PROTEINS INHIBITING ANGIOTENSIN CONVERTING ENZYME DERIVED FROM *PLEUROTUS PULMONARIUS* MYCELIUM (FR.) QUÉL

BADJIE XIETAQIEUALLAH BINTI IBADALLAH

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ABSTRACT

Pleurotus pulmonarius (grey oyster mushroom) is a well-known edible mushroom that has been acknowledged as a curative agent for many diseases. The antihypertensive potential of *Pleurotus pulmonarius* mycelium was investigated *in vitro* via angiotensin converting enzyme (ACE) inhibitory activity. The study was commenced with cultivation of *P. pulmonarius* mycelium using small scale liquid submerged fermentation for seven days in which brown sugar-spent yeast medium was used as substrate. The mycelial yield was separated from the whole broth to produce mycelial aqueous extract and broth extract, respectively. A preliminary ACE inhibitory assay was performed on the mycelial aqueous extract and broth extract. The result demonstrated almost twofold higher anti-ACE activity of the mycelial aqueous extract at the IC_{50} value of 0.72 mg/ml. Ammonium sulphate precipitation was therefore carried out on the mycelial aqueous extract for protein extraction and fractionations. Proteins were fractionated into ten fractions based on gradual ammonium sulphate saturation and labelled as F10 to F100. Each fraction was tested for their ACE inhibitory activity and result showed the highest activity by F40 with the IC_{50} value obtained was 0.022 mg/ml, which was 32 times stronger than the IC_{50} value of mycelial aqueous extract. Fraction F40 was subjected to RP-HPLC for protein purification using a semi-preparative column C18 (10 × 100 mm). Eight apparent chromatogram peaks were observed and labelled as P1 to P8. All peaks were evaluated for their ACE inhibitory activity and P6 demonstrated the highest ACE inhibitory activity at the IC_{50} value of 0.012 mg/ml. The IC_{50} value of P6 was 60 times stronger than that of the mycelial aqueous extract, and considered among the strongest IC_{50} values for ACE inhibitory activity discovered from mushrooms. Proteins from P6 were further analysed via SDS-PAGE. Consequently, there were four protein bands emerged via silver staining method. Each band was then

excised and treated with trypsin in-gel digestion prior to MALDI-TOF/TOF MS. Database search from MALDI-TOF/TOF MS has nominated three potential antihypertensive proteins that work via different mechanisms; serine proteinase inhibitor-like protein, nitrite reductase-like protein and DEAD/DEAH box RNA helicase-like protein. MRPS5-like protein could also be one of the desired proteins due to the highest protein score among all other proteins in MALDI database. Fraction P6 was also subjected to LC-MS/MS to support the protein identification. DEAD/DEAH box RNA helicase-like protein was the only protein that has been identified in both mass spectrometries. This study demonstrated the first reported ACE inhibitory activity from the mycelium of *P. pulmonarius* (Fr.) Quél cultivated locally.

ABSTRAK

Pleurotus pulmonarius (cendawan tiram kelabu) adalah salah satu cendawan yang boleh di makan dan mengandungi pelbagai khasiat penyembuhan. Kebolehan cendawan ini sebagai agen penurun tekanan darah diuji secara *in vitro* menggunakan kaedah perencatan aktiviti enzim angiotensin converting enzyme (ACE). Kajian dimulakan dengan pengkulturan miselium *P. pulmonarius* menggunakan teknik fermentasi terendam berskala kecil yg dilakukan selama tujuh hari dengan menggunakan media gula merah dan yeast terpakai (brown sugar-spent yeast) sebagai substrat. Hasil kultur iaitu miselium dan larutannya diasingkan bagi menghasilkan ekstrak miselium terlarut (mycelial aqueous extract) dan ekstrak larutan medium (broth extract). Aktiviti perencatan enzim ACE telah dilakukan ke atas kedua-dua ekstrak tersebut. Hasil kajian mendapati ekstrak miselium terlarut menunjukkan aktiviti perencatan enzim yang lebih baik dengan bacaan IC_{50} sebanyak 0.72 mg/ml, iaitu dua kali ganda lebih baik daripada ekstrak larutan medium. Oleh itu, ekstrak miselium terlarut dipilih untuk meneruskan ujikaji seterusnya iaitu pengekstrakan dan fraksionasi protein melalui kaedah pemendakan ammonium sulphate (ammonium sulphate precipitation). Melalui kaedah ini, protein telah dikategorikan kepada sepuluh kumpulan dan di label sebagai F10 hingga F100. Setiap kumpulan protein diuji aktiviti perencatan enzim ACEnya, dan ujian mendapati F40 menunjukkan kadar perencatan enzim tertinggi iaitu pada bacaan IC_{50} sebanyak 0.022 mg/ml, yang mana mencatatkan sebanyak tiga puluh dua lebih rendah nilainya berbanding bacaan yang diperolehi daripada ekstrak miselium terlarut. Protein-protein dari F40 kemudiannya dibersihkan menggunakan teknik RP-HPLC dengan menggunakan turus C18 (10 × 100 mm). Sebanyak lapan puncak (peaks) telah diperolehi dan setiap puncak telah diuji aktiviti perencatan enzim ACEnya. Puncak P6 telah menunjukkan aktiviti perencatan enzim tertinggi iaitu sebanyak IC_{50} 0.012 mg/ml.

Nilai ini adalah enam puluh kali lebih rendah daripada bacaan yang direkodkan oleh ekstrak miselium terendam. Malah, nilai ini adalah antara nilai terendah yang dicatatkan bagi aktiviti perencatan enzim ACE oleh cendawan. Puncak P6 seterusnya melalui analisa SDS-PAGE untuk proses pemisahan lanjut protein-protein. Hasil analisa mendapati empat gelang (band) terhasil daripada P6. Setiap gelang telah dicerna menggunakan trypsin dan dihantar untuk analisa MALDI-TOF/TOF. Hasil analisa mendapati tiga protein yang terlibat di dalam proses penurunan tekanan darah, iaitu protein-protein yang menyamai serine proteinase inhibitor, nitrite reductase, dan DEAD/DEAH box RNA helicase. Protin MRPS5-like juga perlu diambil kira sebagai protein yang dikehendaki berhubung dengan peratusan tertinggi skor protin yang ditunjukkan berbanding protein-protein lain di dalam pangkalan data MALDI. Puncak P6 juga telah melalui analisa LC-MS/MS sebagai mengukuhkan hasil carian protein-protein perencat ACE daripada MALDI-TOF/TOF. Hasil kajian mendapati kewujudan DEAD/DEAH box RNA helicase di dalam kedua-dua analisa. Kajian ini membuktikan potensi strain tempatan miselium *P. pulmonarius* (Fr.) Quél terhadap aktiviti penurunan tekanan darah, sekaligus mencatat kajian pertama terhadap perencatan enzim ACE bagi miseleum spesis tersebut.

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Yours sincerely,

Badjie Xietaqieuallah Binti Ibadallah.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

Å	Angstrom
cm	Centimetre
°C	Degree Celsius
CO_2	Carbon dioxide
Da	Dalton
ELISA	*enzyme-linked immunosorbent assay*
g	Gram
g/L	gram per litre
HCL	Hydrochloric acid
IC_{50}	Half maximal inhibitory concentration
kDa	kilo Dalton
lux	Illuminance
LC-MS/MS	Liquid chromatography- tandem mass spectrometry
MALDI-TOF/TOF	Matrix-assisted laser desorption/ionization-time of flight
µg/ml	microgram per millilitre
µl	Microliter
µm	Micrometre
mAU	milli absorbance units
mg/kg	milligram per kilogram
mg/ml	milligram per millilitre
ml/min	millilitre per minute
mm	Millimetre
mmHg	millimetre of mercury

ml	Millilitre
MW	Molecular weight
m/z	mass-to-charge ratio
n/a	Not available
nm	Nanometre
p*I*	Isoelectric point
ppm	parts per millions
rpm	Revolution per minute
RT	Retention time
S.E.M	Standard error of mean
t	Time
V	Volt
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1: INTRODUCTION

Hypertension is a chronic medical condition in which the blood pressure level in the arteries is consistently above the normal range. It is also referred to as high blood pressure. The Sixth Report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure defines hypertension as systolic blood pressure (SBP) of 140 mm Hg or greater and diastolic blood pressure (DBP) of 90 mm Hg or greater, or anyone prescribing antihypertensive medication (Barbosa-Filho *et al.*, 2006).

According to the World Health Organization (WHO., 2011), hypertension has consecutively contributed to the first leading cause of death worldwide. It is known to be a common and progressive disorder that causes a major risk for cardiovascular and renal diseases due to increasing longevity and prevalence of contributing factors such as obesity and unhealthy lifestyle. Even though the treatment of hypertension has proven to prevent cardiovascular diseases and to enhance life, hypertension remains scantily managed everywhere.

There are many types of drug currently used to treat hypertension. Angiotensin converting enzyme (ACE) inhibitors are among the most widely prescribed clinical drugs to effectively treat hypertension and certain kidney-related diseases (Wong *et al.*, 2004). ACE inhibitors offer various advantageous for patients with multiple cardiovascular disorders. Their main purpose is to lower blood pressure by promoting vasodilatation. Prevention of angiotensin II by ACE inhibitor prevents generation of

toxic radicals. Constant treatment with ACE inhibitors resulted in significant improvement of endothelial function of arteriosclerosis patients. Mortality rate of cardiovascular patients with coronary artery disease and chronic heart failure is reduced by long-term treatment with ACE inhibitors because of the blood pressure lowering effect and cholesterol reduction (Tiefenbacher, *et al*., 2003). Other exclusive effects of ACE inhibitors include regression of ventricular hypertrophy and vascular smooth muscle cell proliferation, enhancement of nitric oxide effects and anti-macrophage function (Wong *et al*., 2007).

Formulation of ACE inhibitors as antihypertensive drugs has evolved enormously. From the first discovery of ACE inhibitor in a toxic venom of a Brazillian viper (*Bothrops jararaca),* Cushman and Ondetti had later succeeded on the formulation of a synthetic ACE inhibitor (captopril), which has become a revolution in the treatment of hypertension (Cushman et al., 1999). As much as it has proven to be remarkably efficacious at lowering blood pressure and show other cardiovascular benefits, it renders deleterious side effects to patients. Skin rash, taste disturbance and proteinuria were reported as common side effects caused by the presence of sulphydryl binding group in synthetic ACE inhibitors (Reid, 1997). Other reported common side effects are persistent cough (Mukae, 2000), angioderma and dizziness (Gibbs et al., 1999).

The search for safer ACE inhibitors with less or no side effects is highly anticipated to improve the quality of antihypertensive drug-therapy, thus mitigates adverse reactions and prevents unnecessary complications caused by the medicine. Hence, natural products and food sources provide a vast resource for the development of harmless antihypertensive drugs (Kumar et al., 2010). Natural products and their derivatives have been a remarkable source of human medicines, both in the mainstream medicine

practices and the homeopathic medicine. The advent of technologies in biochemistry allows scientists to specify their study on the bioactive compounds of natural resources and discover their medicinal properties (Haustedt et al., 2006).

Pleurotus pulmonarius is one of the most well-known edible mushrooms in the South East Asian countries including Malaysia. It is renowned for the delectable and succulent taste and often added to various dishes. In addition, *P. pulmonarius* contains high vitamins, minerals, proteins and other bioactive compounds thus make it a nourishing choice of food for dietary supplements as well as the eye-opening curative resources in drug therapies against many devastating diseases (Shahid *et al*., 2006).

Pleurotus pulmonarius mycelium exhibits an equivalent therapeutic importance as its fruiting body. Many scientific research reported contribution of *P. pulmonarius* mycelium in promoting health, especially via its bioactive compounds and proteins activities. Advance in current biotechnologies develop extensive coverage of research in mycelia study from crude extract *in vitro* bioassay to specific analyses up to protein identification, relationship edifice between enzymes and their targets, and more in depth protein-structure relationship, thus allowing better understanding of the mushroom and maximum exploitation in scientific research.

Despite the available discoveries done by scientist vis-à-vis medicinal properties of *P. pulmonarius*, there is still inadequate information available regarding bioactive peptides derived from local strains. As the medicinal value and peptide composition of a mushroom may be affected by the growth environment, it is important to further investigate the medicinal properties of *P. pulmonarius* in Malaysia, especially towards the novel and undiscovered therapeutic value. Hitherto, there is no report on the ACE

inhibitory activity from *P. pulmonarius* mycelium both from local and foreign strains. Hence, this study was intended to investigate antihypertensive activity via ACE inhibitory action on the commercial strain of *P. pulmonarius* mycelium.

OBJECTIVES

The aim of this study was to investigate ACE inhibitory activity of *P. pulmonarius* mycelium cultivated by liquid fermentation. More specific objectives were:

1) To produce mycelial biomass of *P. pulmonarius* by liquid fermentation and to prepare protein extracts,
2) To investigate ACE inhibitory activity of protein extracts from the mycelium and cultured broth,
3) To identify potential ACE inhibitors using a series of protein purification methods and followed by proteomic tools, i.e. MALDI-TOFF/TOFF and LC-MS/MS.

CHAPTER 2: LITERATURE REVIEW

2.1 Hypertension

Hypertension can be classified into two types; essential or primary hypertension and secondary hypertension. Essential hypertension is the condition where the underlying causes of high blood pressure are unjustified. Approximately, up to 95% of people with high blood pressure are in this category. On the other hand, secondary hypertension occurs as a result of other disorders, such as kidney disease, diabetes, obstructive sleep apnea and aortic coarctation (Oparil *et al*., 2003).

2.1.1 Causes and symptoms of hypertension

Hypertension is synonymous with the term 'a silent killer' because people with this pathological condition can be asymptomatic for years, before leading to serious disorders such as a sudden fatal heart attack or stroke if it is left untreated for a long time. In other words, the majority of people suffering from hypertension may possess a late onset condition. However, there are many risk factors that can lead to essential hypertension. Obesity is one major factor of hypertension. Also, sedimentary lifestyles, increased level of stress, high sodium and alcohol intake, vitamin D deficiency and aging are among the factors for essential hypertension. People with a family history of hypertension and some genetic inherited mutations may have higher risk of developing hypertension (Calhoun *et al*., 2008).

Studies have elucidated that some disorders such as rennin elevation, insulin resistance and metabolic syndrome are thought to cause hypertension (Segura *et al*., 2007). Recently, low birth weight has been observed as a risk factor for adult essential

hypertension. In addition, several classes of drugs can increase blood pressure, though the effects are highly individualized (Bagby, 2007).

People with mild to moderate essential hypertension are normally asymptomatic. Severe hypertension is associated with somnolence, headache, visual disturbance, confusion, nausea and vomiting. In children, hypertension may cause fatigue, blurred vision headache, epitasis and bell palsy (Pitts & Adams, 1998).

On the contrary, the cause of secondary hypertension is recognizable. Pheochromocytoma and hyperaldosteronism are diseases often related to secondary hypertension. There are also certain medications that can lead to secondary hypertension. Long term intake of corticosteroids group drugs like prednisone and prednisolone can lead to Cushing's syndrome and hence increase the risk of secondary hypertension. Likewise, non-steroidal anti-inflammatory drugs (NSAIDs) can cause kidney damage and lead to secondary hypertension if used regularly and continuously (Malaysia Hypertension Guideline Working Group, 2008). Other uncommon secondary causes of hypertension consist of hyperparathyroidism, aortic coarctation and intracranial tumours. It is important to identify the underlying causes and factors in secondary hypertension because the treatment is specific (Anderson *et al.*, 1994).

2.1.2 Treatment and medication of hypertension

Hypertension is commonly multifactorial in aetiology. In benign cases, lifestyle modifications including weight loss, regular exercise, moderate alcohol intake, and ingestion of a high-fibre, low-fat and low-salt diet is highly recommended, prior to drug therapy. If the hypertension is malignant enough to justify immediate use of medications, lifestyle changes are initiated concomitantly (Barbosa-Filho *et al.*, 2006).

The majority of resistance hypertension patients require antihypertensive agents for pharmacological treatment and medication. The aim of treatment is to reduce the high morbidity and mortality chances. As well as lowering blood pressure and maintaining at <140/90 mmHg for most patients, it must also be able to reduce certain circumstances often related to hypertension such as diabetes, kidney diseases and other cardiovascular risk factors (Kim *et al*., 2004).

There are alternatives available to treat hypertension, such as monotherapy and/or combined therapy. However, numerous researches demonstrate antihypertensive advantage by combining different classes of drugs (Burgess, 2002). The commonly used drugs are the typical groups of angiotensin I-converting enzyme (ACE) inhibitors, calcium channel blockers, alpha blockers, beta blockers, vasodilators, centrally acting agents and diuretics. The choice of drugs used for the treatment is based on certain criteria such as age, severity of hypertension and occurrence of other complications (Ali *et al*., 2006).

2.1.3 Mechanism of hypertension on the renin-angiotensin-aldosterone system (RAAS)

Blood pressure is regulated by a variety of different interacting biochemical mechanisms. The increase or decrease of blood pressure relies on which pathway predominates at a time. Naturally, regulation of blood pressure is highly related with the RAAS, which plays a vital role in the maintenance of vascular tone with regard to peripheral resistance (Figure 2.1) (Hong *et al*., 2008).

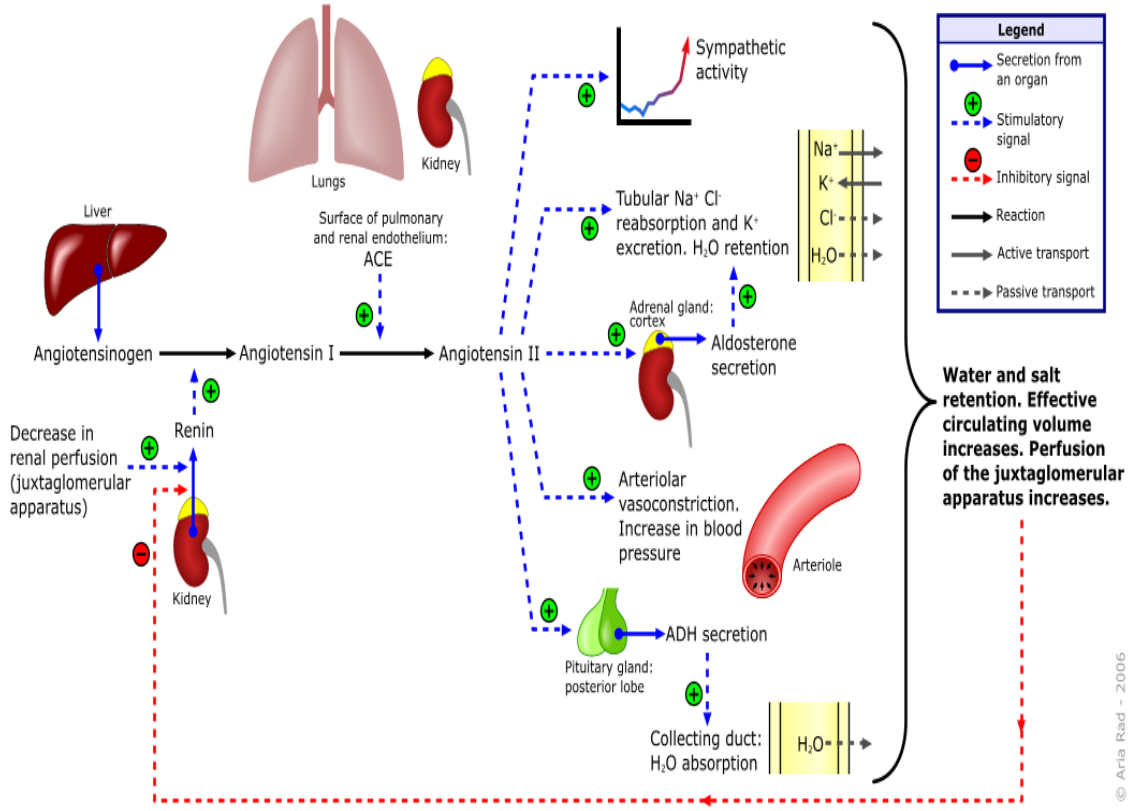


Figure 2.1: Schematic diagram of renin-angiotensin-aldosterone system (Rad, 2006). RAAS plays a vital role in the regulation of blood pressure. The system includes series of enzymes and hormones, mainly the conversion of angiotensin I to angiotensin II. Angiotensin II takes part in many pathways, such as the vascular tone mechanism (vasoconstriction) and superoxide formation (endothelial dysfunction). Angiotensin II also stimulates the secretion of aldosterone, which increases the absorption of sodium and water in the blood, thus increases blood pressure.

Renin is secreted by the juxtaglomerular apparatus of the kidney when blood pressure is decreasing. It splits angiotensinogen to produce the inactive decapeptide angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu). In the presence of ACE, angiotensin I is converted to an active octapeptide vasoconstrictor and cardiovascular trophic factor, angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) (Figure 2.2). Simultaneously, kallikrein enzyme catalyzes the inactive bradykininogen into an active hypotensive bradykinin, which will turn into inactive fragments by the action of ACE, as another course that contributes to the hypertensive effects of ACE activity (Choi *et al.*, 2001).

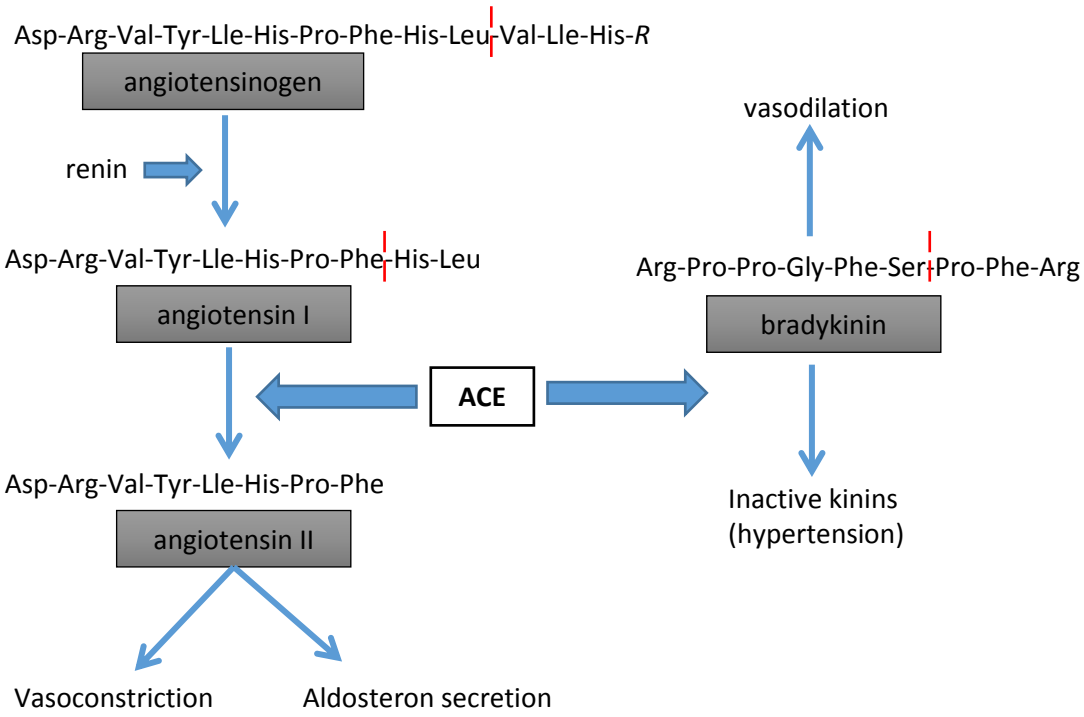


Figure 2.2: The suggested hypertensive mechanism of angiotensin. ACE triggers hypertensive effects via conversion of angiotensin I to angiotensin II, and inactivation of hypotensive peptide bradykinin (Adapted from Barbosa-Filho *et al.*, 2006).

Angiotensin II plays a role in stimulating the synthesis and release of aldosterone (ADH) from the adrenal cortex, as well as triggering vascular smooth muscle constriction. Aldosterone secretion is responsible in increasing blood pressure by promoting sodium retention (and thereby water retention) in the distal tubules (Figure 2.2) (Packer *et al*., 1986). Angiotensin II also promotes the production of superoxide anion and hydrogen peroxide in the polymorphonuclear leucocytes, which inactivates the endothelial vasodilatory compounds nitric oxide (NO) and prostacyclin (PGI_2). Production of superoxide rather than NO inhibits cGMP pathway and thus conducts vascular dysfunction (Barbosa-filho *et al*., 2006).

In general, angiotensin II is a hormone that has many effects on the cardiovascular system, such as to constrict arteries, raise blood pressure and thicken several cardiovascular structures. For the case of cardiovascular disorders, angiotensin II is

secreted in abnormally high amount (Catt *et al.*, 1971). A profusion of angiotensin II triggers persistent complications to the cardiovascular system thus causes the heart to work more intensely to pump blood into the body's main arteries. The excessive force received by the heart muscle tends to raise problems if the heart has been weakened by other problems such as heart attack. High levels of circulating angiotensin II thickens the heart and blood vessels, a condition known as hypertrophy. Together with the occurrence of vessel constrictions, this is thought to incline accumulation of cholesterol level and blockages in the arteries, which can aggravate to strokes and heart attacks (Nancy, 2003).

