-innovation, technological dissemination and marketing. *Sustainability, 6*(5), 2961.

Zhong, J.-J., & Tang, Y.-J. (2004). Submerged cultivation of medicinal mushrooms for production of valuable bioactive metabolites. In J. J. Zhong (Ed.), *Biomanufacturing* (Vol. 87, pp. 25-59). Berlin Heidelberg: Springer

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