2.1.4 Angiotensin Converting Enzyme (ACE)

Angiotensin converting enzyme (ACE; kiniase II; EC 3.4.15.1) is an exopeptidase that catalyzes the conversion of angiotensin I to angiotensin II by removal of the C-terminal dipeptide. ACE is produced predominantly in lung and male reproductive tissues, as well as on the plasma membranes of various cell types including microvillar brush border epithelial cells (e.g. renal proximal tubule cells), endothelial cells, and neuroepithelial cells. Other organs that produce ACE are kidney, brain, intestine and adrenal cortex. ACE is an acidic protein with an approximate p*I* of 4.5, and a zinc dependent dipeptidase which demonstrates reliance upon a metal ion for full enzymatic activity (Ondetti & Cushman, 1984).

ACE is well known to be a key part of the rennin-angiotensin-aldosteron system (RAAS) that regulates blood pressure. Besides acting as a catalyst for the production of angiotensin II, it metabolizes several other peptides such as bradykinin and kallidin into inactive metabolites. As the ACE activity gives rise to vasoconstriction and decreases

vasodilation, the inhibitors of ACE could therefore exhibit potency of treating hypertension (Atlas, 2007).

2.1.5 Angiotensin I-converting enzyme (ACE) inhibitors

Studies with peptidic inhibitors of ACE have been performed since early 60's. In depth research comprising the structure-activity relationship, enzyme interactions and peptide analogue inhibitors have been studied extensively to design potent antihypertensive agents that competitively block ACE activity, hence the conversion of angiotensin I to angiotensin II, thereby reducing angiotensin II level and followed by reduction of blood pressure (Ondetti & Cushman, 1984). Although the structure-function relationship of ACE inhibitors is still not clear, they share some similarities in the structures. They are rich in hydrophobic amino acids and many of them contain proline, lysine, or arginine as C-terminal residues. Common ACE inhibitors are di- or tripeptides, which are resistant to the digestive tract endopeptidases and can be easily absorbed to the blood (Iwaniak *et al*., 2008).

Developments of potent derivatives ACE inhibitors as antihypertensive drugs have become imperative and are widely used for the treatment of cardiovascular diseases due to their ability to improve blood pressure, control patients with hypertension and prolong survival rate in patients. Prescription of ACE inhibitors can avert or reverse hypertrophy of the heart and vessel walls. They also work to prevent constriction of arteries, lower blood pressure and reduce the energetic heart pumping of the patients (Loizzo *et al.*, 2007).

Clinical studies have demonstrated that ACE inhibitors significantly reduce the morbidity and mortality of patients with myocardial infarction or heart failure, and

delay the onset of symptoms in heart failure patients (Tsai *et al.,* 2007). They also reduce the frequency of hospitalization. Studies have shown that ACE inhibitors prevent imminent heart attacks and deaths from heart disease for patient that is at high risk for having heart attacks or developing heart failure. ACE inhibitors are also used to reduce heart attacks in patients with diabetes and mild kidney disease (Nancy, 2003)

2.1.6 Angiotensin I-converting enzyme inhibitors from natural products

The first discovery of ACE inhibitors was from snake venom, which is involved in smooth muscles contractions in the guinea pig ileum because of the bradykinin activity. The first ACE inhibitor was called bradykinin potentiator due to its mechanism (Cheung *et al,*. 1980; Iwaniak *et al*., 2008). Later, many synthetic ACE inhibitors such as captopril, enalapril and lisinopril have been formulated and used clinically as first line antihypertensive drugs.

Albeit the remarkable effect in treating hypertension, synthetic inhibitors can cause deleterious side effects. For some patients, they may undergo dizziness, cough and allergies (Jang *et al*., 2011). Others may get worse adverse effects such as taste disturbance and disruption of kidney function. Hence, the quest for harmless and cost-effective naturally occurring ACE inhibitors are highly coveted for the remedies of hypertension (Li *et al.*, 2007).

Recent interests have focused on the isolation and identification of ACE inhibitors from natural products and foods for the development of more effective ACE inhibitors. These sources are known to carry a wide range of functional and biological properties. Several ACE inhibitors derived from natural and food products have demonstrated potent antihypertensive effects and other positive effects on the metabolism of glucose and

lipid, in increasing exercise tolerance and on reducing insulin requirements in diabetes (Lee *et al*., 2004).

ACE inhibitors from milk products are the most intensely studied so far. To date, numerous ACE inhibitory peptides have been isolated from milk products. For instance, two fermented sour-milk products containing the ACE inhibitory tripeptides, Ile-Pro-Pro (IPP) and Val-Pro-Pro (VPP) have been launched commercially in Finland and Japan, respectively. These products have proven to effectively reduce blood pressure in mildly hypertensive human subjects, and reported to cause no side effects (FitzGerald *et al*., 2000).

Several natural products from plant extracts [broccoli (Lee *et al.,* 2006), spinach (Yang *et al.,* 2003)], fungi [*Grifola frondosa* (Choi *et al.,* 2001), *Ganoderma lucidum* (Morigawa *et al*., 1986), *Flammulina velutipes* (Melzig *et al*., 1996)], and algae [*Sargasum* sp. (Tierney *et al*., 2010), *Ishige okamurae* (Yoon *et al*., 2009)], and many more that act specifically by inhibiting ACE have also been discovered and proven to be amongst the most powerful known vasodilators.

2.5 Mushrooms

Mushrooms can be defined as macrofungus, a type of fungi that belong to the Fungi Kingdom. In general, the life cycle of a mushroom consists of vegetative and reproductive phases. Vegetative growth denotes fungal mycelial growth. At this stage, mycelium absorbs nutrients from the substrate by the secretion of enzymes and then colonizes them. In an optimum source of light, humidity, temperature, and oxygen, the mycelial colony ceases vegetative phase to later grow into fruit bodies, commencing the

reproductive phase. The fruit bodies are called mushrooms, which can grow into different sizes and structures (Cho, 2004).

Mushrooms generally belong to basidiomycetes, and are estimated to be over 140,000 of species available on earth, but only 14,000 to 22,000 are yet known (Hawksworth, 2001). There are at least 2,000 species showing various degrees of edibility, with over 200 species have been collected from the wild and used for traditional medical purposes, and about 35 mushroom species have been cultivated commercially (Sánchez, 2004).

In general, mushrooms are divided into four major categories, (1) edible mushrooms, which are normally fleshy and used as dietary products, e.g. many *Pleurotus* species, (2) medicinal mushrooms, which are considered to have medicinal properties and used as active ingredients in many medical treatments, e.g. *Ganoderma tsugae*, (3) poisonous mushrooms, which are proven or suspected to be harmful and poisonous, e.g. *Amanita muscaria*, and (4) other mushrooms, which include a large number of mushrooms whose properties remain less identified. However, many types of mushrooms could be classified into more than one category, especially the edible mushrooms where they could be edible and also medicinal (Stamets, 2000).

Mushrooms play a vital role in nature. They are the premier recyclers in nature that work by secreting enzymes to digest organic wastes and returning them back to the natural ecosystem. Like other fungi, mushrooms produce digestive exoenzymes by their hyphae, in which they digest the food into small molecules that can be absorbed and used by the fungus (Archer & Wood, 1994).

Mushrooms have been appreciated as scrumptious and nourishing foods, due to their fine, unique and subtle flavours, as well as good textures. Known as the meat of vegetable world, edible mushrooms are used extensively in many cuisines especially in the Asian countries such as Japan, Korea, China, Indonesia and Malaysia, as well as some European countries. In general, these fungi contain high nutritional value such as protein, fibre, minerals and vitamins, thus make them as a healthy dietary source (Shahid *et al*., 2006).

2.5.1 Medicinal properties of mushroom

Mushrooms share a long history with human civilization. Some mushrooms had been exploited in ancient spiritual beliefs and rituals due to their psychoactive and hallucinogenic properties (Smith *et al.*, 2002). The psychoactive mushrooms had been long employed in native medicine traditions in cultures all around the world. Also known as magic mushrooms or shrooms, these mushrooms had been claimed as facilitating profound and life-changing insights often articulated as mystical experiences. Recent scientific study done by Griffiths and his co-workers has supported the claim, in which the significance of enhanced spiritual experiences is correlated with psilocybin, the principal psychoactive component of various hallucinogenic mushrooms (Griffiths *et al*., 2011).

Many societies worldwide recognize that certain mushrooms contain intense health-promoting benefits, and hence are called medicinal mushrooms. In Oriental countries, both edible and non-edible mushrooms have been used for medicinal purposes ever since ages ago. Ying *et al.,* has reported that there are approximately 270 species of mushrooms that are recognized to have various recuperative properties. This number is

thought to increase as the interest in medicinal mushroom studies continues to evolve (Smith *et al.*, 2002).

According to scientific studies, mushrooms constitute potent therapeutic activities and help in the prevention and treatment of various diseases. Scientists have discovered several bioactive compounds in medicinal mushrooms, which demonstrated curative efficacy to many diseases, particularly in anticancer and antitumor therapy. The research of anticancer and antitumor potential from mushrooms has been extensively studied *in vitro* and *in vivo* (Ikekawa, 2001). There are also bioactive compounds from mushrooms extracts that have already been commercialized and are currently produced by Asian pharmaceutical companies. For instance, the main anticancer polysaccharides that have been subjected to early clinical tests are lentinan (*Lentinus edodes*), PSK and PSP (*Trametes versicolor*), Schizophyllan (*Schizophylum commune*) and Grifon-D (*Grifola frondosa*) (Wasser, 2002).

Mushrooms have also benefited the administration of cardiovascular health complications. Some mushroom components such as lipids, fibres, vitamins, protein, phenolic compounds and minerals have shown positive results in the treatment and/or prevention of cardiovascular diseases. Several mechanisms including the anti-atherosclerosis effect of some edible mushrooms have been reported, mainly toward the improvement of vascular reactivity, anti-oxidative effects and anti-platelet aggregating effects. Kaneda and Tokuda (1966) were among the first to report hypocholesterolemic effect of *Lentinula edodes, Auricularia polytricha, Flammulina velutipes* and *Agaricus bisporus.* Whereas for the blood pressure treatment, the low concentration of sodium but high amount of potassium in mushrooms substantiates mushrooms intake within an

antihypertensive diet, which currently directed to more profound research on the antihypertensive study (Guillamón *et al*., 2010).

Mushrooms also demonstrate their pharmacological and therapeutic actions against other numerous diseases and metabolic disturbances as serious as cancer or degenerative diseases. Other verified pharmacological activities studied in medicinal mushrooms are anti-inflammatory effect, antioxidant activity, antimicrobial activity, immunostimulating activity, and activities on metabolic syndrome and its related diseases such as diabetes and hypertension (Poucheret *et al*., 2006).

Medicinal mushrooms have contributed to the main mushrooms cultivation in Asian countries and currently penetrating the Western market place, due to a flourishing demand for healthier foods and their increased consumption in the mainstream medical practices (Sullivan *et al.,* 2006). Throughout the years, more mushroom species are studied for their medicinal prospects. This has led to better understanding of mushrooms in their physiological and morphological characterizations. In fact, medicinal mushroom studies have become a rapidly developing area of biotechnology for biological activities, especially in promoting health.

2.6 *Pleurotus pulmonarius* (Fr.) Quél

Pleurotus pulmonarius represents a circumscribed group of higher white rot fungi of the phylum Basidiomycota. Categorized under the family of Pleurotaceae, it is recognized by a grey colour fruit body with decurrent hymenium, bare stipe and eccentric stalk attached to the pileus, which opens up like an oyster shell during the morphogenesis. Hence, it is often called the “Grey oyster mushroom”. It has cylindrical, smooth white

spores print attached to the gills and produces white velvety mycelia when grown on agar media (Rajarathnam *et al.*, 1987).

Pleurotus pulmonarius is often erroneously identified as *Pleurotus sajor-caju* by mycologists and mushroom growers. Although they are considered as two closely related cultivated oyster mushroom species, they are different in morphological and reproductive approach. *Pleurotus pulmonarius* is categorized under the Order of Agaricales which has gills on the hymenium and has monomitic type of hyphae. In contrast, *P. sajor-caju* is categorized under the Order of Polyporales which has pores on the hymenium and has trimitic or dimintic hyphae. Hence, in 1975, *P. sajor-caju* was returned to the genus *Lentinus* by Pegler and addressed as *Lentinus sajor-caju*. The name *P. sajor-caju* was then removed from the Index Fungorum. However, the name *P. sajor-caju* has been misapplied by mushroom growers and even in scientific papers, thus contributed to the persistent confusion (Stamets, 2000). Figure 2.3 shows (a) *P. pulmonarius* fruiting body and (b) *P. pulmonarius* mycelium growing on an agar plate.

(a)  (b) 

Figure 2.3: (a) *P. pulmonarius* fruiting body, (b) *P. pulmonarius* mycelium.

The first discovery of *P. sajor-caju* (*pulmonarius*) was reported on the succulent tissues of *Euphorbia royleans* Boiss., in the foothills of the Himalayas. Like other *Pleurotus* mushrooms, *P. pulmonarius* is naturally found as a saprophyte, a primary decomposer

of hardwood trees. It grows naturally on dead standing trees or on fallen logs, mostly under shady or diffused light. However, due to its high saprophytic colonizing ability, it can grow over a wide range of temperatures on different natural resources and agricultural wastes (Madan *et al.*, 1987). The growth parameters of *P. pulmonarius* are shown in Table 2.1.

Table 2.1: Growth parameters environmental requirement of *P. pulmonarius* (Stamets, 2000)

Spawn run	**Primodial formation**	**Fruit body development**
Incubation temperature: 24 – 29°C	Initiation temperature: 10 – 27°C	Temperature: 18 – 24°C
Relative humidity: 90 – 100%	Relative humidity: 95 – 100%	Relative humidity: 95 – 100%
Duration: 18 – 14 days	Duration: 3 – 5 days	Duration: 3 – 5 days
CO_2: < 5000 ppm	CO_2: 400 – 800 ppm	CO_2: 400 – 800 ppm
Fresh air exchanges: 1 per hour	Fresh air exchanges: 5 – 7 per hour	Fresh air exchanges: 5 – 7
Light requirements: n/a	Light requirements: 1000 – 1500 (2000) lux	Light requirements: 1000 – 1500 (2000) lux

As compared to other edible mushrooms, *P. pulmonarius* is one of the most successfully cultivated mushrooms (Zhang *et al.*, 2002). It is among the easiest, fastest and cheapest to grow and requires less preparation time and technology. It can grow in various by-products as substrates such as banana leaves, peanut leaves, sugarcane leaves, wheat and rice straw, thus makes it very economical and significant for commercial purposes. Its fruiting body is fairly resistant to diseases and pests attacks. Because of its easiness

in cultivation and high yields, the demand for *P. pulmonarius* is increasing, thus making its way to supermarket shelves during any season (Mandeel *et al*., 2005). Owing to its succulent taste and suitability to almost any Asian cuisine, *P. pulmonarius* has become the most popular edible mushroom in Malaysia.

Besides the fruiting body, *P. sajor-caju* mycelium plays comparable important role in human and animal life, mainly in culinary and food products such as food additives and mushroom-flavour agents (Chahal, 1989). Having the ability to absorb inorganic microelements, degradation of phenolic compounds and lignocellulosic materials, *P. sajor-caju* mycelium has proven its ability in bioremediation and decreasing environmental pollution. In fact, mycelia products are believed to be 'the wave of the future' because they ensure standardized quality by way of rapid and undemanding production (Lindequist *et al.*, 2005).

Research performed by Ferreira and his co-workers in 2008 showed that *P. sajor-caju* mycelium exhibits potential in the treatment of olive oil mill wastewater. This mycelium is also involved in bio pulping, as well as recycling process of environmental products such as unused wood residues and its by-products. The advantages of *P. sajor-caju* mycelium and its contributions to the waste management enhance economic returns needed to support ecosystem management. These values include reducing fuel for fire, decreasing pests and disease outbreaks, and increasing biodiversity (Croan, 2000).

2.6.1 Medicinal values of *Pleurotus pulmonarius*

Pleurotus pulmonarius is one of the commonly recognized and therapeutically valuable Basidiomycetes mushrooms. Generally, it contains low fat but abundance of carbohydrates, thus making it an ideal food source for weight conscious people and patients suffering from cardiovascular diseases. It also contains high level of mineral elements (such as calcium, copper, iron, cuprum, magnesium, phosphorus, potassium and sodium) and vitamins (B_1, B_2, B_{12} and C). Apart from having a very high amount of fibre, it also has high content of proteins and peptides. The amount of crude proteins found in this species is among the highest in the group of cultivated edible mushrooms. Like other *Pleurotus* species, this mushroom generally contains crude protein that ranks below most animal meats, eggs and cheese, but is proved to be similar to, or even higher than in vegetables (Bonatti *et al.,* 2004).

A study made by Chahal *et al*., (1989) over 20 years ago discovered that the mycelia biomass of *P. sajor-caju* consists of essential amino acids that meet the Food and Agricultural Organization (FAO) requirements. A preliminary feeding trial experiment on rats showed that about 50% of protein in the diet can be replaced by the mycelial biomass of *P. sajor-caju* without any deleterious effects. To date, much recent research has been carried out on *P. pulmonarius* crude extract and the isolated compounds such as polysaccharides, protein, peptide and other substances, focusing on various medicinal properties.

The crude extract of this species produced low cytotoxicity effects, which may be suitable for food and oral therapeutic substances. Also, it has shown efficacy in combating aging problems due to the high antioxidant activity. For instance, a study by Kanagasabapathy *et al.,* (2011) showed that the crude extract from the fresh fruiting

body of *P. sajor-caju* exhibits good antioxidant activity *in vitro*, albeit no genoprotective effects was detected since it does not prevent hydrogen peroxide-induced oxidative damage to cellular DNA (Shi *et al.*, 2002). Numerous research has been done on the antiviral properties which this species has indicated low antiviral activity *in vivo* when tested against pox virus and infectious bursa disease virus (Kidukuli *et al.,* 2010), but good activity against Human Immunodeficiency virus (HIV)-1 reverse transcriptase (Gregori *et al*., 2007) and potent antiviral activity against Tobacco Mosaic Virus (Verma *et al.,* 2001).

Research has proven that the translation-inhibiting and ribonuclease activities of *P. sajor-caju* peptides are considerably higher than that of *Pleurotus ostreatus* and *Calvatia caelata* (Ng *et al.*, 2002). Also, an ubiquitin-like peptide has been isolated from this mushroom and the result demonstrated positive N-glycosidase activity and good inhibition of cell-free translation. The ribonuclease from this species has been reported to have antibacterial and antifungal activities, and exerts antiproliferative activity on hepatoma and leukemia cells, as well as anti-mitogenic effect on mouse spleen cells (Ng *et al.,* 2004).

2.7 Bioactive peptides

Components of proteins in food are known to have nutritional, functional and biological properties, and contain sequences of bioactive peptides encrypted within their primary structures, which could exert a physiological effect in the body. These active peptides are also known to contribute to the physicochemical and sensory properties of various protein-rich foods, which make these components potential ingredients for health enhancement purposes (Korhonen *et al*., 2003).

In the past few decades, scientists have intensified their research on medicinal properties extracted from crude natural products. Currently, in-depth studies of medicinal properties have been developed, focusing more on bioactive proteins and peptides derived from both natural products and food bases. Due to the increasing commercial interest in the production of bioactive peptides from various sources, industrial-scale technologies suitable for their commercial production have also been developed and are upgrading concurrently to the development of bioactive peptide research and findings (Korhonen *et al*., 2006).

According to Hartmann *et al.*, (2007), proteins and peptides could be physiologically active or bioactive through its natural form or via hydrolysis *in vivo* or *in vitro* for their release from the host protein, particularly by the three methods: (a) *in vivo* hydrolysis by digestive enzyme like trypsin, (b) *in vivo* hydrolysis by proteolytic microbial enzymes, and (c) *in vitro* hydrolysis via food ripening and processing such as fermentation by isolated or microbial enzymes (Möller *et al.,* 2008). The term food-derived bioactive peptide refers to plant or animal-derived peptides that conduct a regulatory function in the human body system and may ultimately influence health (Hartmann *et al,* 2007). The naturally occurring food-derived bioactive peptides in traditional foods were consumed years ago. Some of them were used as traditional medicines for treating various diseases.

Research in the field of bioactive peptides has discovered many food-derived bioactive peptides with potent biological activities that are potential to be the active ingredients for pharmaceuticals and may contribute to enhancing healthier nutrition for the public. A wide range of bioactivities have been identified, including those that have effect on the major body systems such as the cardiovascular system (antioxidative,

antihypertensive, hypocholesterolemic and antithrombotic), nervous system (opioid agonist and antagonist activities), gastrointestinal system (anti-appetizing, mineral-binding and antimicrobial), and immune system (antimicrobial, immunomodulatory and cytomodulatory). Some peptides are multifunctional and can exert various effects and properties on different regulatory mechanisms (Meisel, 2004).

2.7.1 Bioactive peptides from mushrooms

Over the centuries, the medicinal values of mushrooms have been studied and they have been found to possess abundant proteins and peptides. Most of the proteins and peptides have potentially applicable activities, regardless of whether they are intra-cellular or extra-cellular. Thus, whole mushroom extracts (from fruit bodies or mycelium) or isolated compounds like peptides may be used as sources of medically valuable compound (Ng, 2004).

Numerous researches worldwide have demonstrated that mushroom-derived peptides play an important role in the prevention and treatment of perturbing diseases such as cancer and hypertension. In depth *in vivo* studies demonstrating the anti-cancer activity in animal models have been done on the extracted, purified glucan polysaccharides and polysaccharide-peptides derived from mushrooms. These studies strongly suggest an immunomodulating mode of action (Smith *et al*., 2002). The fruiting body of *Tricholoma giganteum* has shown potent antihypertensive activity in spontaneously hypertensive rats (SHR), at a dosage of 1 mg/kg. The peptide was sequenced as Gly-Glu-Pro (GPP), a novel tripeptide with very low similarity to other ACE inhibitory peptide sequences (Lee *et al.*, 2004). Likewise, other medicinal mushrooms such as *Ganoderma lucidum*, *Flammulina veluptipes* and *Grifola frondosa* have been reported to demonstrate potent antihypertensive activity (Barbosa-Filho *et al.*, 2006).

Mushroom-derived bioactive peptides exhibit antioxidant activity in many species. For instance, novel antioxidant peptides from fermented mushroom *Ganoderma lucidum* have been discovered and research has shown that the *G. lucidum* peptide (GLP) inhibits lipid peroxidation in biological systems through its antioxidant, metal chelating and free radical scavenging activities (Sun *et al*., 2004). A polysaccharide-peptide complex from *Pleurotus abalonus* fruiting body has been shown to increase the antioxidant enzymes activities including glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD), thus reduces lipid peroxidation activity when tested on senescence-accelerated mice (Li *et al*., 2007).

Various antifungal peptides that act against different microorganisms have been exploited from mushrooms. Agrocybin, Eryngin, Ganodermin and Pleurostin were isolated from *Agrocybe cylindracea*, *Pleurotus eryngii*, *Ganoderma lucidum* and *Pleurotus ostreatus* respectively. These peptides have shown inhibitory activities on mycelia growth of fungal plant pathogens such as *Fusarium oxysporum* and *Mycosphaerella arachidicola* (Wang *et al*., 2004; Ngai *et al*., 2005)

Mushrooms also contain bioactive peptides that demonstrate antiproliferative activity. *Agrocybe cylindracea* and *Hypsizigus marmoreus* are among the mushrooms that have shown positive antiproliferative activity, together with other different medicinal activities discovered from the same peptides (Ngai *et al*., 2003; Wong *et al*., 2008). Overall, there are abundant research on other bioactive peptides from mushrooms that contribute to improve health problems and its recuperation.

CHAPTER 3: MATERIALS AND METHODS

In this study, *P. pulmonarius* mycelium was investigated for its antihypertensive activity. Preliminary assay was performed on the mycelial aqueous extract and broth extract and the most active sample was chosen for further analyses. Different steps of protein purifications were done in this study. Each purification step was re-assayed to determine the most active ACE inhibitors, and finally protein identification was done to discover the proteins responsible for the antihypertensive activity. Figure 3.1 shows a schematic diagram of the overall process in this study.

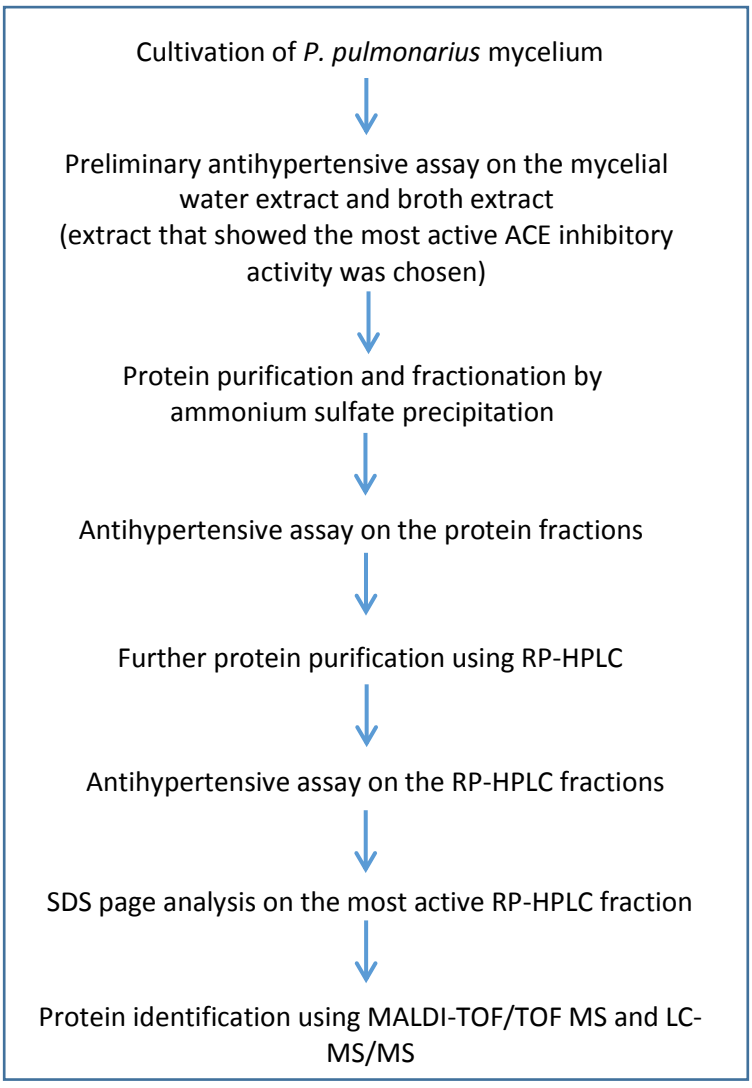


Figure 3.1: Schematic diagram of overall process took part in this study. The experiment incorporated cultivation process, bioassay activity and superficial protein study.

3.1 Cultivation of *Pleurotus pulmonarius* mycelium

3.1.1 Fungal strain

Pleurotus pulmonarius mycelium stock culture (KUM 61119) was obtained and authenticated by Mushroom Research Centre, University of Malaya. Mycelium stock cultures were maintained on Malt Extract agar (MEA) plate (Appendix A (1), Table 1, page 94) at 25 ± 2°C and sub-cultured routinely.

3.1.2 Preparation of *P. pulmonarius* inoculum

Pleurotus pulmonarius mycelium was cultured on the MEA plates under sterile condition to avoid contamination. Mycelial plugs (5 mm diameter) were punctured from the periphery of the mycelium stock/previous culture by using a sterile cork borer. A mycelial plug was placed onto the centre of a MEA plate and incubated for 7 days at 25 ± 2°C.

3.1.3 Growth of *P. pulmonarius* mycelium by submerged fermentation

Following plate culture, *P. pulmonarius* mycelium was then cultivated by fermentation using liquid medium consisting of brown sugar and spent yeast at the percentage ratio of 1:2 (Appendix A (2), Table 2, page 94). Each 100 ml of the sterile medium was inoculated in 500 ml Erlenmeyer flask with 10 mycelial plugs (diameter size of 5 mm) cut from the periphery of 7-day old mycelia cultures. The inoculated flasks were incubated at 25 ± 2°C for 7 days on rotary shaker with constant shaking at 150 rpm. At harvest, mycelium was separated from the broth medium by filtration. Mycelium was washed gently with running tap water and both mycelium and broth medium were then freeze dried separately. The freeze dried products were ground and stored at 4 ± 2°C prior to use.

Freeze dried crude mycelium was immersed in distilled water for an hour with constant stirring and centrifuged briefly to remove the mycelium and any debris. The supernatant, i.e. mycelial aqueous extract was analysed for protein estimation (as shown in section 3.4). Freeze dried broth was dissolved in distilled water as stock concentration for broth extract and protein estimation was also determined. The mycelial aqueous extract and broth extract were used in the preliminary antihypertensive assay. Sample that showed better antihypertensive activity was also used for further protein extraction by ammonium sulphate precipitation.

3.2 Antihypertensive assay by inhibition of ACE

Antihypertensive assay was performed by using ACE Kit WST (Dojindo Laboratories, Japan). The kit assay was designated based on the amount of 3-hydroxybutyric acid (3HB) generated from 3-hydroxylbutylyl-Gly-Gly-Gly by the action of ACE inhibitory activity detected in the samples. The activity was evaluated in percentage of inhibition (Lam *et al.*, 2007 & 2008).

The ACE Kit WST consists of working solutions (indicator working solution and enzyme working solution) and substrate buffer (Appendix A (3), page 95). For the ACE inhibitory assay, blank 1, blank 2 and 20 µl of samples were prepared. Then, 20 µl of substrate buffer was added to each sample and blanks, followed by 20 µl of deionized water to blank 2 only. Next, 20 µl of enzyme working solution was added to each sample and blank 1. The microplate was incubated at 37°C for one hour. Following the incubation, 200 µl of Indicator working solution was added to each sample and blanks. The microplate was incubated at room temperature for 10 minutes, allowing variance intensity of yellow colour to develop. The absorbance value was read at 450 nm with

ELISA microplate reader. The ACE inhibitory activity was calculated by the following equation:

$$\textbf{ACE inhibitory activity (inhibition rate \%)} = \frac{\textbf{(Blank 1 – Sample)}}{\textbf{(Blank 1 – Blank 2)}} \times 100$$

For the preliminary assay, mycelial aqueous extract and broth extract at the concentration of 0.2 mg/ml, 0.5 mg/ml, 1.0 mg/ml, 2.0 mg/ml and 3.0 mg/ml were tested for the assay. The IC_{50} value was evaluated and compared. The most active extract was selected and subjected to protein extraction and purification. Captopril was used as a positive control using five different concentrations ranging from 1.0×10^{-10} mg/ml to 1.0×10^{-6} mg/ml. All samples were tested in triplicate values.

3.3 Preparation of crude protein extract and protein fractions obtained by ammonium sulphate precipitation

3.3.1 Extraction of *P. pulmonarius* mycelial proteins

The crude extract of *P. pulmonarius* mycelium was dissolved in distilled water in the ratio of 1:10. The extract was blended and then stirred vigorously for one hour on ice to achieve homogeneity. The supernatant was collected by centrifugation at 5,000 rpm for 10 minutes at 4 ± 2°C. The supernatant was designated as the source for mycelial protein extract.

3.3.2 Protein fractionation by salting out method

The protein extract of *P. pulmonarius* mycelium was subjected to salting out process by using ammonium sulphate. The process was carried out by fractioning the mycelial proteins using 10 to 100% ammonium sulphate saturation (Apendix A (4), Table 3, page 96). Each of the ammonium sulphate saturation was poured gradually into the protein

extract solutions and was constantly stirred for 30 minutes on ice bath before underwent centrifugation at 10,000 rpm for 15 minutes at 4 ± 2°C. At each stage of saturation, the precipitated protein yield was collected and dissolved in 4 ml distilled water. The supernatant was then used for the subsequent increasing salt saturations, until the 100% of salt saturation was achieved. The precipitated proteins were categorized according to the concentration of the salt saturations at which they were formed, and these protein fractions were labelled as F10 to F100.

The protein fractions were then dialyzed four times against distilled water for 48 hours by using Snakeskin Dialysis Tubing™ (Thermo Fisher Scientific, Rockford, IL, USA), with 3,500 molecular weight cut off (MWCO). The experiment was done at 4 ± 2°C. The dialyzed protein fractions were aliquot into 1.5 ml Eppendorf tubes, freeze-dried and stored at -20 ± 2°C prior to use.

3.4 Protein estimation

Concentration of proteins in each fraction was estimated using bicinchoninic acid (BCA) method, a detergent-compatible formulation based on bicinchoninic acid for the colorimetric detection and quantification of total protein. The assay was carried out using BCA™ Protein Assay Kit (PIERCE, Thermo Fisher Scientific, Rockford, IL, USA). Bovine serum albumin (BSA) at 2.0 mg/ml in 0.9% saline and 0.05% sodium azide was used as standard. Protein concentrations for each protein fraction were determined with reference to the BSA standard curve graph (Apendix A (5), Table 4, page 97).

3.5 Antihypertensive assay of the fractionated mycelial proteins (F10 to F100)

Mycelial protein fractions were tested for their ACE inhibitory activity at the concentration of 50 µg/ml, with the same procedure stated in section 3.2. Protein fraction that demonstrated the highest percentage of ACE inhibition was evaluated for its IC_{50} value and selected for further purification by RP-HPLC analysis.

3.6 Purification of the active protein fraction via reverse phase high performance liquid chromatography (RP-HPLC)

3.6.1 Preparation of protein fraction sample for RP-HPLC analysis

Protein fraction that gave the highest ACE inhibitory activity was subjected to RP-HPLC for further purification. Five tubes of the freeze dried protein fraction was dissolved with 200 µl distilled water respectively and pooled into an Eppendorf tube to make a total of 1 ml protein solution. The protein was spun briefly and the supernatant was collected for further analyses. The protein was subjected to protein estimation (Section 3.4). Protein fraction at the concentration of 2 mg/ml was then filtered with 0.45 µm, 4 mm disposable syringe filter.

3.6.2 RP-HPLC analysis of F40 protein fraction

F40 protein fraction was analyzed using a Shimadzu HPLC system equipped with System Controller SCL-10Avp, Array detector SPD-10Avp, Liquid Chromatograph LC-10AT and degasser DGU-12A. The HPLC protocol was optimized several times before the final protocol was achieved. HPLC grade acetonitrile and 0.1% TFA in distilled water were used as the mobile phase (Appendix A (6), page 98). The C18 Chromolith® SemiPrep column (C18, 100 × 10 mm, 2 µm, 130 Å) coupled with guard column was used for the fractions separation.

Two hundred microlitre of 2 mg/ml F40 protein sample was injected into the injector and allowed to run for 30 minutes. Elution of fractions was achieved by the use of binary gradient from 0% to 40% acetonitrile at the constant flow rate of 3.9 ml/min. Fraction peaks were detected at 220 nm and 254 nm wavelengths. The baseline for each wavelength was obtained using the same protocol. Every single symmetrical peak shown by the chromatogram was collected and labelled as P1 to P8 for the apparent peaks and L1 to L3 for the ambiguous peaks. The collected fractions were then freeze dried and stored at -20°C. During series of run extending over several days, column precision was maintained by flushing the column with 100% acetonitrile at isocratic flow rate of 3.9 ml/min for 40 minutes each time before running the sample. Upon completion, the column was also flushed with the aqueous solvent for 10 minutes, followed by flushing with acetonitrile for 40 minutes using the same flow rate.

3.7 Re-evaluation of antihypertensive assay on RP-HPLC peaks (P1 to P8)

The collected RP-HPLC peaks labelled as P1 to P8 were re-evaluated for their ACE inhibitory activity at the concentration of 15 µg/ml, using the same protocol as stated in Section 3.3. The peak that showed the most active anti ACE activity was evaluated for its IC_{50} value and selected for protein identification assays consisting SDS PAGE analysis, MALDI-TOF/TOF MS and LC-MS/MS.

3.8 SDS-PAGE analysis of the most active protein fractions

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed on the fractions that demonstrated the highest ACE inhibitory activity in each protein fractionation stage. Preparation of stock solutions and buffers is explained in Appendix A (7), page 99.

3.8.1 Preparation of SDS-PAGE gels

Recipes for the preparation of SDS-PAGE gel solutions are presented in Appendix A (7c), Table 4 & 5, page 101. A set of vertical glass plates gel unit was assembled vertically, with 0.75 mm spacer strips were placed at both edges in between the glass plates. The glass plates were clamped correctly to avoid leaking. The separating gel solution was poured carefully into the space by using a micropipette. The top of the gel was then overlaid with distilled water. The gel was allowed to polymerize for a minimum of one hour. The overlying distilled water was discarded before the stacking gel was casted. The separating gel surface was rinsed briefly with sufficient volume of stacking gel solution to remove any unpolymerized gel residue. The stacking gel solution was poured carefully and a desired size of comb was then inserted into the gel formation with concern not to trap any bubble below the teeth of the comb. The gel was allowed to polymerize for a minimum of one hour. The polymerized gel with the comb in place could be stored overnight at 4 ± 2°C.

3.8.2 Preparation of loading sample

The 4× sample buffer was first allowed to equilibrate to room temperature before it was then mixed with the protein fraction samples in the ratio of 1:3. The samples mixtures were heated for three minutes at 100°C before it was loaded into the wells of SDS-PAGE gel. The samples mixtures were run within two hours to prevent re-oxidation.

3.8.3 SDS-PAGE electrophoresis

The glass plates unit was placed and clamped into a vertical electrophoresis apparatus with the shorter glass plate facing inward of the chamber. The comb was removed earlier and rinsed with a little amount of running buffer once or twice. The running buffer was then added to the upper and lower buffer chambers. Then, 7 µg in 12 µl of

protein samples were loaded into the wells, followed by 4 µl of protein marker to another well. The apparatus lid was placed on the apparatus, and the leads were plugged into the power supply. Electrophoresis was performed using Bio-Rad Pac 300 at a constant voltage of 60 V for the first 15 minutes and followed by a gradual increase of voltage from 80 to 100V, to avoid excess heat. When the blue dye of each sample and protein marker reached approximately 1 cm from the bottom of the gel, the power supply was turned off and the apparatus was dissembled. The gel was then silver stained to visualize the proteins.

3.8.4 Silver staining procedure

The preparation of silver staining solutions is explained in Appendix A (8), page 102. The SDS gel was soaked in fixation solution for 30 minutes or overnight. It was then replaced by incubation solution for another 30 minutes. The gel was washed three times with distilled water for 5 minutes respectively. The gel was then soaked in silver solution for 20 minutes, and rinsed with distilled water two times for 2 minutes. Developing solution was then poured into the container and the gel was incubated for 4 minutes, or until the bands appeared. Developing solution was replaced by stopping solution for 10 minutes before the gel was washed with distilled water three times for 10 minutes respectively. The gel was stored in preserving solution.

3.9 Identification of ACE inhibitor proteins from the most active RP-HPLC fraction (P6) by MALDI-TOF/TOF MS analysis

The P6 RP-HPLC fraction was subjected to MALDI-TOF/TOF analysis. Previously, the fraction was analyzed with SDS-PAGE analysis and visualized with silver staining. The emerging bands were cut and placed into 200 µl Eppendorf tubes individually. Each band was subjected to trypsin in-gel digestion at 37°C overnight, followed by desalting

using Zip Tip (C18) (EMD Milipore Co., MA, USA). The samples were analyzed using 4800 Plus MALDI-TOF/TOF MS Proteomics Analyser (Applied Biosystems, CA, USA) combined with Mascot database software (Matrix Science Ltd., London, UK). Protein identification was carried out using the following acquisition settings: Databases- Fungi (SwissProt), Fungi (NCBI), and *P. ostreatus* (NCBI); protein mass range- 0kDa to 100kDa; mass tolerance between 0.5 Da to 1.15 Da- one missed cleavage.

3.10 Identification and re-confirmation of ACE inhibitor proteins by LC-MS/MS analysis

The P6 RP-HPLC fraction was diluted at 3 µg/ml and sent to ITSIBiosciences USA, for LC-MS/MS analysis. Sample was subjected to in-solution tryptic digestion. The raw data files were searched using Proteome Discoverer 1.3 (Thermo Scientific) and the SEQUEST algorithm. Protein identification was performed using the following parameter: Databases- Fungi (SwissProt), Fungi (NCBI), and *P. ostreatus* (NCBI); Modification; Carbamidomethyl Cysteine (static), Oxidation of Methionine (variable), three missed cleavage per peptide.

3.11 Statistical analysis

Mean values for all the parameters tested were obtained and subjected to one-way analysis of variance (ANOVA). The mean values were tested for significance using the multiple range tests at 95% at least significant difference (LSD). The least significant difference was conducted to detect any significant difference in the antihypertensive assays of *P. pulmonarius* mycelial protein fractions and *P. pulmonarius* mycelial RP-HPLC fractions.

CHAPTER 4: RESULTS

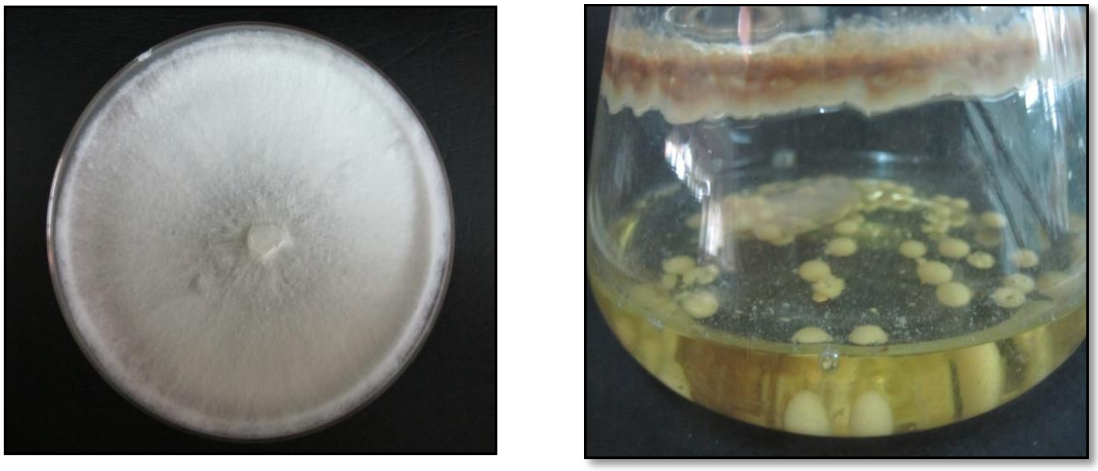
4.1 Yields of *P. pulmonarius* mycelium grown in submerged fermentation

Cultivation of *P. pulmonarius* mycelium was successfully performed for at least thirty batches of sub-cultivation on the MEA plate at 25°C. The mycelium used as inoculums showed steady growth on the MEA plate. White-velvety form of mycelium was observed to completely colonize the MEA agar surface on day-7 (Figure 4.1 (a)).

Following the plate cultivations, small scale submerged fermentations of *P. pulmonarius* mycelium was then carried out in Brown sugar-spent yeast medium. Ten mycelial plugs (5 mm diameter) were pierced from the periphery of the culture using a sterile cork borer and inoculated into sterilized 500 ml Erlenmeyer flasks, each containing 100 ml of brown sugar-spent yeast liquid media in the percentage ratio of 2:1 (w/v). Incubation period was 7 days with constant agitation at 150 rpm. From the observation, the mycelium grew either in pellet forms or a clump depending on the dispersion of the mycelial plugs during shake incubation (Figure 4.1 (b)). The mycelial yield was separated from the whole broth. Both the mycelial biomass and the whole broth were freeze dried and ground, then stored at 4°C for subsequent analyses. The yield of crude mycelial extract was 4 ± 0.3 g/L per batch.

4.2 Preliminary antihypertensive assay of *P. pulmonarius* mycelial aqueous extract and broth extract

Antihypertensive assay was performed on *P. pulmonarius* mycelial aqueous extract and broth extract at five different concentrations, i.e. 0.2 mg/ml, 0.5 mg/ml, 1.0 mg/ml, 2.0 mg/ml and 3.0 mg/ml (Appendix B, Table 1 & 2, page 104). Captopril was used as positive control at the concentration from 1.0×10^{-10} mg/ml to 1×10^{-6} mg/ml.



(a) (b)

Figure 4.1: Fungal cultures of *P. pulmonarius* mycelium used as inoculums (a) *P. pulmonarius* mycelium grown on MEA plate at day-7, (b) submerged fermentation of *P. pulmonarius* mycelium in brown sugar-spent yeast media at day-7. The mycelium grew in pellet form.

The result indicated different outcome of antihypertensive assay between *P. pulmonarius* mycelial aqueous extract and broth extract, but with compatible pattern of ACE inhibitory percentage via graph analysis (Figure 4.2). The IC_{50} values were obtained by extrapolation of graph at the 50% ACE inhibitory activity. *P. pulmonarius* mycelial aqueous extract exhibited its IC_{50} value at 0.72 mg/ml whereas the IC_{50} value for broth extract was at 1.32 mg/ml. The ACE inhibitory activity of *P. pulmonarius* mycelial aqueous extract was almost two times higher than that of the broth extract, by means of the IC_{50} values. However, the IC_{50} value of captopril was 1×10^{-8} mg/ml, which was much lower than that of the mycelial aqueous extract. *Pleurotus pulmonarius* mycelial aqueous extract showed better activity than the broth extract, thus was subjected to ammonium sulphate precipitation for protein extraction and fractionation.

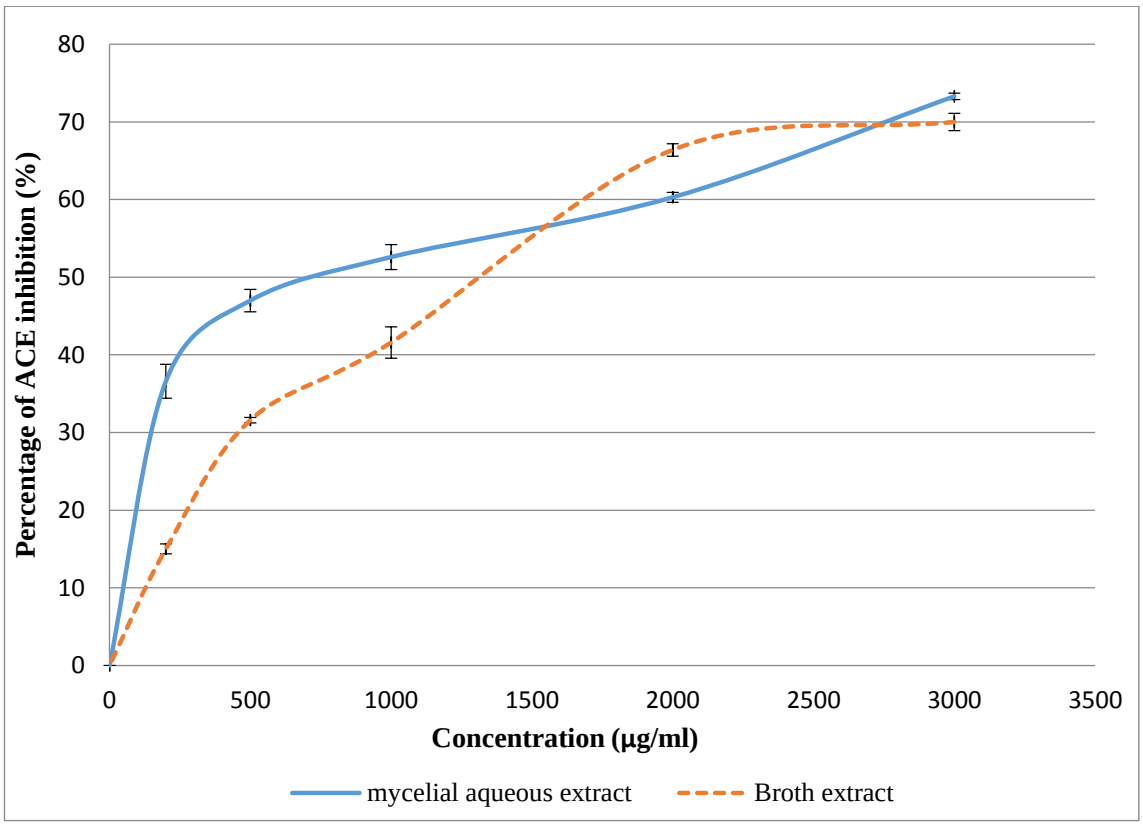


Figure 4.2: Antihypertensive assay of *P. pulmonarius* mycelial aqueous extract and broth extract. Results are expressed as the mean of triplicates values (n=3). The IC_{50} values measured were 0.72 mg/ml for mycelia aqueous extract and 1.32 mg/ml for broth extract.

4.3 Antihypertensive assay of *P. pulmonarius* mycelial protein fractions

Mycelial aqueous extract was fractionated by ammonium sulphate precipitation and later was tested for antihypertensive activity at 50 µg/ml. As shown in Figure 4.3, F40 demonstrated the highest ACE inhibitory activity at 61.2%. In contrast, F30, F80, F90 and F100 indicated comparably low ACE inhibitory activity. Fraction F40 was chosen for further purification by RP-HPLC as the ACE inhibitory result was significantly different (p<0.05) from the other protein fractions. The raw data of the experiment is presented at Appendix B, Table 3 & Figure 1, page 106.

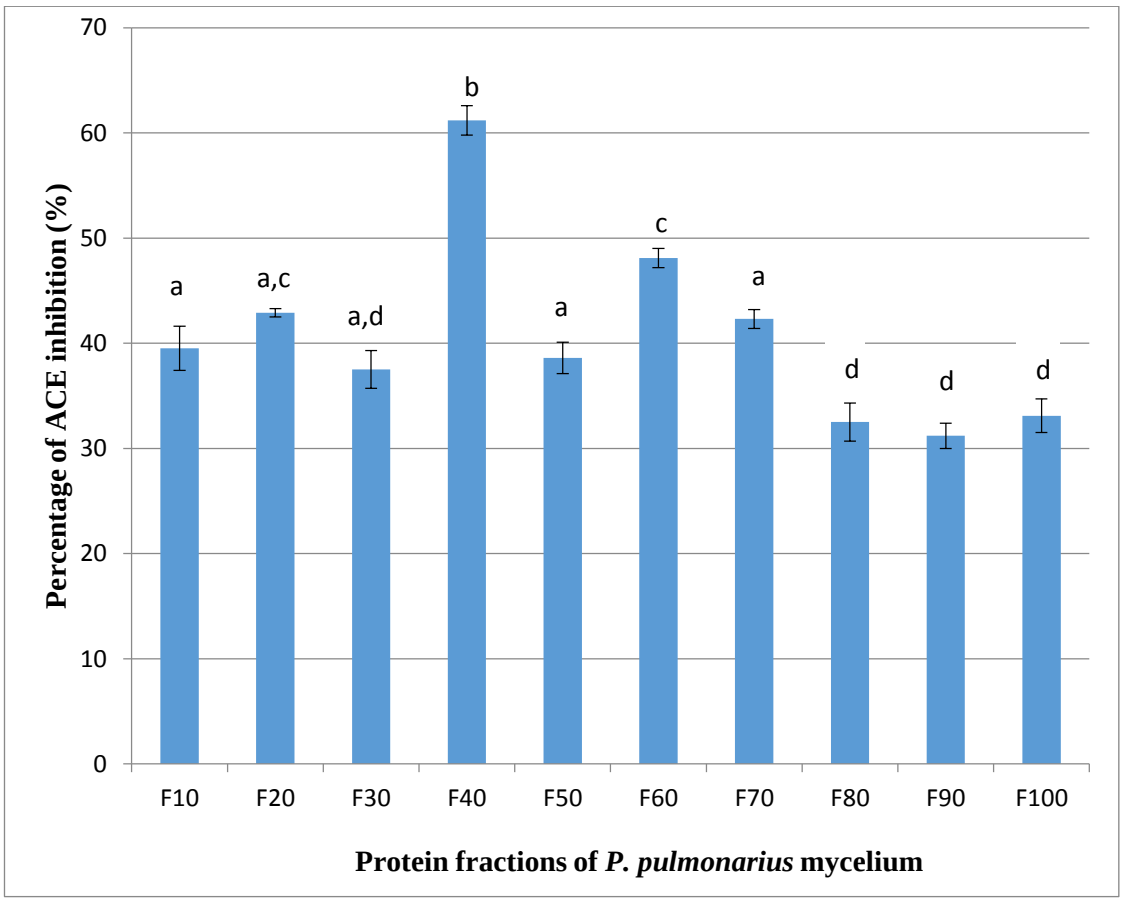


Figure 4.3: ACE inhibition by *P. pulmonarius* mycelial protein fractions (F10 to F100) at the concentration of 50 µg/ml. Results are expressed as the mean of triplicates values (n=3). Means with different alphabetical letters denotes the ACE inhibitory activity of the samples are significantly different (p<0.05).

The IC_{50} value of F40 was determined at the concentration of 5 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml and 150 µg/ml (Appendix B, Table 4, page 107). The result showed an IC_{50} value at 22 µg/ml as shown in Figure 4.4. Fraction F40 was proven to demonstrate greater ACE inhibitory activity with an approximately thirty two times higher activity than that of *P. pulmonarius* mycelial aqueous extract by means of the IC_{50} value.

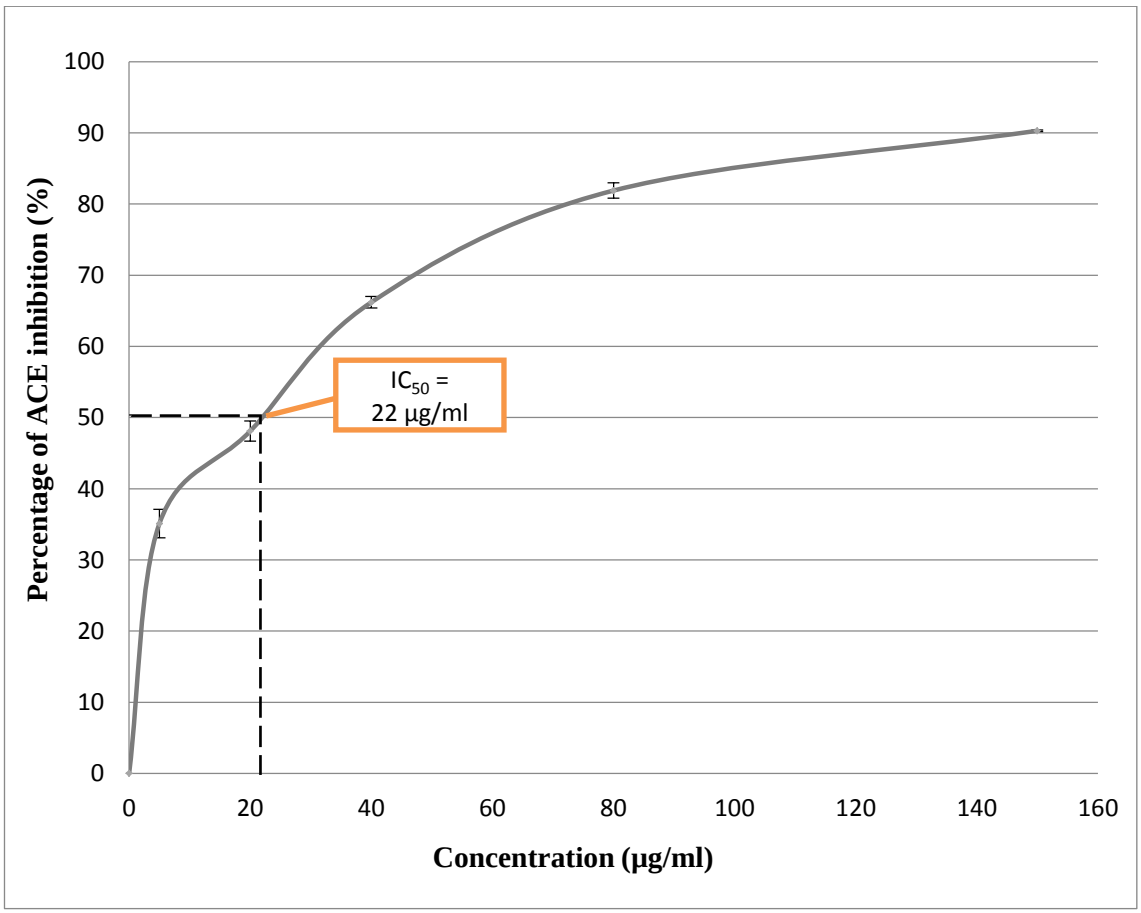


Figure 4.4: Evaluation of IC_{50} value for fraction F40. Results are expressed as the mean of triplicates values (n=3). The IC_{50} value determined was 22 µg/ml.

4.4 Further purification of fraction F40 via RP-HPLC

Fraction F40 of *P. pulmonarius* mycelium was subjected to RP-HPLC analysis for further protein purification. In order to ensure no false or contaminant peaks, good baselines with no significant peak detected by the same method used for sample running must be achieved before every sample collection session. After several optimizations involving different flow rates and percentages of mobile phases, an optimum chromatogram of eight apparent peaks was detected at the wavelength of 220 nm at the constant flow rate of 3.9 ml/min with binary gradient of 0% to 40% acetonitrile in 30 minutes (Figure 4.5). Preliminary runs during optimization established that there was no fraction eluted above 40% acetonitrile for the chosen wavelength. Only 200 µl of fraction F40 was loaded for every cycle due to the size of sample loop used in the

HPLC analysis, which resulted in only about 5 µl of 40 to 70 µg of HPLC fractions yield for every 2 mg/ml sample run.

The first peak of the RP-HPLC profile showed a high intensity peak at 340 mAU merging with a low peak. The two peaks were then pooled and labelled as P1, whereas the other peaks were collected individually and labelled as P2, P3, P4, P5, P6, P7 and P8. All peaks were eluted within 2 to 11 minutes of the retention time. Apart from the mentioned peaks, three ambiguous peaks with low intensity were detected within the timeline. The peaks showed inconsistency in every running cycle, hence were pooled accordingly and labelled as L1, L2 and L3. The sample was also analysed using the wavelength of 254 nm, with the same flow rate, percentage of mobile phases and retention time. The result indicated three apparent peaks concurrent to the peaks detected by 220 nm wavelength but with different intensity, thus were labelled according to the matched peaks from the 220 nm RP-HPLC profile (Figure 4.6).

Antihypertensive assay was performed on peaks P1 to P8. To avoid any predicament of results, peaks L1, L2 and L3 were ruled out from the assay due to the inconsistency and consistently low intensity of the peaks detected in every running cycle. The most active ACE inhibitory bioassay guided RP-HPLC peak was then subjected to subsequent procedures intended for protein identification and profiling.

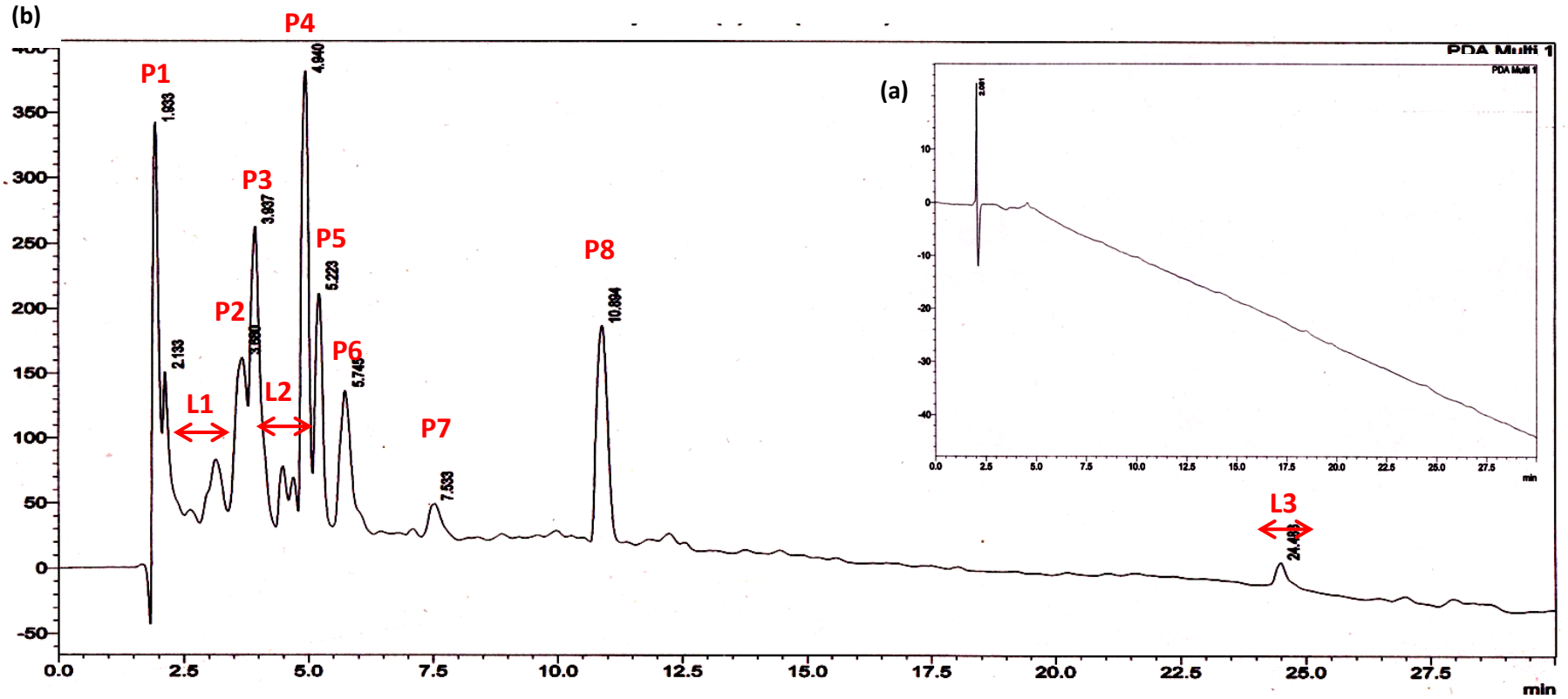


Figure 4.5: HPLC analysis of fraction F40 performed in 30 minutes at constant flow rate of 3.9 ml/min at 220 nm (a) Baseline with no significant peak detected (b) Elution of sample peaks. For each run, 200 µl of 2 mg/ml sample was injected at a binary gradient of 0% to 40% acetonitrile. Eluents were labelled as P1, P2, P3, P4, P5, P6, P7 and P8 for the apparent peaks. On the other hand, the ambiguous peaks which show no consistent in emergence were labelled as L1, L2 and L3.

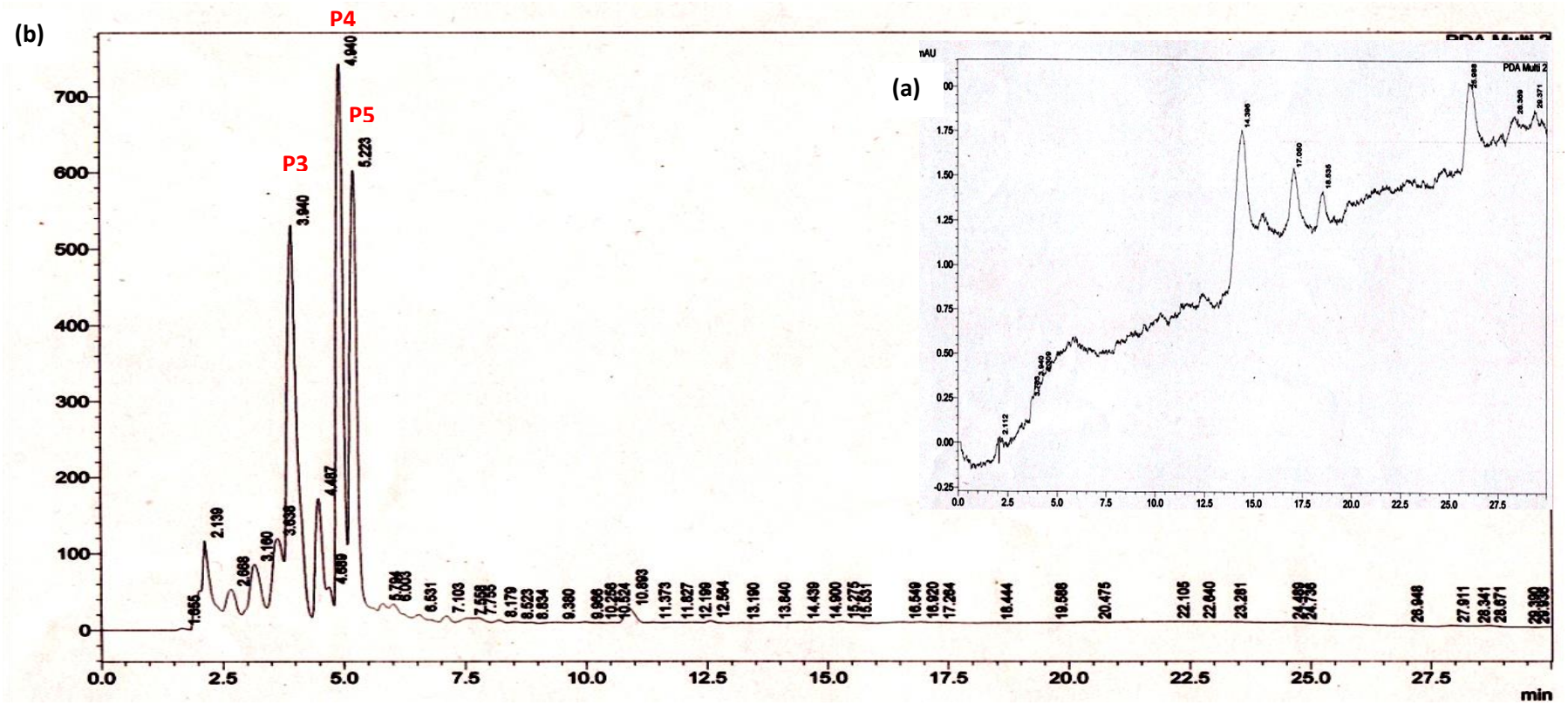


Figure 4.6: HPLC analysis of fraction F40 performed in 30 minutes at constant flow rate of 3.9 ml/min at 254 nm (a) Baseline with no significant peak detected (b) Elution of sample peaks. For each run, 200 µl of 2 mg/ml sample was injected at a binary gradient of 0% to 40% acetonitrile. Three highest peaks were detected and labelled as P3, P4 and P5 according to their resemblance with the peaks detected at 220 nm.

4.5 Antihypertensive assay of the RP-HPLC fractions

Fraction F40 of *P. pulmonarius* mycelium was further resolved by RP-HPLC. As a result, eight peaks were observed and labelled as P1 to P8. Antihypertensive assay was then carried out on all of the RP-HPLC fractions. For these fractions, 15 µg/ml samples were used for the assay as the percentage of ACE inhibition was postulated to increase with the increase of protein purity. As shown in Figure 4.7, P6 showed the highest ACE inhibitory activity at 57.7%, which the result was significantly different from the other fractions (p<0.05). In contrast, all other fractions exhibited comparable activity of ACE inhibition. The lowest ACE inhibitory activity for the RP-HPLC fraction was shown by fraction P1 at 19.6%. The raw data of the experiment is presented in Appendix B, Table 5 & Figure 2, page 108.

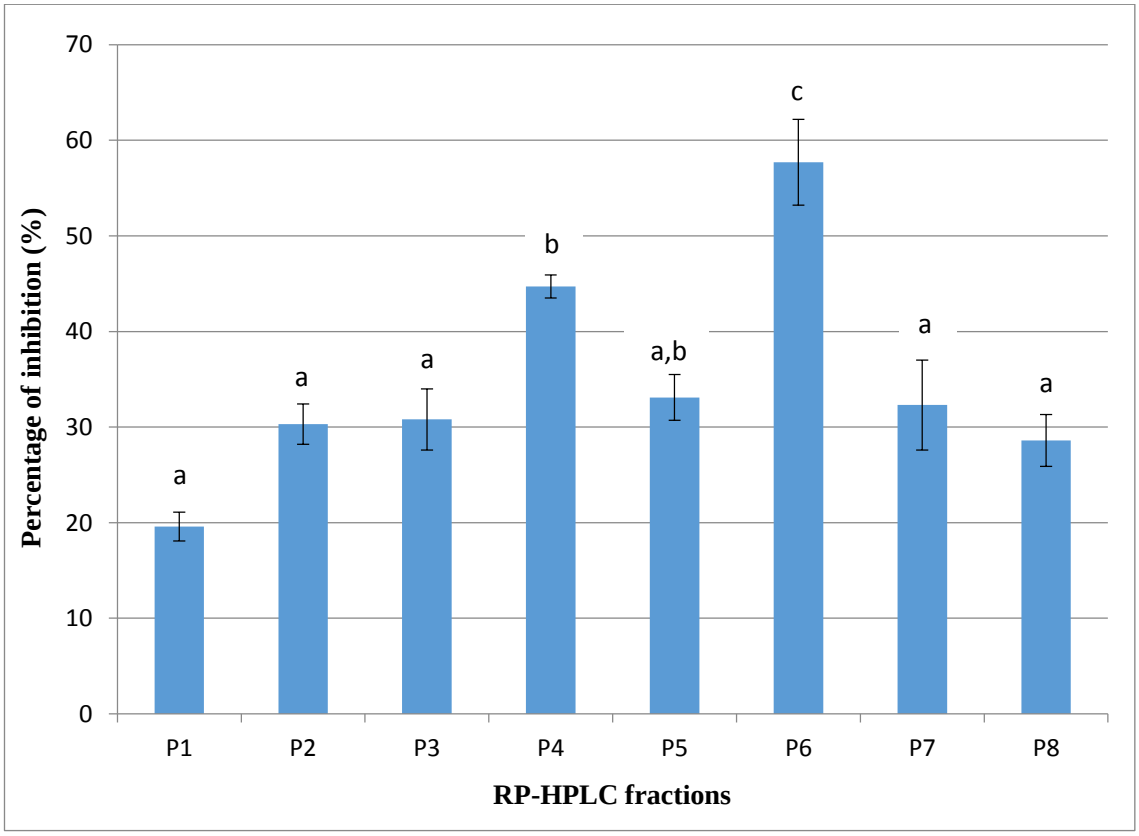


Figure 4.7: ACE inhibition by RP-HPLC fractions, P1 to P8. Results are expressed as the mean of duplicates values (n=2). Means with different alphabetical letters denotes the ACE inhibitory activity of the samples are significantly different (p<0.05).

The IC_{50} value of P6 was evaluated at the concentration of 5 µg/ml, 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml and 150 µg/ml. The lowest ACE inhibitory activity was detected at 30.1% while the highest activity was at 96.8% (Appendix B, Table 6, page 109). As shown in Figure 4.8, the IC_{50} value was obtained by extrapolation of graph and the value was depicted at 12 µg/ml.

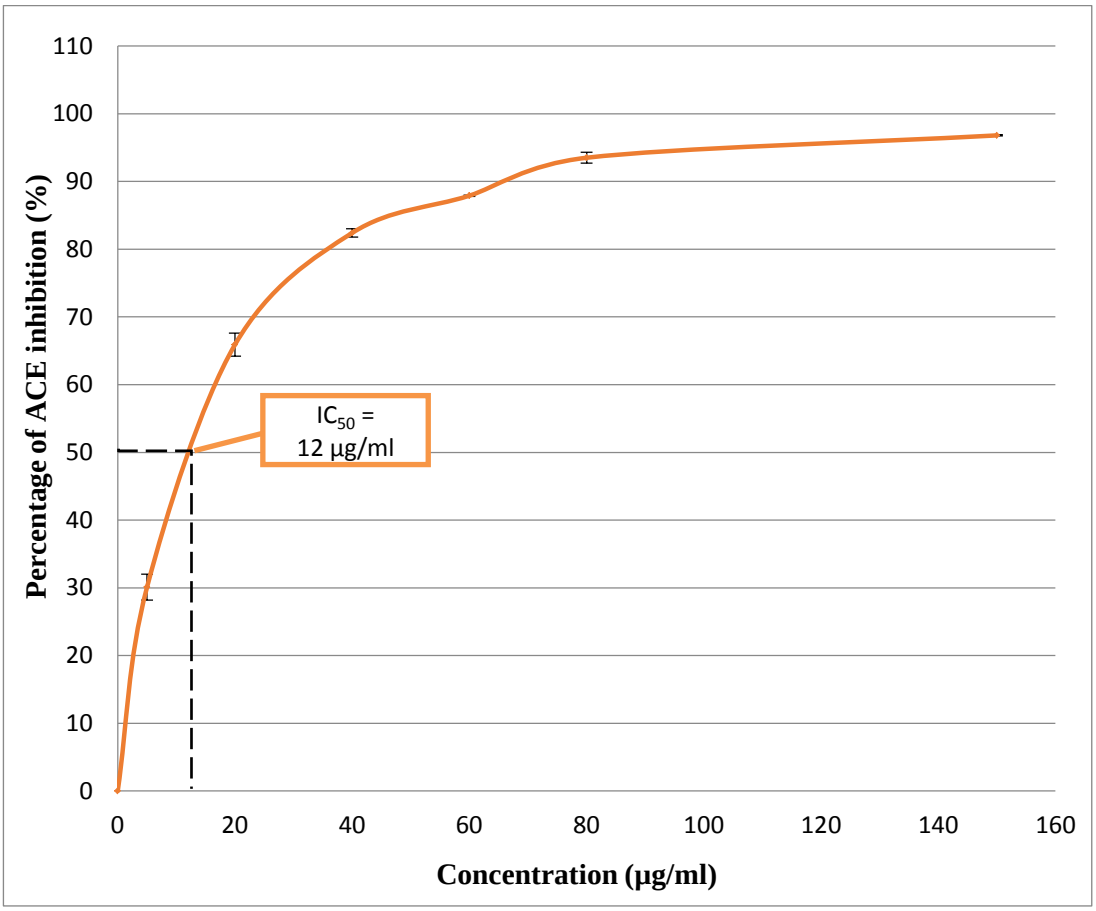


Figure 4.8: Evaluation of IC_{50} value for RP-HPLC fraction P6. Results are expressed as the triplicates of mean values (n=3). The IC_{50} value determined was 12 µg/ml.

The IC_{50} values of *P. pulmonarius* mycelial aqueous extract, F40 and P6 were compared. There was an increase of ACE inhibitory activity following every purification step. In detail, P6 showed almost two times higher inhibitory activity than that of the F40, and sixty times greater ACE inhibitory activity when compared to the mycelial aqueous extract. Thus, it was confirmed that further protein purifications show greater efficacy of the ACE inhibitory activity. Table 4.1 shows the comparison of IC_{50} values of ACE inhibitory activity in every purification step.

Table 4.1 Comparison of IC_{50} values in different stages of ACE inhibitory assay

Protein source	IC_{50} value (μg/ml)
Mycelial aqueous extract	720
Fraction F40 (Ammonium sulphate)	22
Fraction P6 (RP-HPLC)	12

The IC_{50} values were determined within tested concentration of 0 to 3 mg/ml (n=3) for mycelia aqueous extract and 0 to 150 μg/ml for F40 and P6 (n=3).

4.6 SDS-PAGE analysis of the bioactive RP-HPLC fraction P6

Fractions F40 and P6 were screened by SDS-PAGE for three reasons (a) to observe any analogous band between the two fractions, (b) to determine sample purity, and (c) to separate proteins based on their molecular mass prior to MALDI-TOF/TOF MS. Because P6 was a further purified product from F40, it was postulated that P6 should demonstrate if not single, several bands similar to F40.

From the SDS-PAGE result, F40 sample was resolved into several protein bands by the 18% polyacrylamide gel. There were four bands with the estimated molecular mass of 62 kDa, 57 kDa, 36 kDa and <7000Da, appeared in both samples. The bands were labelled as H1, H2, H3 and H4 respectively. Silver-stained SDS-PAGE gel is shown in Figure 4.9. The bands were cut and saturated in distilled water for protein identification by MALDI-TOF/TOF MS.

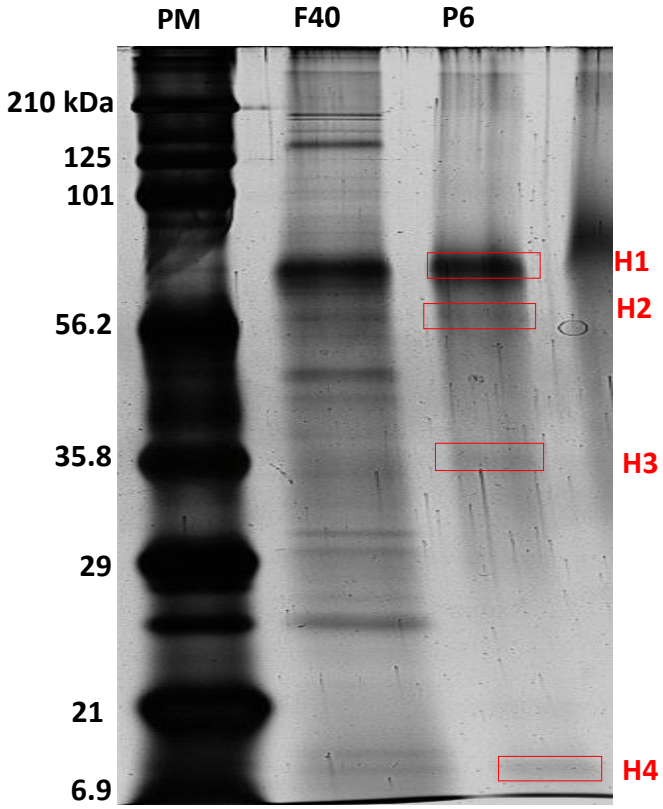


Figure 4.9: SDS-PAGE of F40 and P6. PM; Prestained broad range protein marker, F40; fraction F40 of *P. pulmonarius* mycelium, P6; bioassay guided RP-HPLC fraction. Protein bands of interest are labelled as H1, H2, H3 and H4, respectively.

4.7 Identification of proteins obtained from P6 by MALDI-TOF/TOF MS

The H1, H2, H3 and H4 bands from the polyacrylamide gel were cut out and sent for MALDI-TOF/TOF MS for identification of proteins responsible for the antihypertensive effect in the sample. Mascot search engine was used to identify the protein. Three databases used in the protein search were Fungi (NCBI), Fungi (SwissProt) and *Pleurotus ostreotus* (SwissProt). The protein profile for each protein band is presented in Table 4.2. Each protein identified by the databases was investigated for their correlation with antihypertensive mechanism based on the earlier reported studies. As a result, three proteins related to antihypertensive mechanisms were identified. As shown in Table 4.3, the proteins were serine proteinase inhibitor-like protein from H4 (*P. ostreatus*; SwissProt), DEAD/DEAH box RNA helicase-like protein from H4 (Fungus; NCBI), and nitrite reductase-like protein from H3 (Fungus; SwissProt).

4.8 Protein validation by LC-MS/MS

Fraction P6 was sent for LC-MS/MS analysis to validate proteins identified by MALDI-TOF/TOF MS. The databases used for protein profiling by LC-MS/MS were Fungus (NCBI) and *P. ostreatus* (SwissProt). As a result, five proteins were identified by *P. ostreatus* (SwissProt) database, i.e. manganese peroxidase, DNA polymerase, DNA-directed RNA polymerase, RNA polymerase II second largest subunit and putative ribosomal protein (Appendix B, Table 7.1, page 110). In addition, there were 865 proteins identified from Fungi databases, nine of which demonstrated two uni que peptides (Appendix B, Table 7.2, page 110). Out of the total number, only five proteins were similar to the proteins identified by MALDI-TOF/TOF MS. However, only one antihypertensive protein presented in both mass spectrometry analyses, which was DEAD/DEAH box RNA helicase. A detail of the proteins is presented in Table 4.4.

Table 4.2 Protein profiles from MALDI-TOF/TOF MS

DATABASE: NCBI (fungus)					
Protein band	**Description**	**Accession**	**Protein score**	**MW (kDa)**	**Peptide matched**
H1	predicted protein	gi|154319345	54	8579.4	6
	hypothetical protein SNOG_14294	gi|169622156	52	8668.6	5
	PHD transcription factor	gi|327354476	48	106605	14
	lea domain containing protein	gi|320588713	48	15309.9	7
	hypothetical protein Kpol_526p14	gi|156843100	48	36116.4	8
	hypothetical protein UM00912.1	gi|71004786	48	46294.6	9
	Pc20g03180	gi|255944205	47	61667.3	10
	dynamin GTPase	gi|70992441	46	91991.2	12
	predicted protein	gi|170111974	46	39129.7	8
	interferon-induced GTP-binding protein Mx	gi|331243271	46	77328.5	11

DATABASE: NCBI (fungus)					
Protein band	**Description**	**Accession**	**Protein score**	**MW (kDa)**	**Peptide matched**
H2	predicted protein	gi|154276998	56	46321.8	9
	translation elongation factor 2	gi|169806469	51	97744.2	12
	nucleolar complex protein 2	gi|331225687	51	50584.8	9
	hypothetical protein SS1G_10595	gi|156043185	51	150374.2	15
	DEHA2B11836p	gi|294655336	51	64389.1	10
	hypothetical protein FOXB_01093	gi|342889206	51	11455.1	6
	hypothetical protein BC1G_02690	gi|154318602	50	73416.3	11
	hypothetical protein PTT_19968	gi|330946371	49	55944.7	9
	pyruvate carboxylase	gi|225562373	47	132181.7	13
	pyruvate carboxylase	gi|325092290	47	131444.3	13

DATABASE: NCBI (fungus)					
Protein band	**Description**	**Accession**	**Protein score**	**MW (kDa)**	**Peptide matched**
H3	hypothetical protein TRAVEDRAFT_42575	gi|392572024	83	17104.1	9
	hypothetical protein FG01414.1	gi|46109064	83	84247.8	18
	Pc12g12820	gi|255933239	77	137849.2	23
	predicted protein	gi|302882311	69	205521.3	21
	predicted protein	gi|115433306	69	57075.5	13
	hypothetical protein FG08719.1	gi|46128683	66	276017	30
	orotidine-5'-phosphate decarboxylase	gi|344301052	65	29532.1	10
	predicted protein	gi|340520552	65	28370.9	10
	ZYRO0G03300p	gi|254585155	64	116056.9	17
	hypothetical protein PGTG_10451	gi|403168926	64	56455.5	13

DATABASE: NCBI (fungus)					
Protein band	**Description**	**Accession**	**Protein score**	**MW (kDa)**	**Peptide matched**
H4	MRPS5-like protein	gi|401840118	85	35112.1	14
	mrps5p	gi|401626773	78	35027.2	13
	Mrps5p	gi|365761907	75	35093.1	13
	hypothetical protein FOXB_08951	gi|342879236	73	276051	31
	hypothetical protein FG08719.1	gi|46128683	70	276017	31
	hypothetical protein TPHA_0G02630	gi|367003601	69	78569.5	15
	DEAD/DEAH box RNA helicase	gi|242767802	67	129739.3	18
	hypothetical protein PGUG_05568	gi|190348916	66	97199.7	15
	intronic ORF at intron of small subunit ribosomal RNA gene	gi|400201847	65	46421.3	12
	probable 26S proteasome non-ATPase regulatory subunit p58	gi|319411607	64	66945.9	13

DATABASE: SwissProt (fungus)					
Protein band	**Description**	**Accession**	**Protein score**	**MW (kDa)**	**Peptide matched**
H1	ATPase synthesis protein 25, mitochondrial	sp|D4B478|ATP25_ARTBC	40	78896.4	10
	Uncharacterized zinc-finger protein C4F10.19c	sp|O36031|YEKJ_SCHPO	38	17814.8	5
	Mitochondrial import inner membrane translocase subunit TIM16	sp|Q6C331|TIM16_YARLI	38	16546.7	5
	Autophagy-related protein 11	sp|Q75B79|ATG11_ASHGO	36	122401.2	13
	Ribosome biogenesis protein ERB1	sp|P0CS34|ERB1_CRYNJ	35	93635.9	11
	40S ribosomal protein S3	sp|O60128|RS3_SCHPO	34	27763.7	7
	ARS-binding factor 2, mitochondrial	sp|Q02486|ABF2_YEAST	33	21548.4	6
	Guanine nucleotide-binding protein subunit gamma	sp|Q6FJ50|GBG_CANGA	33	10416.2	4
	40S ribosomal protein S1	sp|P0CQ58|RS3A_CRYNJ	32	29382.7	7
	tRNA (guanine(37)-N1) methyltransferase	sp|Q5KBP2|TRM5_CRYNJ	31	61617.9	8

DATABASE: SwissProt (fungus)					
Protein band	**Description**	**Accession**	**Protein score**	**MW (kDa)**	**Peptide matched**
H2	Ribosome production factor 2 homolog	sp|Q9UUG1|RPF2_SCHPO	44	36049.1	8
	Protein SDS24	sp|P38314|SDS24_YEAST	40	57493.5	8
	Vacuolar membrane protein PEP3	sp|P27801|PEP3_YEAST	39	108014.4	12
	ATP-dependent RNA helicase DBP10	sp|Q6FNA2|DBP10_CANGA	34	109608	10
	Uncharacterized mitochondrial protein urf-LM	sp|Q35138|URFLM_NEUCR	31	36924.2	6
	RNA exonuclease 3	sp|Q6CJB5|REXO3_KLULA	30	55606.7	8
	Decaprenyl-diphosphate synthase subunit 1	sp|O43091|DPS1_SCHPO	29	42419.1	6
	Peptidyl-prolyl cis-trans isomerase B	sp|Q5B4R3|PPIB_EMENI	29	23392.1	5
	Saccharopine dehydrogenase	sp|Q870G1|LYS1_EMENI	29	41357.1	6
	114 kDa U5 small nuclear ribonucleoprotein component	sp|P36048|SN114_YEAST	29	114482.5	9

DATABASE: SwissProt (fungus)					
Protein band	**Description**	**Accession**	**Protein score**	**MW (kDa)**	**Peptide matched**
H3	rRNA biogenesis protein rrp36	RRP36_ASPOR	61	40140.3	12
	ATP synthase subunit 4, mitochondrial	ATPF_NEUCR	54	26317.1	8
	Heat shock protein homolog SSE1	HSP7F_LACK1	49	77140.5	12
	Acetyl-coenzyme A synthetase	ACSA_PHYBL	49	75298.4	11
	Nitrite reductase	NIR_EMENI	48	123994.2	15
	Phosphatidylinositol transfer protein sfh5	SFH5_ASPNC	46	52350.1	10
	Structure-specific endonuclease subunit SLX4	SLX4_AJEDR	45	93607.9	13
	Orotidine 5'-phosphate decarboxylase	PYRF_ASPFU	43	30362.5	7
	Multiprotein-bridging factor 1	MBF1_KLULA	43	16435.9	7
	Formation of crista junctions protein 1	FCJ1_CANTT	43	62715.9	10

DATABASE: SwissProt (fungus)					
Protein band	**Description**	**Accession**	**Protein score**	**MW (kDa)**	**Peptide matched**
H4	E3 ubiquitin-protein ligase BRE1	BRE1_CANGA	48	80348.8	14
	Ubiquinone biosynthesis protein COQ4	COQ4_LACBS	45	34155.3	8
	DNA replication complex GINS protein PSF1	PSF1_PHANO	42	23788.9	7
	Orotidine 5'-phosphate decarboxylase	PYRF_ASPFU	42	30362.5	7
	Altered inheritance of mitochondria protein 9	AIM9_CANGA	40	73930.2	12
	Polynucleotide 5'-hydroxyl-kinase	GRC3_DEBHA	39	78024.3	10
	Orotidine 5'-phosphate decarboxylase	PYRF_KODOH	38	28888.9	7
	Regulator of rDNA transcription protein 5	RRT5_CANGA	38	49790.4	8
	Serine/threonine-protein kinase TEL1	ATM_YARLI	38	259222.8	18
	Pre-mRNA-splicing factor SYF2	SYF2_YARLI	38	23171	7

DATABASE: SwissProt (*P. ostreatus*)					
Protein band	**Description**	**Accession**	**Protein score**	**MW (kDa)**	**Peptide matched**
H3	Ostreolysin	sp|P83467|OSTL_PLEOS	8	5491.9	1
	Calmodulin	sp|O94739|CALM_PLEOS	5	16813.9	1
H4	Ostreolysin	sp|P83467|OSTL_PLEOS	8	5491.9	1
	Serine proteinase inhibitor	sp|Q7M4T5|PIA2_PLEOS	6	8190.1	1

Table 4.3 Putative antihypertensive proteins

Protein band/database	Protein name	Peptide sequence*	Accession	Protein score (%)	Theoretical MW
H3 (SwissProt; fungus)	nitrite reductase-like protein	GIEGSKPTR YSVIFYIR DFGLIATEK LGIAAEMER LMEFAANHK GGFVIDESLR TIADVKSCTK SCVGTTWCR SGTCKTIADVK GDIDQDPNRR VGLSSYFEHR KIEDLYLNPK AMTDLEDFGSVK	NIR_EMENI	48	123,994.2

		GDVVESVKSGTCK RNFELNGEQAGR			
H4 (SwissProt; *P. ostreatus*)	serine proteinase inhibitor-like protein	NGGVSDDKIR	sp|Q7M4T5|PIA2_PLEOS	6	8190.1
H4 (NCBI; fungus)	DEAD/DEAH box RNA helicase-like protein	GLDVKQLK LEAWKQK LSRAGVMR DCKPFLK ELMRLLK KGEEIFAK NMEALARK DQIAELKR ARWAVTNR KPSMTKSAK	gi|242767802	67	12,9739.3

		ANRLSVAPSK AVCAYGGAPIK YSVDIAKALK YSVDIAKALK DDHNYRPLK GAEIVVCTPGR SPSPVGSTYSSSK ITVHKTEKPAGGK AASAIQSASAPTPGIPK			

* Peptide sequences denote sequences matched by MASCOT search engine with the databases used in MALDI TOF/TOF MS.

Table 4.4 Analogous proteins identified in LC-MS/MS[1] and MALDI-TOF/TOF MS

Protein name	Peptide sequences[2]	Accession	Theoretical MW (kDa)
DEAD/DEAH box RNA helicase	FIKTVSEDEVPTHLR	358374223	72.4
Pre-mRNA-processing ATP-dependent RNA helicase	YDTDDASPRSAHSGSGTGRSSR	342319919	168.1
serine/threonine-protein kinase	QDRPRSRQSSPDHEGPSR	322699933	89.5
peptidyl-prolyl isomerase	ADELNSKNTmFGRVAGDTIYNLQK	351642550	62.7
translation elongation factor 2	KDVVFNmLDKLEVTLK	358030862	66.5

[1] Proteins details shown are according to data obtained from LC-MS/MS.

[2] Peptide sequences denote sequences detected by LC-MS/MS.

CHAPTER 5: DISCUSSION

5.1 Cultivation of *P. pulmonarius* mycelium via liquid submerged fermentation

Production of mycelial biomass is increasingly being dominated by species that is edible and having medicinal properties. Many technologies and cultivation techniques are invented to achieve the high demand of mycelial biomass production for scientific research on nutritional and pharmaceutical products. Submerged fermentation is among the most popular technique that has been widely developed especially for large scale production of mycelial biomass. It is a technique whereby fermentation is performed using liquid medium as a nutrient source and mycelium is used in the active physiological state. This technique is employed in mycelium production to achieve such purposes: (1) liquid spawn for fruiting body production on solid substrate; (2) biomass aimed for its nutritional values; and (3) biomass and/or extruded metabolites that exhibit pharmaceutical potentials (Smith *et al.*, 2002).

In this research, submerged liquid fermentation was selected for *P. pulmonarius* mycelial biomass production mainly due to its ability to retain protein content and to preserve nutritional composition of the biomass, as well as its benefit in producing mycelium at shorter time as compared to solid state fermentation. This study reported 4 ± 0.3 g/L of dried mycelial biomass obtained by small scale submerged fermentation using flasks. Kim *et al*., (2002) reported maximum dry mycelial biomass of *P. sajor-caju* between 2.84 g/L to 7.82 g/L within five to ten days in an automated 5-L bioreactor, whereas Confortin *et al*., (2008) reported dry mycelial biomass at 5.5 g/L

and 8.18 g/L on different carbon sources when cultivated in 5-L bioreactor. Therefore, the yield of mycelial biomass in this study was slightly above average for small scale fermentation. In particular, crude protein extract that composed of 5 g dried mycelial biomass produced up to 2 mg/ml fractionated proteins out of the salting-out process. After RP-HPLC, the proteins from each RP-HPLC peak reached the utmost concentration at around 0.07 mg/ml only. Hence, several cycles of cultivations were fulfilled to accommodate sample stock throughout the study.

5.2 Antihypertensive activity of *P. pulmonarius*

Medicinal and/or edible mushrooms have gained interest in bioactive protein and peptide studies for various therapeutic effects including antihypertensive activity. In this study, *P. pulmonarius* mycelium exhibited positive *in vitro* antihypertensive activity. Hence, the study has underlined the importance of mushroom mycelia cultivated by liquid fermentation as natural ACE inhibitor resources. Extensive study was performed in which intracellular proteins in the mycelial aqueous extract and extracellular proteins that were released in the broth were tested separately for ACE inhibitory activity. The result showed approximately twice more potent ACE inhibitory effect in the intracellular proteins of *P. pulmonarius* mycelium (IC_{50} = 0.72 mg/ml). The anti-ACE activity reported was immensely stronger than water extracts of mycelia from six different mushrooms, i.e. *Catathelasma ventricosum*, *Lactarius camphorates*, *Pholiata adipose*, *Phellinus linteus*, *Thelephora ganbajun* and *Tricholoma giganteum*, with their IC_{50} values ranged from 1.28 to 5.25 mg/ml (Yan *et al*., 2013).

As a way to unearth the proteins that contributed to the anti-ACE activity, a series of protein purifications were performed in this study. The aim of protein purifications was to focus on proteins with ACE inhibitory property, thus eliminate unrelated proteins and

other compounds. Accordingly, proteins from mycelial aqueous extract were fractionated via salting-out method by means of ammonium sulphate concentration ranging from 10% to 100%. Amongst ten fractions, F40 showed the best ACE inhibitory activity with the IC_{50} value of 22 µg/ml. This fraction was estimated to consist of proteins with high to moderate hydrophobicity as it required 40% (w/v) of salt in order to precipitate out the proteins. This result corresponded to earlier studies which discovered potent anti-ACE activity in the presence of abundant hydrophobic amino acid residues (Wu *et al.,* 2006).

Fraction F40 was further purified by RP-HPLC. A semi-preparative C_{18} HPLC (10 × 100 mm) column was used for further purification of F40 due to its advantage to separate small molecular proteins, peptides or small molecules (Hearn *et al*., 1979). From the RP-HPLC protocol employed in this experiment, an optimum chromatogram of eight apparent peaks was eluted at 220 nm, and labelled as P1 to P8. Evaluation of ACE inhibitory activity at 15 µg/ml of samples demonstrated the strongest anti-ACE activity by P6, in which the value was also significantly different compared to the other peaks ($p<0.05$). The IC_{50} value determined was 12 µg/ml.

Interestingly, an active ACE inhibitor P6 in this study demonstrated among the strongest inhibitory effect compared to the naturally occurring ACE inhibitory peptides discovered from other medicinal mushrooms. The IC_{50} value of P6 was stronger than an active tripeptide of *Tricholoma giganteum* (IC_{50} = 0.04 mg/ml) (Lee *et al.,* 2004), *Grifola frondosa* (IC_{50} = 0.13 mg/ml) (Choi *et al.,* 2000) and an active pentapeptide of *Pholiota adiposa* (IC_{50} = 0.044 mg/ml) (Koo *et al.,* 2006). P6 also showed better ACE inhibitory activity compared to two oligopeptides ACE inhibitors analyzed from

Pleurotus cornucopiae which showed the IC_{50} values of 0.46 and 1.14 mg/ml (Jang *et al.*, 2011).

In addition, this study has demonstrated an increase in ACE inhibitory effects correspond to each protein purification step. The IC_{50} values of the inhibitory activity showed thirty times reduction between the mycelial aqueous extract and fraction F40 from salting-out method by ammonium sulphate precipitation, and sixty times reduction when compared to the RP-HPLC fraction P6 (IC_{50} = 720 mg/ml > 22 µg/ml > 12 µg/ml). This suggests potent ACE inhibitory proteins that can exhibit an independent inhibitory action and may indicate specific protein inhibitor for ACE. This characteristic is vital for the discovery of good hypotensive agents (Hong *et al*., 2008). Likewise, captopril was used as positive control for ACE inhibitory activity in this study. The result showed an extremely low IC_{50} value compared to the activity shown by *P. pulmonarius* mycelium. This result was well inferred since captopril is an established clinical drug currently used for the treatment of hypertension, despite the commonly reported adverse effects.

An active ACE inhibitor P6 was analysed by SDS-PAGE as a prerequisite for protein identification using MALDI-TOF/TOF MS. Besides, fraction F40 was also screened along with P6 for two supplementary reasons; a) to observe any analogous band between the two fractions, and b) to determine sample purity. Result showed several bands migrated along the resolving gel in F40 with four similar bands reoccurred in P6. This substantiated the role of reversed phase HPLC in further purifying the fraction as fewer bands emerged in fraction P6 as compared to fraction F40. The four bands from P6 were excised out and subjected to protein identification by MALDI-TOF/TOF MS.

5.3 Protein profiling by MALDI-TOF/TOF MS and LCMS/MS

Mass spectrometry (MS) has become one of the key technologies in the proteomics field due to its high sensitivity, automation and throughput for protein analysis. Among the MS platforms, MALDI-TOF/TOF mass spectrometry offers high sample throughput and the flexibility to connect with different off-line sample fractionation techniques. The identification of isolated protein is characterized by the matching of peptide mass fingerprint (PMF) against the protein sequence database (Wojcik *et al*., 2003). The quest for antihypertensive proteins in this study has utilized this technique supported by tandem mass spectrometry (MS/MS), in which polypeptides sequence tagging was performed to allow protein identification by sequence similarity screening of classical bioinformatics databases subsequent to PMF.

Fraction P6 demonstrated enhanced anti-ACE activity by sixty times compared to the mycelial aqueous extract, and was selected for protein profiling and identification. The fraction was subjected to SDS-PAGE for protein separation prior to MALDI-TOF/TOF MS. SDS-PAGE result showed four distinct bands labelled as H1, H2, H3 and H4. All bands were then subjected to MALDI-TOF/TOF MS. Result from the Mascot search using three different databases hit overall proteins with an average to low protein scores.

In proteomic study, the success of protein identification from mass spectrometry i.e. MALDI-TOF/TOF and LC-MS/MS is highly reliant on the size of database and the error rate within, the mass accuracy, the control over the digest chemistry and the number of matching peptides and their molecular weight (Suckau *et al.,* 2003). In case of fungal protein database, a curated database is extremely required as the available databases are inadequate for both genomic and proteomic study of fungal proteins. In particular, protein database for mushrooms species is still poorly covered. For instance,

only 25 to 50 proteins are found under *P. pulmonarius* via high quality databases such as Swiss-Prot and UniProt. This may contribute to low scores mass spectrometry result. Another consideration is the databases used in this study are not from the same species, also due to the same frailty. Thus, the proteins are not totally similar in comparison and this may cause inaccuracy of the matched sequences and again contributed to low protein scores.

In this study, only one-dimensional chromatography was used for protein separation, i.e. RP-HPLC. Hence, sample P6 was expected to still contain mixture of proteins and peptides. When the sample underwent 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.

According to Petsko *et al*., (2004), a protein that shows more than 40% sequence similarity is hypothetically of correct match. Several proteins with scores more than 40% have been identified from MALDI profile, but the highest score was shown by MRPS5-like protein from band H4, at only 85%, supported by two more hits of mrps5p proteins that scores 78% and 75% from the same protein band (Table 4.2, page 52). MRPS5 is a mitochondrial ribosomal protein (MRP) from the 5SP family. Generally, this protein is known to involve in protein synthesis within the mitochondrion. Proteins comprising the mitochondrial ribosome display a wide variation in sequence and some biochemical properties when compare with different species. This causes recognition of the protein by sequence homology is quite tricky. Also, individual MRPs were characterized functionally by mutational studies due to the same reason (Graack *et al*., 1998).

Several disorders associated with MRP genes are Leigh Syndrome, multiple mitochondria dysfunctions, and non-syndromic hearing loss (O'Brien *et al*., 2005). However, the connection of this protein with antihypertensive property is beyond our comprehension as there is so far no information reported. Further investigations including protein purification, verification of this protein as an ACE inhibitor by bio-assay and *in vivo* studies and in depth proteomic analyses on the protein are highly required to confirm this finding.

Given the drawback vis-à-vis protein impurity and implausible protein scores, to identify antihypertensive proteins based on the protein score alone is somewhat precarious. Hence, the search for antihypertensive proteins were expanded by narrowing down the protein profile based on the earlier antihypertensive studies reported by other researchers. However, it is noted that some information regarding the other proteins, especially the hypothetical proteins might be missing due to this methodology. Accordingly, three proteins pertaining antihypertensive activity were discovered, i.e. Serine proteinase inhibitor-like protein and DEAD/DEAH box RNA helicase-like protein from band H4 and nitrite reductase-like protein from band H3. There was as yet no ACE inhibitory or antihypertensive related protein identified from band H1 and H2.

Fraction P6 was also subjected to LC-MS/MS to validate the proteins identified from MALDI-TOF/TOF MS. The high sensitivity and reliability of LC-MS/MS, plus the identification method that based on independent sequencing of peptide has made it suitable to analyse very complex mixture. The result showed an abundance of proteins identified by LC-MS/MS, with all of them showed low protein scores and most of them hit only one unique peptide sequenced by MS/MS. Out of the total hits, there were five proteins appeared in both proteomic tools, i.e. DEAD/DEAH box RNA helicase, Pre-

mRNA-processing ATP-dependent RNA helicase, serine/threonine-protein kinase, peptidyl-prolyl isomerase, and translation elongation factor 2. Intriguingly, only one protein which has been shown to have antihypertensive property was identified by both mass spectrometries, which was DEAD/DEAH box RNA helicase-like protein.

Although theoretically a LC-MS/MS is commonly used for proteomic analysis of complex mixtures or samples, the ability is limited by one-dimensional RP-HPLC method used in this study, due to the difficulty to separate a very large number of proteins and peptides in the sample. Again, an inadequate database is another limiting factor for protein identification by LCMS/MS.

5.4 Proteins with antihypertensive activity found in *P. pulmonarius* mycelium

5.4.1 Serine proteinase inhibitor-like protein

Serine proteinases (synonym as serine protease) are serine-type endopeptidases that are well-known as a group of proteolytic enzymes. The classical mechanism of serine proteinases involves a catalytic triad of Serine, which is activated by a proton relay involving acidic residue (e.g. aspartic acid and glutamate) and basic residue (usually histidine). The serine proteinases include digestive enzymes and generally participate in development, blood coagulation, inflammation, and various other processes (Gan, 1994).

In human, serine proteinases play an important role in the RAAS system (figure 5.1). Among the serine proteinases, chymase is a major serine proteinase implicated for the formation of cardiac Angiotensin II via ACE-independent enzymatic pathway. Chymase is an Angiotensin II-forming enzyme as efficient as ACE. It cleaves Angiotensin I at the same site as ACE to form Angiotensin II, but is highly resistant to ACE inhibitors.

According to Wolny *et al*., (1996), earlier studies have shown the incomplete blockade of RAAS via ACE inhibitors, as the plasma Angiotensin II levels return to normal despite ACE inhibitor therapy. Wolny and colleagues suggested that chymase was responsible for the incomplete blockade. The same notion coincided with Park *et al*., (2009), who further suggested that ACE-independent mechanisms predominate in diabetic kidneys thus accountable for the ineffectiveness of ACE inhibitors in some diabetic-related hypertension patients. As chymase is completely inhibited by serine proteinase inhibitors, discovery of serine proteinase inhibitors could be a potentially important therapeutic target in the treatment of hypertension, especially diabetic-related hypertension and nephropathy. As ACE-independent Angiotensin II is strongly upregulated in the human diabetic kidney, particularly in mesangial cells and in vascular smooth muscle cells, serine proteinase inhibitors could also contribute to the healing of structural remodelling associated with cardiovascular diseases such as vascular injuries and peripheral vascular disease, as well as protection from renal vascular disease (Lorenz, 2010). Serpin and aprotinin are among the well-known serine proteinase inhibitors that have been studied for the restorative purposes of hypertension.

The 6% protein score corresponds to the similarity of sample sequence with fragment 11 to 19 of a total 76-amino acid of serine proteinase inhibitor from *P. ostreatus*, with two different amino acids present in the second and fifth residues. According to Erjavac *et al*., (2012), information about proteases from mushrooms is limited. Many proteases found in mushrooms exhibit unique characteristics and could be exclusive to Basidiomycetes. This opens the opportunity for more discoveries of novel proteases, and also a good source of protease inhibitors, albeit the even lesser information available on the protein inhibitors.

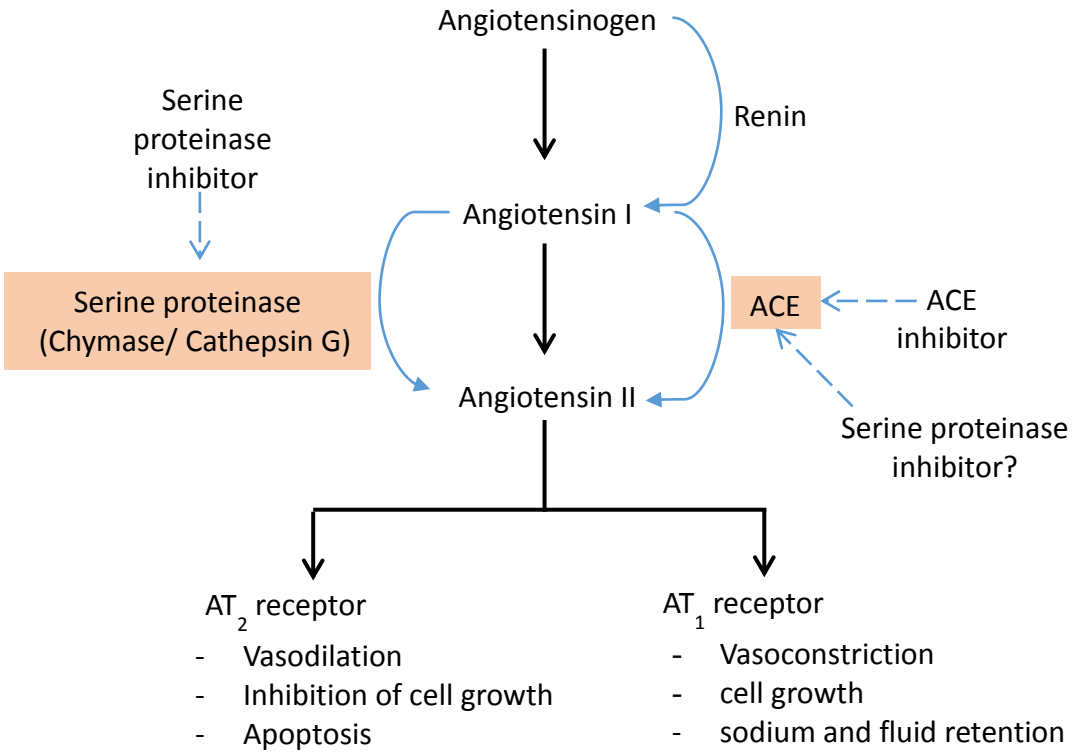


Figure 5.1: The RAAS system that includes conversion of angiotensin I to angiotensin II by ACE and serine proteinases (The illustration is adapted from Peterson *et al*., 2002)

Detailed study on serine proteinase inhibitor from mushroom is yet to be enhanced. Two low-molecular-weight inhibitors of endogenous serine proteinase from *P. ostreatus* has been the most comprehensively studied so far. Discovered by Dohmae and colleagues in 1995, these proteinase inhibitors with respective molecular weights of 8307 and 8244 Da showed concise similarity with the long-known yeast proteinase B inhibitor. These proteinase inhibitors showed similar mechanism of action to propeptides of subtilisin family proteinases due to their structural resemblance (Dohme *et al*., 1995). Another serine proteinase inhibitor was found from the fruiting body of *Lentinus edodes*. This proteinase inhibitor showed inhibition of activated factor XI when tested against microbial and mammalian serine proteinases, including human enzymes of blood coagulation and fibrinolysis. This inhibitor did not show any significant similarity to any major groups of serine proteinase inhibitors, also to non-

inhibitor proteins in the available databases, and thus was hypothesised to represent a novel serine proteinase inhibitor family (Odani *et al.*, 1999).

The identification of a sequence corresponds to serine proteinase inhibitor is beyond expectation, as our objective was to investigate antihypertensive activity via ACE inhibitory mechanism. Yet, it is interesting to discover another antihypertensive potential with comparable advantages. As the mechanism of angiotensin II formation is similar between ACE and serine proteinase, it is possible that the *in vitro* experiment is valid for both ACE inhibitory and serine proteinase inhibitory assay. Although serine proteinase is fully resistant to ACE inhibitor, there is no report affirming the resistance of ACE towards serine proteinase inhibitor. In fact, a neutral serine proteinase inhibitor called leupeptin was reported to inhibit ACE activity in human T-lymphocytes though no effects were shown by chymostatin (chymase inhibitor) and E-64 (calpain inhibitor) (Petrov *et al*., 2000). Hence, it is possible if the protein plays synergistic inhibitor effect for serine proteinase and ACE. Further investigation is necessary to attest this inference.

5.4.2 Nitrite reductase-like protein

Nitric oxide (NO) has been identified as the endothelium-derived relaxing factor, and later was characterized as a vasoregulatory molecule, thus featured in the regulation of blood pressure and flow, smooth muscle cell proliferation and migration, platelet aggregation, and leucocytes adhesion to the endothelium. Many studies reported benefits of NO mediated mechanisms for the treatment of cardiovascular disorders including hypoxia, pulmonary or systemic hypertension, ischemia, atherosclerosis and vasospasm (Alef *et al*., 2011).

In short, the production of NO is mediated by two pathways. The first endogenous NO pathway discovered decades ago is the NOS-dependent pathway. NO is synthesized by nitric oxide synthases (NOS). There are two constitutively expressed NOS isoforms (endothelial NOS; eNOS, and neuronal NOS; nNOS) and one inducible isoform (iNOS). eNOS is the most important isoform in regulating NO production to influence the cardiovascular system. More recently, scientists have demonstrated that NO formation can take place independently of the NOS pathway (NOS-independent pathway). This pathway involves the conversion of nitrate (NO_3^-) to nitrite (NO_2^-) and then to NO, via reduction activity by both non-enzymatic and highly regulated enzymatic mechanisms (Zuckerbraun *et al*., 2011). Figure 5.2 is illustrated to understand the NOS-dependent and NOS-independent pathways for the production of NO, as well as the connection of NO to other mechanisms.

The reduction of nitrite to NO in the NOS-independent pathway occurs both in the blood and tissues. The non-enzymatic conversion is mediated by acidic reduction, while the enzymatic conversion is regulated by nitrite reductase enzymes. This enzyme was reported to show maximum reductase activity with faster response especially during the hypoxic condition, thus underpins vasodilation, maintain blood flow and oxygen demand to the cells (Gladwin *et al*., 2006). Other study done by Pinder *et al.,* (2009) demonstrated maximum dilation on the aortic ring of rabbit under hypoxic condition when treated with plasma nitrite, as the result of nitrite reductase activity. Several nitrite reductase enzymes have been discovered in human including xanthine oxidoreductase (XOR), aldehyde oxidase, deoxyhemoglobin, deoxymyoglobin and cytochrome c oxidase, while Webb *et al*., (2008) reported the ability of eNOS to mediate nitrite reductase activity within circulation under hypoxic condition.

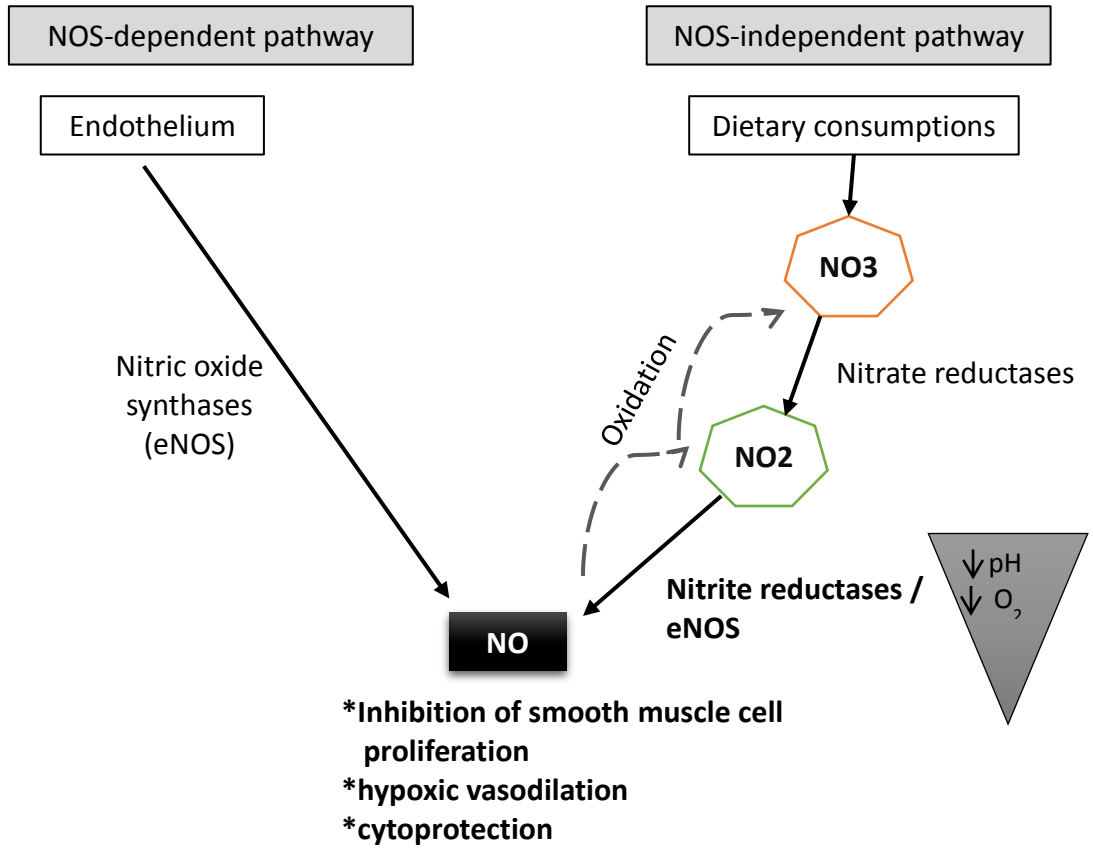


Figure 5.2: NOS-dependent and NOS-independent pathways for the production of nitric oxide (NO). Reduction process takes place in the NOS-independent pathway include nitrate reductases and nitrite reductases enzymes. Conversion of nitrite (NO_2^-) to NO by nitrite reductase enzymes can occur under hypoxic condition (low pH and oxygen supply), where the NOS-dependent pathway works very limited. NO can then have a myriad of biological effects, including vasodilation, modulation of smooth muscle cell proliferation, and cytoprotective effects (The illustration is adapted from John *et al*., 2009)

Ghosh *et al*., (2013) reported enhanced vasodilator activity of nitrite and lowering blood pressure effect in the spontaneously hypertensive rats (SHR). A clinical study was also implemented and result showed improved efficacy of inorganic nitrate and nitrite as a consequence of increased erythrocytic (XOR)-nitrite reductase activity. This study suggests the concept of dietary nitrate supplementation as an effective and economical antihypertensive therapy. Although there are overlapping and integrated mechanisms

involving NO pathways alongside the other undiscovered pathways that put physiological role of nitrite and nitrite reductases only in its infancy, studies suggest that nitrite reductase mechanism and the global role of nitrite may be a major stable reservoir of NO in the circulation and being harness to treat many diseases especially the ones associated with deleterious cardiovascular consequences.

It is known that eNOS/NO pathway interacts with RAAS system in many circumstances. The most studied system involving both pathways is the mechanisms underlying vascular (endothelial) dysfunction by oxidative stress and its related diseases such as aging, diabetes, arteriosclerosis and hypertension. Generally, Angiotensin II stimulates superoxide (O_2^-) formation from the vascular NADPH oxidase via AT_1 receptor. This results in uncoupling of eNOS, which lead to more superoxide production rather than NO. This inhibits cGMP pathway and thus conducts endothelial dysfunction. Presence of ACE inhibitor could not only reduce Angiotensin II, but also increase in eNOS expression, which may be mediated via another vasodilator and NO release device, a bradykinin-mediated mechanism (Imanishi *et al.*, 2009).

In regard to our result, the presence of nitrite reductase enzyme-like protein might be the responsible protein for the ACE inhibitory activity in addition to the known function studied by other researchers. Although there is no supporting data about the synergistic effect of nitrite reductase to ACE inhibitor, there is still room for further investigation due to overlapping and complicated mechanisms liaised in cardiovascular system.

A concern due to variation of estimated molecular mass for nitrite reductase between MALDI and the SDS-PAGE band (H3) is fathomed. A study by Prodouz *et al.*, (1981) has shown that native nitrite reductase was denatured into a single homodimeric subunit

in the presence of guanidine HCl and ß-mercaptoehtanol in SDS-PAGE. Another SDS-PAGE analysis by Steensma *et al*., (2001) has indicated degradation of cytochrome cd_1 nitrite reductase. The result was confirmed by mass spectrometric analysis that showed reduced molecular mass and the cleavage of the c domain residues, in which resulted the presence of no full-length protein domain. Similarly, Bowsher *et al*., (1988) also found a degradation product of nitrite reductase screened by SDS-PAGE. Certainly, degradation of protein samples are among the most common problems that contribute to variation of protein mass in SDS-PAGE. Large protein size or protein with subunits may also indicate partial existence in the gel due to denaturation (Hjelmeland *et al*., 1981).

5.4.3 DEAD/DEAH box RNA helicase-like protein

RNA helicases are known mostly for RNA metabolisms such as pri-mRNA splicing, ribosome biogenesis and translation initiation, as well as in the mediation of antiviral immune response. Defective RNA helicases have been linked to serious disorders like cancer, neurodegenerative disorders and infectious diseases (Steimer *et al.*, 2012). Recently, hypertensive related diseases such as cardiomyocyte hypertrophy has been linked to the regulation of RNA helicase. Sahni *et al*., (2010) has postulated antihypertensive effect of DEAD/DEAH box helicase through the regulation of cardiac cellular mechanisms. The process is regulated by CHAMP (cardiac helicase activated by MEF2 protein), a type of RNA helicase that acts through general cell cycle machinery by inhibiting cell cycle progression.

MEF2 protein is a cardiac transcription factor that responsible to activate CHAMP. Other than MEF2, cardiac transcription factors are GATA4 and homeobox transcription factor Csx/Nkx^{2-5} (Akazawa *et al.*, 2003). CHAMP is localized primarily to the

cytoplasm of cardiomyocetes and control proliferation of cardiomycete by regulating the mRNAs of proteins involved in cell cycle progression. According to Liu and Olsen (2002), antiproliferative activity of CHAMP was mediated by a cell cycle inhibitor $p21^{CIP1}$ and ATPase domain. The upregulation of $p21^{CIP1}$ inhibited cyclin-dependent kinase (CDK) activity thus suppressed cell growth progression and proliferation of hypertrophic coronary. In regard to hypertrophy, this antiproliferative effect would weaken the wall stress, prevent or reverse the hypertrophic phenotype and thus circumvent the subsequent development of heart failure. This would also improve diastolic function and pressure overload in hypertensive patients (Frey *et al.,* 2003).

Given that multiple pathways can elicit a similar molecular response or vice versa, it is postulated that DEAD/DEAH box helicase-like protein characterized here is responsible for ACE inhibitory activity as well as other antihypertensive effect via cellular mechanism. More detail investigation on the molecular pathway, structural and pharmacological properties of this protein is necessary to ensure the ACE inhibitory property.

Protein identification by LC-MS/MS has discovered DEAD/DAH box RNA helicase as one of the protein substances in *P. pulmonarius* mycelium. The unsatisfactory protein scores and coverage given by MALDI-TOF/TOF (67%) and LC-MS/MS (2%) may due to different species databases used as citation for protein identification, as postulated earlier. Additionally, the conserved core regions of DEAD family proteins are normally flanked by extension of sequences at both N-terminal and C-terminal of the proteins, which determine specific function of the individual protein but cause less common in sequences comparisons within the protein family (You *et al*., 1999). Nevertheless, detection of DEAD/DEAH box RNA helicase-like protein in both proteomic tools may

substantiate the presence of this protein as one of the ACE inhibitors in *P. pulmonarius* mycelium.

Noticeably, there was discrepancy of estimated molecular masses of DEAD/DEAH box RNA helicase demonstrated in MALDI and band H4 of SDS-PAGE. This may due to protein degradation that happened intracellulary or during SDS-PAGE procedure. Apparently, You *et al*., (1999) has observed the degraded form of HCV core protein, a member of DEAD box family proteins via indirect immunofluorescence staining.

CHAPTER 6: CONCLUSION AND FUTURE WORK

P. pulmonarius mycelium protein, specifically the intracellular group of protein derived from mycelial biomass demonstrated potential *in vitro* antihypertensive assay. Our present study elucidated antihypertensive effect based on the ability to inhibit ACE activity at the IC_{50} value of 0.012 mg/ml, which indicated among the lowest IC_{50} value of ACE inhibitory activity derived from mushrooms. Protein identifications by MALDI-TOF/TOF and LC-MS/MS have brought about three proteins that were possibly responsible for the anti-ACE activity based on earlier studies reported; serine proteinase inhibitor-like protein, DEAD/DEAH box RNA helicase-like protein and nitrite reductase-like protein. The MRPS5-like protein should also be considered due to the highest protein score from MALDI. Some limitations are considered in this study, such as the inability to further purified the identified ACE inhibitory proteins and limited of existing curated mushroom databases for mass spectrometry analyses.

This research may become groundwork to more in depth investigations on the ACE inhibitory proteins derived from *P. pulmonarius* mycelium. Detailed and comprehensive proteomic approaches such as 2D gel electrophoresis, 2D chromatography and ECI-LCMS/MS are anticipated in future protein works. An anti-ACE bioassay guided study of each identified protein and their peptide sequence is a fine approach to uncover ACE inhibitory proteins/peptides prior to more detailed *in vivo* study. A thorough mechanistic research is necessary to elucidate the blood pressure machinery involved in the identified proteins. Evaluations on the gastrointestinal digestion, physiochemical, cytotoxicity and other pharmaceutical considerations are anticipated prior to the ratification of new antihypertensive products. Nevertheless, this research has discovered potential naturally occurring ACE inhibitors that should cause minimal or no side

effects and has offered better understanding of *P. pulmonarius* and its medicinal benefits.

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APPENDIX A: MATERIALS & METHODS

1) Preparation of Malt Extract agar (MEA) plate

Table 1 Recipe for the preparation of MEA agar

Material	Amount
Malt Extract Agar	25 g
Distilled water	400 ml

Malt extract agar powder was mixed with distilled water. The solution was stirred homogeneously and then autoclaved at 121 ± 2°C for 15 minutes. The MEA was let cooled at approximately 45 to 55 ± 2°C before poured into sterile Petri dishes. The MEA plates were stored at 25± 2°C prior to use.

2) Preparation of Brown sugar-spent yeast medium (per 1000 ml)

Table 2 Recipe for the preparation of Brown sugar- spent yeast medium broth

Material	Amount
Brown sugar	10.0 g
Spent Yeast	20.0 g
Malt Extract	20.0 g
Distilled water	1000 ml

The mixture was slightly heated to dissolve all of the ingredients and stirred concurrently to achieve homogeneity. Aliquot of each 100 ml of the medium was transferred into 500 ml Erlenmeyer flasks. The sealed flasks were autoclaved at 121 ± 2°C for 15 minutes.

3) Antihypertensive assay

Antihypertensive assays were performed according to the technical manual provided in the ACE Kit (http://www.dojindo.eu.com/TechnicalManual/Manual_A502.pdf).

a) Preparation of Enzyme working solution

Enzyme B was dissolved with 2 ml distilled water to make Enzyme B solution. Then, 1.5 ml of Enzyme B solution was added to Enzyme A to make Enzyme working solution. Enzyme A and B vials were capped under vacuum pressure. Therefore, disposable syringes were used when adding deionized water and Enzyme B solution to the respective vial. The enzyme working solution was stable at -20°C for two weeks and stable for three days if stored in a refrigerator.

b) Preparation of Indicator working solution

Enzyme C and Coenzyme was dissolved with 3 ml deionized water. In order to prepare Indicator working solution, each 2.8 ml of Enzyme C solution and Coenzyme solution was added to Indicator solution. Enzyme C and Coenzyme vials were capped under vacuum pressure. Deionized water was added through a rubber septum with a disposable syringe. The indicator working solution was stable at -20°C for two weeks and stable for three days if stored in a refrigerator.

4) Semi-purification of protein by salting out method

Table 3 Amount of ammonium sulphate required for protein precipitation

Initial percentage of saturation for ammonium sulphate (%)	**Percentage saturation (%) at 4°C**									
	10	20	30	40	50	60	70	80	90	100
	Solid ammonium sulphate (grams) to be added to 1 litre of solution									
0	53	110	170	233	301	373	450	533	621	717
10		55	113	175	241	311	386	466	552	645
20			57	117	181	249	322	400	483	573
30				58	120	187	257	333	414	502
40					60	124	193	266	345	430
50						62	129	200	276	358
60							64	133	207	287
70								67	138	215
80									69	143
90										71.7
100										-

This table indicates the amount of ammonium sulphate (at 4 °C) to be added to one litre of solution to produce a desired change in the presence saturation of ammonium sulfate. Saturated solution is 4.1 M and required 716.8 g of salt per litre. This table was calculated from the provided formula from http://www.encorbio.com/protocols/AM-SO4.htm

5) Protein estimation by using Bicinchoninic acid (BCA) assay kit

a) Preparation of Diluted Albumin (BSA) Standards

The Albumin Standard (BSA) at the concentration of 2 mg/ml per ampule was diluted into several vials. Distilled water was used as diluents. Preparation of the standards was as shown in Table 4.

Table 4 Preparation of Bovine Serum Albumin (BSA) Standards for protein estimation using BCA assay kit

Vial	Volume of diluents (µl)	Volume and Source of BSA	Final BSA concentration (µg/ml)
A	0	300 µl of stock	2000
B	125	375 µl of stock	1500
C	325	325 µl of stock	1000
D	175	175 µl of vial B dilution	750
E	325	325 µl of vial C dilution	500
F	325	325 µl of vial E solution	250
G	325	325 µl of vial F solution	125
H	400	100 µl of vial G solution	25
I	400	0	0

b) Preparation of the BCA™ Working Reagent (WR)

i. The following formula was used to determine the total volume of WR required:

(# Standards + # sample) × (# replicates) × (volume of WR per sample) = total WR

In this study, the Microplate Procedure was used for each protein estimation assay. Therefore, 200 µl of WR per sample was used in the calculation.

ii. Preparation of WR Reagent was done by mixing 50 parts of BCA™ Reagent A with 1 part of BCA™ Reagent B (Reagent A:B, 50:1).

c) Microplate procedure (Sample to WR ratio = 1:8)

Microplate wells were loaded with 25 µl of standards and samples respectively. Then, 200 µl of WR was added to each well. The plate was mixed thoroughly on a plate shaker for thirty seconds. The plate was then incubated at 37°C for thirty minutes. The plate was cooled to room temperature before the optical density was read by an ELISA Plate Reader at the absorbance value of 562 nm. A standard curve graph was plotted by means of the BSA Standards reading. Protein concentrations were obtained via extrapolation from the standard curve graph.

6) Preparation of mobile phases for RP-HPLC

(a) Organic solvent

HPLC grade acetonitrile was filtered using Whatman® nylon membrane filter (0.45 µm, 47 mm).

(b) Aqueous solvent

For the aqueous solvent, 0.1% TFA was added into distilled water. The solution was filtered using Whatman® cellulose membrane filter (0.45 µm, 47 mm).

The mobile phases were degassed to purge any trapped bubble in the tube prior to every HPLC analysis

7) Preparation of SDS-PAGE stock solutions and buffers

(a) Preparation of stock solutions for glycine – SDS-PAGE system

- **Solution A (monomer acrylamide and bisacrylamide [30.8%T, 2.7%C_{bis}])**

Thirty grams of acrylamide and 0.8 g of N, N'-methylenebisacrylamide were dissolved in 100 ml distilled water. In order to remove trace of ionic impurities from non-ionic acrylamide solution, the solution was deionised by small amount of amberlite IRN-150L, a homogenous mixture of strong cationic and anionic resins. The solution was stirred for an hour, and then filtered to remove the amberlite. Solution A was stored in dark and kept at 4 ± 2°C. The solution was used within 3 months only.

- **Solution B (4× running buffer [1.5 M Tris-HCl, pH 8.8])**

In order to prepare the stock of solution B, 18.17 g of Tris base was dissolved and made up to 100 ml with distilled water. The solution was adjusted to pH 8.8 using concentrated HCl, and kept at 4 ± 2°C.

- **Solution C (10% (w/v) SDS)**

To prepare the stock of solution C, 10 g of SDS was dissolved and made up to 100 ml with distilled water. The solution was kept at room temperature.

- **Solution D (stacking gel buffer [0.5 M Tris-HCl, pH 6.8])**

For making solution D, 6.06 g of Tris base was dissolved and made up to 100 ml with distilled water. The solution was adjusted to pH 6.8 using concentrated HCl, and kept at at 4 ± 2°C.

- **10% APS (10% (w/v) ammonium persulfate)**

In order to prepare the 10% of APS solution, 0.1 g ammonium persulfate was dissolved into 1 ml distilled water. The solution was prepared freshly prior to use.

- **TEMED (N, N, N', N'-tetramethylenediamine)**

TEMED was stored at room temperature and took directly from the bottle when needed.

- **4× SDS-PAGE sample buffer (0.1% (w/v) bromophenol blue, 10% (w/v) glycerol, 2% (w/v) SDS, 1% (w/v) DTT 62.5 Mm Tris-HCl pH 6.8)**

Twenty milligrams of bromophenol blue, 2 ml of glycerol, 400 mg of SDS and 200 mg of dithiothreitol (DTT) were mixed homogenously. Then, 2.5 ml of this solution was made up to 20 ml with distilled water and stored at -20 ± 2°C in aliquots.

- **Running buffer (tank buffer [25 mM Tris, 198 mM glycine, 0.1% (w/v) SDS, pH 8.3])**

To prepare the stock of running buffer, 3.03 g Tris base, 14.4 g glycine and 1 g SDS were mixed and made up to 1 L with distilled water. The solution was stored at room temperature. The buffer was filtered and reused a few times.

(b) Preparation of Prestained SDS-PAGE Protein Marker (Broad range, Bio-Rad®)

The Protein Marker was shaken well and aliquoted into Eppendorf tubes to avoid possibilities of contamination. The tubes were kept in -20 ± 2°C as stocks. The marker to be used was heated to 95 – 100 ± 2°C for 3 to 5 minutes. The tube was spun shortly prior to use.

(c) Preparation of glycine SDS-PAGE gels solutions

- **Recipe for the preparation of separating gel (18% gel)**

For the preparation of 18% separating gel with 0.75 mm thick, the related stock solutions were mixed together following their respective concentration as stated in Table 4 below. The 10% APS and TEMED were added freshly into the solution before it was poured into the gel – casting apparatus.

Table 4 Recipe for 18% separating gel for SDS-PAGE analysis.

Stock solution	**Volume of stock solution (ml)**
Solution A	5.34
Solution B	2.50
Solution C	0.10
Distilled water	2.01
10% APS	0.005
TEMED	0.0033
Total volume	**10.00**

Solution A, B, and C was used for the making of separating gel.

- **Recipe for the preparation of 4% stacking gel**

A 0.75 mm thick of 4% stacking gel was used for the SDS-PAGE analysis. To make the gel, solution A, D and C was mixed together (Table 5). Distilled water was then added into the solution. The freshly prepared 10% APS and TEMED were added into the solution right before the solution was poured into the gel – casting apparatus.

Table 5 Recipe for 4% stacking gel for SDS-PAGE analysis.

Stock solution	**Volume of stock solution(ml)**
Solution A	0.33
Solution D	0.63
Solution C	0.03
Distilled water	1.53
10% APS	0.013
TEMED	0.0025
Total volume	**2.5**

Solution A, D, and C was used for the making of stacking gel.

8) Preparation of silver staining solutions

(a) Fixing solution (40% (v/v) ethanol, 10% (v/v) acetic acid)

Hundred millilitre of ethanol and 25 ml of acetic acid were mixed and made up to 250 ml with distilled water. The solution was kept at room temperature.

(b) Incubation solution (30% (v/v) ethanol, 0.5 M sodium acetate, 8 mM sodium thiosulphate)

Ethanol (75 ml) was mixed with 0.5 g sodium thiosulphate and 17 g sodium acetate. The solution was made up to 250 ml with distilled water. The solution was kept at room temperature.

(c) Silver solution (5.9 mM silver nitrate)

To prepare the silver solution, 0.625 g silver nitrate was added into 250 ml distilled water. The solution was kept at room temperature.

(d) Developing solution (0.24 M sodium carbonate, 0.04% (v/v) 37% formaldehyde, 2.8% (v/v) 5% sodium thiosulfate)

Sodium carbonate (6.25 g) was mixed with 0.1 ml 37% formaldehyde and 7 µl of 5% sodium thiosulfate. The solution was made up to 250 ml. The solution was freshly prepared prior to use and kept at room temperature.

(e) Stopping solution (40 mM EDTA-Na_2.2H_2O)

Stopping solution was made by adding 3.65 g of EDTA-Na_2.2H_2O into distilled water and was made up to 250 ml. The solution was kept at room temperature.

(f) Preserving solution (30% (v/v) ethanol, 4.6% (v/v) 87% glycerol)

In order to prepare the preserving solution, 75 ml of ethanol was mixed with 11.5 ml of glycerol. The solution was made up to 250 ml with distilled water, and kept at room temperature.

APPENDIX B: RAW DATA

Table 1 Antihypertensive assay of *P. pulmonarius* mycelial aqueous extract

Sample	**Conc. (µg/ml)**	**% of ACE inhibition**	**Min of ACE inhibitory (%) ± S.E.M.**
P. pulmonarius mycelia crude extract	3000	73.7	**73.4 ± 0.70**
		72.6	
		73.9	
	2000	61.4	**60.3 ± 1.1**
		59.2	
		60.4	
	1000	50.4	**52.6 ± 2.8**
		55.8	
		51.7	
	500	49.8	**47 ± 2.5**
		45.6	
		45.5	
	200	33.8	**36.6 ± 3.9**
		34.95	
		40.96	
BLANK 1		**1.486 ± 0.03**	**B1-B2= 1.416**
BLANK 2		**0.07 ± 0.00**	

S.E.M = Standard error of means for triplicate values (n=3).

Table 2 Antihypertensive assay of *P. pulmonarius* whole broth crude extract

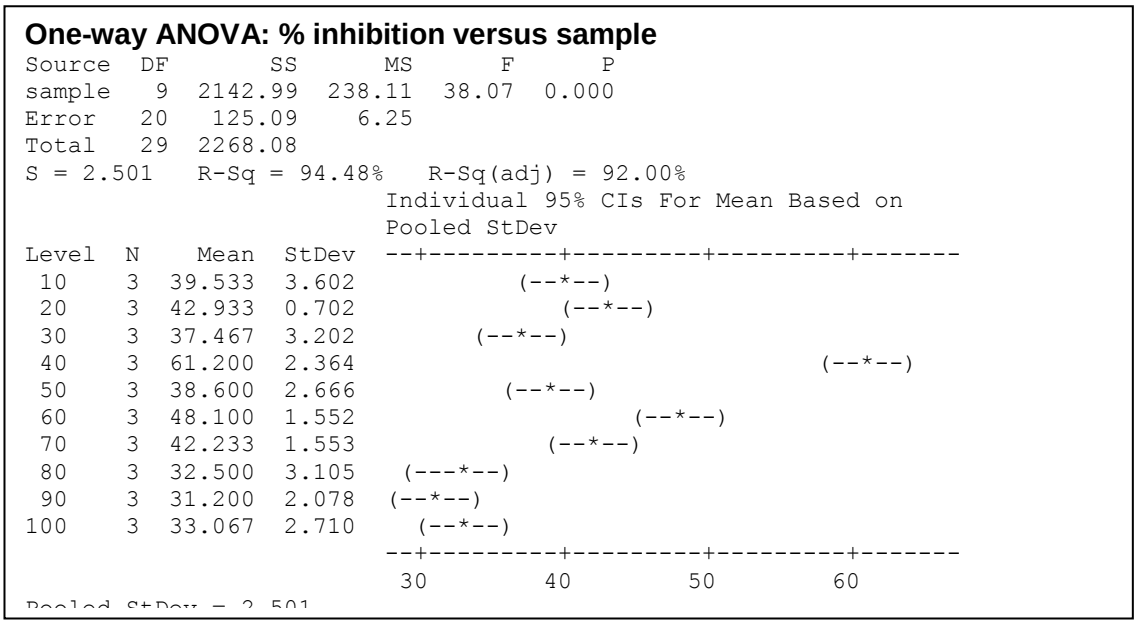
Sample	Conc. (µg/ml)	% of ACE inhibition	Min of ACE inhibitory (%) ± S.E.M.
P. sajor-caju mycelia broth crude extract	3000	72	**70 ± 1.9**
		69.6	
		68.2	
	2000	67.9	**66.4 ± 1.4**
		66.1	
		65.1	
	1000	39.2	**41.6 ± 3.5**
		40	
		45.7	
	500	31.4	**31.6 ± 0.6**
		32.3	
		31.2	
	200	16.3	**15 ± 1.1**
		14.8	
		14.1	
BLANK 1		**1.486 ± 0.03**	**B1-B2= 1.416**
BLANK 2		**0.07 ± 0.00**	

S.E.M = Standard error of means for triplicate values (n=3).

Table 3 Antihypertensive assay of *P. pulmonarius* mycelial protein fractions

Fraction	% of ACE inhibition	Min of ACE inhibitory (%) ± S.E.M.
F10	39.4	**39.5 ± 3.60**
	43.2	
	36	
F20	43	**42.9 ± 0.70**
	42.2	
	43.6	
F30	37.6	**37.5 ± 3.20**
	34.2	
	40.6	
F40	60.9	**61.2 ± 2.36**
	63.7	
	59	
F50	40.7	**38.6 ± 2.67**
	39.5	
	35.6	
F60	48	**48.1 ± 1.55**
	49.7	
	46.6	
F70	43.5	**42.3 ± 1.55**
	40.5	
	42.7	
F80	35.7	**32.5 ± 3.10**
	32.3	
	29.5	
F90	28.8	**31.2 ± 2.08**
	32.4	
	32.4	
F100	35.9	**33.1 ± 2.71**
	30.5	
	32.8	
BLANK 1	**1.609 ± 0.02**	**B1-B2= 1.55**
BLANK 2	**0.059 ± 0.00**	

S.E.M = Standard error of means for triplicate values (n=3). Samples were protein fractions from ammonium sulphate precipitation tested at 50 µg/ml.



One-way ANOVA: % inhibition versus sample

```
Source  DF       SS      MS      F      P
sample   9  2142.99  238.11  38.07  0.000
Error   20   125.09    6.25
Total   29  2268.08
S = 2.501   R-Sq = 94.48%   R-Sq(adj) = 92.00%
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev  --+---------+---------+---------+-------
 10    3  39.533  3.602           (--*--)
 20    3  42.933  0.702              (--*--)
 30    3  37.467  3.202        (--*--)
 40    3  61.200  2.364                                  (--*--)
 50    3  38.600  2.666          (--*--)
 60    3  48.100  1.552                    (--*--)
 70    3  42.233  1.553             (--*--)
 80    3  32.500  3.105   (---*--)
 90    3  31.200  2.078  (--*--)
100    3  33.067  2.710    (--*--)
                          --+---------+---------+---------+-------
                           30        40        50        60
Pooled StDev = 2.501
```

Figure 1 One-way ANOVA for antihypertensive activity of *P. pulmonarius* mycelial protein fractions, F10 to F100 (n=3).

Table 4 IC_{50} value for antihypertensive assay of fraction F40

Sample	Conc. (µg/ml)	% of ACE inhibition	Min of ACE inhibitory (%) ± S.E.M.
F40	150	90.6	**90.3 ± 0.23**
		90.2	
		90.2	
	80	81.7	**81.9 ± 1.76**
		83.7	
		80.2	
	40	65.6	**66.2 ± 1.31**
		65.3	
		67.7	
	20	49.5	**48.1 ± 2.42**
		49.5	
		45.3	
	5	33.8	**35.1 ± 3.39**
		32.6	
		38.98	
BLANK 1		**1.486 ± 0.03**	**B1-B2= 1.416**
BLANK 2		**0.07 ± 0.00**	

S.E.M = Standard error of means for triplicate values (n=3).

Table 5 Antihypertensive assay of the RP-HPLC fractions

Fraction	% of ACE inhibition	Min of ACE inhibitory (%) ± S.E.M.
P1	21.1	**19.6 ± 2.12**
	18.1	
P2	32.4	**30.3 ± 2.97**
	28.2	
P3	33.9	**30.8 ± 4.38**
	27.7	
P4	43.5	**44.7 ± 1.63**
	45.8	
P5	30.7	**33.1 ± 3.32**
	35.4	
P6	53.2	**57.7 ± 6.29**
	62.1	
P7	37	**32.3 ± 6.65**
	27.6	
P8	31.3	**28.6 ± 3.75**
	26	
BLANK 1	**1.301**	**B1-B2= 1.224**
BLANK 2	**0.077**	

S.E.M = Standard error of means for duplicate values (n=2). The assay was performed at the concentration of 15 µg/ml protein.

One-way ANOVA: % INHIBITION versus SAMPLE

```
Source  DF      SS     MS      F      P
SAMPLE   7  1866.7  266.7  14.81  0.001
Error    8   144.1   18.0
Total   15  2010.7
S = 4.244   R-Sq = 92.84%   R-Sq(adj) = 86.57%
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev  --+---------+---------+---------+-------
P1     2  19.600  2.121  (----*----)
P2     2  30.300  2.970          (---*----)
P3     2  30.800  4.384          (----*---)
P4     2  44.650  1.626                    (----*---)
P5     2  33.050  3.323           (----*----)
P6     2  57.650  6.293                              (---*----)
P7     2  32.300  6.647           (----*---)
P8     2  28.650  3.748        (----*----)
                          --+---------+---------+---------+-------
                           15         30        45         60
Pooled StDev = 4.244
```

Figure 2 One-way ANOVA for antihypertensive activity of RP-HPLC fractions P1 to P8 (n=2).

Table 6 IC_{50} value for antihypertensive assay of RP-HPLC fraction P6

Sample	Conc. (µg/ml)	% of ACE inhibition	Min of ACE inhibitory (%) ± S.E.M.
	150	97.5	**96.8 ± 0.62**
		96.6	
		96.3	
	80	92.9	**93.5 ± 1.37**
		92.6	
		95.1	
	60	87.8	**87.9 ± 0.17**
		88.1	
		87.8	
	40	83.5	**82.4 ± 1.10**
		81.3	
		82.5	
	20	69.2	**65.9 ± 2.86**
		64.2	
		64.3	
	5	33.9	**30.1 ± 3.27**
		28.5	
		28	
BLANK 1		**1.486 ± 0.03**	**B1-B2= 1.416**
BLANK 2		**0.07 ± 0.00**	

S.E.M = Standard error of means for triplicate values (n=3).

Table 7.1 Protein identification by LC-MS/MS (Database NCBI-*P. ostreatus*)

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	MW [kDa]
4512252	manganese peroxidase	6.99	4.83	3	1	36.9
13811419	DNA polymerase	3.75	1.91	1	2	116.8
158251728	DNA-directed RNA polymerase	3.05	1.16	1	2	141.4
354992137	RNA polymerase II second largest subunit	2.42	4.37	1	1	77.5
270056459	putative ribosomal protein	2.24	4.03	1	2	116.8

Table 7.2 Protein identification by LC-MS/MS (Database NCBI & SwissProt-Fungi)

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]
74582443	RecName: Full=Nucleoporin nup211; AltName: Full=Nuclear pore protein nup211	7.41	1.96	1	2	2	3	1837	211.3
406862336	3-isopropylmalate dehydratase [Marssonina brunnea f. sp. 'multigermtubi' MB_m1]	20.79	2.07	106	2	2	8	774	84.6
402076267	hypothetical protein GGTG_10944 [Gaeumannomyces graminis var. tritici R3-111a-1]	9.45	2.39	1	2	2	3	1295	139.7
401623462	phr1p [Saccharomyces arboricola H-6]	5.52	5.66	11	2	2	2	565	66.2
149387660	hypothetical protein PICST_36839 [Scheffersomyces stipitis CBS 6054]	5.30	2.84	1	2	2	2	1761	202.2
351638400	D-lactate dehydrogenase [Magnaporthe oryzae 70-15]	4.59	5.22	2	2	2	2	575	61.6

384489611	hypothetical protein RO3G_05538 [Rhizopus delemar RA 99-880]	7.13	3.54	2	2	2	3	961	108.8
380093319	putative SPO11 protein [Sordaria macrospora k-hell]	5.20	15.36	2	2	2	2	384	42.8
1246898	SAH [Kluyveromyces lactis]	67.38	2.47	1	2	2	18	608	69.2
119400279	ubiquitination network signaling protein, putative [Aspergillus clavatus NRRL 1]	2.96	2.46	1	1	1	1	1018	109.6
325529975	RecName: Full=Phosphatidylethanolamine N-methyltransferase; Short=PEAMT	4.81	1.36	1	1	1	2	953	108.4
74701377	RecName: Full=COP9 signalosome complex subunit 5	2.63	3.69	1	1	1	1	406	44.1
68052138	RecName: Full=DASH complex subunit DAM1; AltName: Full=Outer kinetochore protein DAM1	2.12	4.89	1	1	1	1	307	34.9
338818226	RecName: Full=Genetic interactor of prohibitins 3, mitochondrial; AltName: Full=Found in mitochondrial proteome protein 38; Flags: Precursor	2.77	3.82	2	1	1	1	550	62.9
152032522	RecName: Full=ATP-dependent RNA helicase HAS1	2.70	6.62	1	1	1	1	559	62.6
17368407	RecName: Full=Mating type protein A-1; Short=Mt A-1	2.80	7.69	2	1	1	1	286	32.4
74689911	RecName: Full=Putative nicotinamide N-methyltransferase	2.04	4.76	1	1	1	1	273	30.7
74582917	RecName: Full=FACT complex subunit pob3; AltName: Full=Facilitates chromatin transcription complex subunit pob3	17.66	3.13	1	1	1	6	512	57.4
17380272	RecName: Full=Probable proteasome subunit beta type-3	2.51	10.29	1	1	1	1	204	22.6
74609191	RecName: Full=Mediator of RNA polymerase II transcription subunit 13; AltName: Full=Mediator complex subunit 13	2.01	0.82	1	1	1	1	1345	151.1
380354494	Num1 protein [Candida orthopsilosis]	2.07	0.52	1	1	1	1	2104	237.1

380353899	ABC transporter [Candida orthopsilosis]	2.66	1.19	1	1	1	1	1512	169.6
380352465	Coq6 protein [Candida orthopsilosis]	2.04	2.89	1	1	1	1	485	53.9
380351451	integral peroxisomal membrane protein [Candida orthopsilosis Co 90-125]	2.79	3.11	2	1	1	1	483	54.5
380351275	Nsp1 protein [Candida orthopsilosis Co 90-125]	2.19	1.90	1	1	1	1	897	92.8
380353894	Prp8 protein [Candida orthopsilosis]	3.12	0.75	2	1	1	1	2413	279.5
408397860	hypothetical protein FPSE_02873 [Fusarium pseudograminearum CS3096]	2.84	1.35	1	1	1	1	1406	159.6
408389150	PKS9 [Fusarium pseudograminearum CS3096]	2.80	0.61	1	1	1	1	2642	286.6
407924038	hypothetical protein MPH_05670 [Macrophomina phaseolina MS6]	2.74	16.48	1	1	1	1	182	20.1
407922846	hypothetical protein MPH_06904 [Macrophomina phaseolina MS6]	17.65	7.51	1	1	1	7	173	19.1
407929337	Borealin-like protein [Macrophomina phaseolina MS6]	2.79	4.62	1	1	1	1	346	37.0
407917147	hypothetical protein MPH_12326 [Macrophomina phaseolina MS6]	3.03	1.40	1	1	1	1	2073	229.1
407920196	Sugar/inositol transporter [Macrophomina phaseolina MS6]	2.78	5.45	1	1	1	1	459	52.1
407917439	hypothetical protein MPH_12130 [Macrophomina phaseolina MS6]	5.10	21.54	1	1	1	2	65	7.1
358374223	DEAD box RNA helicase HelA [Aspergillus kawachii IFO 4308]	2.71	2.31	2	1	1	1	648	72.4
358365480	eukaryotic translation initiation factor 2c [Aspergillus kawachii IFO 4308]	2.66	2.67	1	1	1	1	900	101.4
406861659	nuclear condensin complex subunit [Marssonina brunnea f. sp. 'multigermtubi' MB_m1]	2.59	1.18	1	1	1	1	1103	122.2
406867006	hypothetical protein MBM_01997 [Marssonina brunnea f. sp. 'multigermtubi' MB_m1]	3.06	1.55	1	1	1	2	1031	115.6
406865168	bromodomain protein [Marssonina brunnea f. sp. 'multigermtubi' MB_m1]	2.82	1.71	1	1	1	1	701	76.8
406864243	WD repeat-containing protein 5B [Marssonina	2.52	3.08	1	1	1	1	519	57.0

	brunnea f. sp. 'multigermtubi' MB_m1]								
406862792	intracellular protein transport-like protein [Marssonina brunnea f. sp. 'multigermtubi' MB_m1]	2.08	1.15	1	1	1	1	1219	135.0
406859479	hypothetical protein MBM_09300 [Marssonina brunnea f. sp. 'multigermtubi' MB_m1]	3.63	3.62	1	1	1	2	276	28.1
406859978	collagen-like protein 2 [Marssonina brunnea f. sp. 'multigermtubi' MB_m1]	2.16	5.76	1	1	1	1	295	33.6
406699303	Muconate cycloisomerase 1 [Trichosporon asahii var. asahii CBS 8904]	2.78	3.37	1	1	1	1	683	72.8
406699148	hypothetical protein A1Q2_03342 [Trichosporon asahii var. asahii CBS 8904]	2.00	3.72	1	1	1	1	296	30.8
406699110	cleavage and polyadenylation specific protein [Trichosporon asahii var. asahii CBS 8904]	2.80	2.46	2	1	1	1	1339	146.6
406605025	hypothetical protein BN7_3110 [Wickerhamomyces ciferrii]	1.81	2.78	1	1	1	1	395	44.8
406602147	AFG3 family protein [Wickerhamomyces ciferrii]	2.81	2.65	1	1	1	1	905	101.6
405118307	hypothetical protein CNAG_06718 [Cryptococcus neoformans var. grubii H99]	2.74	5.34	1	1	1	1	281	30.7
403418291	predicted protein [Fibroporia radiculosa]	2.54	6.17	1	1	1	2	389	40.5
403174635	hypothetical protein PGTG_15003 [Puccinia graminis f. sp. tritici CRL 75-36-700-3]	2.58	5.69	1	1	1	1	299	33.6
402471066	hypothetical protein EDEG_00911 [Edhazardia aedis USNM 41457]	2.53	3.08	1	1	1	2	876	103.7
402226202	hypothetical protein DACRYDRAFT_103206 [Dacryopinax sp. DJM-731 SS1]	2.64	5.85	1	1	1	1	376	41.4
402221924	FAD/NADP-binding domain-containing protein, partial [Dacryopinax sp. DJM-731 SS1]	2.04	3.08	1	1	1	1	390	43.6
402216504	ARM repeat-containing protein [Dacryopinax sp. DJM-731 SS1]	2.66	1.86	1	1	1	1	1077	118.4
402225957	hypothetical protein DACRYDRAFT_97650	2.27	1.56	1	1	1	1	1092	116.2

	[Dacryopinax sp. DJM-731 SS1]								
372464244	hypothetical protein KAFR_0E03720 [Kazachstania africana CBS 2517]	18.97	5.93	1	1	1	7	236	25.7
402077888	hypothetical protein GGTG_10084 [Gaeumannomyces graminis var. tritici R3-111a-1]	2.11	2.92	1	1	1	1	343	36.3
401884280	hypothetical protein A1Q1_02581 [Trichosporon asahii var. asahii CBS 2479]	2.35	1.18	2	1	1	1	936	102.6
401880699	protein kinase [Trichosporon asahii var. asahii CBS 2479]	3.26	0.81	2	1	1	2	984	106.0
401826144	DNA-directed RNA polymerase subunit beta [Encephalitozoon hellem ATCC 50504]	2.80	2.01	1	1	1	1	1396	156.9
401827927	glycyl-tRNA synthetase [Encephalitozoon hellem ATCC 50504]	2.78	3.11	1	1	1	1	579	66.9
401780129	hypothetical protein NDAI_0G02910 [Naumovozyma dairenensis CBS 421]	2.08	0.83	1	1	1	1	967	110.9
302308091	AER023Cp [Ashbya gossypii ATCC 10895]	3.15	2.02	1	1	1	1	1039	115.1
188219314	unnamed protein product [Podospora anserina S mat+]	2.02	0.95	2	1	1	1	1051	119.8
58270472	hypothetical protein CNH02180 [Cryptococcus neoformans var. neoformans JEC21]	7.96	0.49	1	1	1	3	2638	293.4
58259255	imidazoleglycerol phosphate synthase [Cryptococcus neoformans var. neoformans JEC21]	2.51	3.39	1	1	1	1	619	66.4
401626680	YBL095W [Saccharomyces arboricola H-6]	2.70	6.67	1	1	1	1	270	30.3
401623854	tcb2p [Saccharomyces arboricola H-6]	3.03	1.44	1	1	1	1	1179	132.3
400602476	hypothetical protein BBA_00947 [Beauveria bassiana ARSEF 2860]	2.50	14.29	1	1	1	1	168	18.5
400598811	PP-loop family protein [Beauveria bassiana ARSEF 2860]	2.59	2.89	1	1	1	1	658	75.3
400593204	hybrid PKS-NRPS protein [Beauveria bassiana ARSEF 2860]	2.85	0.55	1	1	1	1	4016	441.0

350297019	hypothetical protein NEUTE2DRAFT_54039 [Neurospora tetrasperma FGSC 2509]	3.06	35.94	1	1	1	1	64	7.1
378733582	prostaglandin-endoperoxide synthase, variant [Exophiala dermatitidis NIH/UT8656]	2.84	1.28	1	1	1	1	1174	132.5
367006661	mitochondrial 54S ribosomal protein YmL25 [Tetrapisispora phaffii CBS 4417]	2.00	7.64	1	1	1	1	157	18.6
387593570	hypothetical protein NEQG_01284 [Nematocida parisii ERTm3]	1.53	5.95	1	1	1	1	168	19.6
356891207	hypothetical protein Ecym_6378 [Eremothecium cymbalariae DBVPG#7215]	23.63	1.01	1	1	1	8	1589	183.4
342320721	Proteophosphoglycan ppg4 [Rhodotorula glutinis ATCC 204091]	2.12	0.88	1	1	1	1	1471	153.7
342319919	Pre-mRNA-processing ATP-dependent RNA helicase PRP5 [Rhodotorula glutinis ATCC 204091]	2.01	1.43	1	1	1	1	1534	168.1
356890435	hypothetical protein Ecym_5213 [Eremothecium cymbalariae DBVPG#7215]	2.61	5.53	1	1	1	1	416	47.4
336375288	hypothetical protein SERLA73DRAFT_69472 [Serpula lacrymans var. lacrymans S7.3]	2.21	7.09	1	1	1	1	141	16.2
311329978	hypothetical protein PTT_07749 [Pyrenophora teres f. teres 0-1]	28.75	3.10	2	1	1	10	549	60.5
398407441	hypothetical protein MYCGRDRAFT_20718, partial [Zymoseptoria tritici IPO323]	2.51	1.79	1	1	1	1	892	97.8
398397549	cellobiohydrolase [Zymoseptoria tritici IPO323]	2.51	3.15	1	1	1	1	444	46.4
398396400	hypothetical protein MYCGRDRAFT_19839, partial [Zymoseptoria tritici IPO323]	2.73	1.31	1	1	1	1	1839	204.5
341038467	xanthine dehydrogenase-like protein [Chaetomium thermophilum var. thermophilum DSM 1495]	2.18	0.71	1	1	1	1	1406	154.5
340959936	KH RNA-binding domain-containing protein [Chaetomium thermophilum var. thermophilum DSM 1495]	2.78	1.83	1	1	1	1	983	109.7

340960865	putative pyridoxal phosphate binding protein [Chaetomium thermophilum var. thermophilum DSM 1495]	2.67	3.84	1	1	1	1	521	57.9
340960631	hypothetical protein CTHT_0036820 [Chaetomium thermophilum var. thermophilum DSM 1495]	2.39	1.17	1	1	1	1	1023	115.6
340959195	palmitoyltransferase-like protein [Chaetomium thermophilum var. thermophilum DSM 1495]	2.76	3.37	1	1	1	1	742	82.9
340939314	ubiquitin carboxyl-terminal hydrolase-like protein [Chaetomium thermophilum var. thermophilum DSM 1495]	2.52	1.49	1	1	1	1	1008	111.6
340923665	putative mitochondrial large ribosomal RNA protein [Chaetomium thermophilum var. thermophilum DSM 1495]	2.70	5.96	1	1	1	1	638	70.3
328857828	hypothetical protein MELLADRAFT_63029 [Melampsora larici-populina 98AG31]	36.35	6.88	1	1	1	13	218	24.3
328850965	family 3 glycosyltransferase [Melampsora larici-populina 98AG31]	2.64	3.70	1	1	1	1	730	82.5
328850574	hypothetical protein MELLADRAFT_112479 [Melampsora larici-populina 98AG31]	2.13	1.83	3	1	1	1	602	67.1
328849478	hypothetical protein MELLADRAFT_113351 [Melampsora larici-populina 98AG31]	2.61	4.64	1	1	1	1	345	39.1
303303007	leucyl aminopeptidase [Encephalitozoon intestinalis ATCC 50506]	2.56	2.45	1	1	1	1	489	52.8
303302660	hypothetical protein Eint_090230 [Encephalitozoon intestinalis ATCC 50506]	4.69	4.41	1	1	1	2	272	31.6
303302623	Willebrand factor type A domain-containing AAA ATPase [Encephalitozoon intestinalis ATCC 50506]	2.62	0.67	1	1	1	1	2818	324.7
347010253	hypothetical protein MYCTH_2304281 [Myceliophthora thermophila ATCC 42464]	2.85	1.17	1	1	1	1	1792	196.5
347006219	amidohydrolase-like protein [Myceliophthora thermophila ATCC 42464]	2.57	4.83	1	1	1	1	580	61.6

346325503	isovaleryl-CoA dehydrogenase IvdA, putative [Cordyceps militaris CM01]	2.52	7.32	1	1	1	1	396	42.5
346322878	hypothetical protein CCM_03849 [Cordyceps militaris CM01]	2.91	3.97	1	1	1	1	504	56.5
346319278	ribosomal RNA-processing protein 12 [Cordyceps militaris CM01]	3.21	1.27	2	1	1	1	1261	137.7
347014196	hypothetical protein MYCTH_2312075 [Myceliophthora thermophila ATCC 42464]	2.59	1.37	1	1	1	1	1754	191.3
346318636	hypothetical protein CCM_08281 [Cordyceps militaris CM01]	2.75	4.01	1	1	1	1	424	47.4
328773405	hypothetical protein BATDEDRAFT_15601 [Batrachochytrium dendrobatidis JAM81]	2.23	3.10	1	1	1	1	548	58.6
326474976	hypothetical protein TESG_06349 [Trichophyton tonsurans CBS 112818]	2.61	4.22	1	1	1	1	379	41.4
327350627	NAD(P) transhydrogenase [Ajellomyces dermatitidis ATCC 18188]	16.68	1.31	9	1	1	6	1067	111.8
326466042	RNA polymerase II mediator complex subunit Nut1 [Trichophyton rubrum CBS 118892]	3.07	1.97	1	1	1	1	1064	117.9
322708927	hypothetical protein MAA_04281 [Metarhizium anisopliae ARSEF 23]	2.23	1.20	1	1	1	1	1003	105.8
322708838	Fungal specific transcription factor, putative [Metarhizium anisopliae ARSEF 23]	2.57	2.52	1	1	1	1	715	81.1
322700132	mitochondrial cytochrome b2, putative [Metarhizium acridum CQMa 102]	2.11	3.11	1	1	1	1	483	52.7
322699933	serine/threonine-protein kinase prp4 [Metarhizium acridum CQMa 102]	2.59	2.26	1	1	1	1	796	89.5
322703396	chitin binding protein [Metarhizium anisopliae ARSEF 23]	2.11	4.11	1	1	1	1	365	41.3
322699684	hypothetical protein MAC_02607 [Metarhizium acridum CQMa 102]	2.80	0.52	1	1	1	1	3442	359.6
322693163	UDP-glucose 4-epimerase, putative [Metarhizium acridum CQMa 102]	2.15	4.04	1	1	1	1	371	40.5

320591518	er glycosyl hydrolase [Grosmannia clavigera kw1407]	2.88	2.23	1	1	1	1	1120	122.5
320591362	FAD-binding domain containing protein [Grosmannia clavigera kw1407]	3.07	4.17	1	1	1	1	672	71.6
320589851	ubiquitin specific peptidase y chromosome [Grosmannia clavigera kw1407]	2.76	3.61	1	1	1	1	665	73.8
320589512	inositol kinase kinase [Grosmannia clavigera kw1407]	2.63	0.57	1	1	1	1	2089	231.9
320587198	c6 zinc finger domain containing protein [Grosmannia clavigera kw1407]	3.61	4.99	1	1	1	1	601	67.1
310800066	acetyltransferase [Glomerella graminicola M1.001]	2.68	6.50	1	1	1	1	369	39.0
310799802	cytochrome P450 [Glomerella graminicola M1.001]	2.13	2.12	1	1	1	1	518	59.0
310798679	hypothetical protein GLRG_08851 [Glomerella graminicola M1.001]	2.52	7.59	1	1	1	1	395	46.6
310796707	TBC domain-containing protein [Glomerella graminicola M1.001]	2.76	2.06	1	1	1	1	924	102.4
310796003	hypothetical protein GLRG_06608 [Glomerella graminicola M1.001]	2.35	3.21	1	1	1	1	405	45.6
310793340	hypothetical protein GLRG_03945 [Glomerella graminicola M1.001]	6.48	1.53	1	1	1	3	1890	206.9
310790676	bZIP transcription factor [Glomerella graminicola M1.001]	2.13	1.81	2	1	1	1	609	67.5
310793983	hypothetical protein GLRG_04588 [Glomerella graminicola M1.001]	5.94	2.63	1	1	1	2	760	82.7
310793445	exonuclease [Glomerella graminicola M1.001]	2.63	3.58	1	1	1	1	699	77.1
310790185	TfdA family Taurine catabolism dioxygenase TauD [Glomerella graminicola M1.001]	2.79	4.11	1	1	1	1	414	46.2
396499553	hypothetical protein LEMA_P008110.1 [Leptosphaeria maculans JN3]	2.19	2.04	1	1	1	1	734	80.6
396480333	predicted protein [Leptosphaeria maculans JN3]	3.11	3.07	1	1	1	1	748	82.2

396478770	hypothetical protein LEMA_P102650.1 [Leptosphaeria maculans JN3]	2.65	2.20	1	1	1	1	998	106.5
396462504	similar to vacuolar protein sorting protein (VPS11) [Leptosphaeria maculans JN3]	2.86	1.74	1	1	1	1	978	109.1
396483807	similar to VanZ domain protein [Leptosphaeria maculans JN3]	2.95	11.00	1	1	1	1	200	22.4
396480355	similar to gi|121919568|sp|Q0U3A4.1|SET9_PHANO RecName: Full=Histone-lysine N-methyltransferase SET9; AltName: Full=SET domain protein 9 [Leptosphaeria maculans JN3]	2.31	1.64	1	1	1	1	670	77.1
396479463	similar to HEAT repeat protein [Leptosphaeria maculans JN3]	3.01	1.61	1	1	1	1	1059	116.9
396459675	similar to transcriptional corepressor of histone genes (Hir3) [Leptosphaeria maculans JN3]	4.14	0.54	1	1	1	2	2054	229.8
344233939	hypothetical protein CANTEDRAFT_96903 [Candida tenuis ATCC 10573]	2.06	2.03	2	1	1	1	541	60.6
336379807	hypothetical protein SERLADRAFT_452101 [Serpula lacrymans var. lacrymans S7.9]	4.62	1.00	7	1	1	2	1103	127.0
300110818	hypothetical protein SCHCODRAFT_231239 [Schizophyllum commune H4-8]	2.89	2.99	1	1	1	1	602	68.6
300109529	cAMP-dependent protein kinase A catalytic subunit [Schizophyllum commune H4-8]	2.02	2.08	1	1	1	1	432	49.5
300110469	EXP1-like protein [Schizophyllum commune H4-8]	2.54	3.23	1	1	1	1	526	58.9
300108223	hypothetical protein SCHCODRAFT_107087, partial [Schizophyllum commune H4-8]	2.82	1.21	1	1	1	1	1072	120.7
238845953	branched-chain amino acid aminotransferase [Arthroderma otae CBS 113480]	2.71	8.54	1	1	1	1	363	39.4
238845915	conserved hypothetical protein [Arthroderma otae CBS 113480]	5.32	2.62	1	1	1	2	1146	128.7
238840370	carbapenem antibiotics biosynthesis protein carD	2.27	2.90	1	1	1	1	483	53.0

	[Arthroderma otae CBS 113480]								
238843813	fibronectin type III domain-containing protein [Arthroderma otae CBS 113480]	8.12	1.97	1	1	1	3	661	72.7
238841621	predicted protein [Arthroderma otae CBS 113480]	2.75	2.35	1	1	1	1	638	73.7
238839487	PIG-P [Arthroderma otae CBS 113480]	2.28	4.30	1	1	1	1	372	41.0
346979415	nitrate transporter [Verticillium dahliae VdLs.17]	2.65	3.68	1	1	1	1	516	55.8
346975189	SNF7 family protein [Verticillium dahliae VdLs.17]	3.02	5.56	2	1	1	1	342	36.9
346973883	NAP1-binding protein [Verticillium dahliae VdLs.17]	2.16	3.97	1	1	1	1	453	49.4
346979146	Sec7 domain-containing protein [Verticillium dahliae VdLs.17]	2.54	1.58	2	1	1	1	1453	158.3
326483341	hypothetical protein TEQG_06257 [Trichophyton equinum CBS 127.97]	2.10	1.87	6	1	1	1	803	87.0
325091847	conserved hypothetical protein [Ajellomyces capsulatus H88]	5.12	5.82	1	1	2	2	739	84.1
311338198	leucine rich repeat protein [Arthroderma gypseum CBS 118893]	1.51	1.16	1	1	1	1	517	58.4
326481992	hypothetical protein TEQG_05010 [Trichophyton equinum CBS 127.97]	2.62	6.27	1	1	1	1	367	40.8
325091992	eukaryotic translation initiation factor 3 [Ajellomyces capsulatus H88]	2.16	1.50	3	1	1	1	868	96.9
325090136	conserved hypothetical protein [Ajellomyces capsulatus H88]	2.80	2.84	3	1	1	1	811	90.1
325087970	predicted protein [Ajellomyces capsulatus H88]	3.70	3.75	2	1	1	1	480	52.5
311344619	calcium-transporting ATPase 3 [Arthroderma gypseum CBS 118893]	5.19	1.14	1	1	1	2	1049	114.6
261360628	homogentisate 1,2-dioxygenase [Verticillium albo-atrum VaMs.102]	2.17	6.83	2	1	1	1	278	30.5
261358560	predicted protein [Verticillium albo-atrum VaMs.102]	2.54	13.01	2	1	1	1	146	16.1

261361269	conserved hypothetical protein [Verticillium albo-atrum VaMs.102]	2.53	1.22	4	1	1	1	1392	155.4
261361263	phenolpthiocerol synthesis polyketide synthase ppsA [Verticillium albo-atrum VaMs.102]	2.54	1.41	1	1	1	1	1911	208.1
240277585	conserved hypothetical protein [Ajellomyces capsulatus H143]	2.12	6.16	2	1	1	1	276	31.5
240281867	hypothetical protein HCDG_00949 [Ajellomyces capsulatus H143]	2.13	5.28	2	1	1	1	284	29.9
239608866	DNA methyltransferase Dim-2 [Ajellomyces dermatitidis ER-3]	2.94	1.62	2	1	1	1	1233	138.3
239586916	PH domain-containing protein [Ajellomyces dermatitidis SLH14081]	2.52	2.20	2	1	1	1	819	89.6
226294680	predicted protein [Paracoccidioides brasiliensis Pb18]	2.54	6.32	1	1	1	1	380	42.7
226292236	conserved hypothetical protein [Paracoccidioides brasiliensis Pb18]	2.76	1.08	2	1	1	1	1858	207.1
226286518	conserved hypothetical protein [Paracoccidioides sp. 'lutzii' Pb01]	2.31	2.66	3	1	1	1	638	70.6
226284292	predicted protein [Paracoccidioides sp. 'lutzii' Pb01]	2.16	6.86	1	1	1	1	175	19.4
226279964	dolichyl pyrophosphate Glc1Man9GlcNAc2 alpha-1,3-glucosyltransferase [Paracoccidioides sp. 'lutzii' Pb01]	5.64	2.70	1	1	1	2	518	58.9
225681880	predicted protein [Paracoccidioides brasiliensis Pb03]	2.80	2.89	1	1	1	2	380	42.0
225679360	DUF500 and UBA/TS-N domain-containing protein [Paracoccidioides brasiliensis Pb03]	2.71	3.32	2	1	1	1	662	72.5
226295345	nucleolar protein NOP58 [Paracoccidioides brasiliensis Pb18]	3.16	2.39	2	1	1	1	880	95.2
226291337	conserved hypothetical protein [Paracoccidioides brasiliensis Pb18]	2.83	0.78	3	1	1	1	2429	266.3
226278925	predicted protein [Paracoccidioides sp. 'lutzii'	3.00	3.53	1	1	1	1	709	79.5

	Pb01]								
225681973	acetate kinase [Paracoccidioides brasiliensis Pb03]	2.96	4.21	1	1	1	1	404	44.7
225678183	hypothetical protein PABG_06554 [Paracoccidioides brasiliensis Pb03]	2.57	4.45	2	1	1	1	292	31.6
218713484	squalene monooxygenase Erg1 [Talaromyces stipitatus ATCC 10500]	2.53	3.46	1	1	1	1	491	53.7
218722129	SNF2 family helicase/ATPase PasG, putative [Talaromyces stipitatus ATCC 10500]	2.06	1.68	1	1	1	1	895	100.7
218720728	TBC domain protein, putative [Talaromyces stipitatus ATCC 10500]	2.11	2.33	1	1	1	1	729	82.0
218720684	uridine nucleosidase Urh1, putative [Talaromyces stipitatus ATCC 10500]	2.68	5.14	1	1	1	1	370	39.5
210072913	DUF1212 domain membrane protein Prm10, putative [Penicillium marneffei ATCC 18224]	3.35	1.78	1	1	1	1	897	97.9
210071983	exonuclease, putative [Penicillium marneffei ATCC 18224]	2.21	4.21	1	1	1	1	309	34.9
210064835	calcineurin Ca2+-binding regulatory subunit CnaB [Penicillium marneffei ATCC 18224]	2.52	8.85	1	1	1	1	192	21.7
210073584	conserved hypothetical protein [Penicillium marneffei ATCC 18224]	2.61	3.53	1	1	1	1	566	63.9
187980898	conserved hypothetical protein [Pyrenophora tritici-repentis Pt-1C-BFP]	2.67	14.45	1	1	1	1	173	19.1
187978532	inner centromere protein, ARK binding region protein [Pyrenophora tritici-repentis Pt-1C-BFP]	15.83	1.11	2	1	1	6	1169	128.7
187973903	4-coumarate-CoA ligase 1 [Pyrenophora tritici-repentis Pt-1C-BFP]	5.91	3.70	1	1	1	2	648	72.3
187972443	pre-mRNA-processing ATP-dependent RNA helicase PRP5 [Pyrenophora tritici-repentis Pt-1C-BFP]	2.06	1.37	1	1	1	1	1165	129.3
187985153	HET domain containing protein [Pyrenophora tritici-repentis Pt-1C-BFP]	2.52	2.83	1	1	1	1	637	68.5

187985045	NAD dependent epimerase/dehydratase family protein [Pyrenophora tritici-repentis Pt-1C-BFP]	2.74	2.92	2	1	1	1	377	43.0
187976373	external NADH-ubiquinone oxidoreductase 1, mitochondrial precursor [Pyrenophora tritici-repentis Pt-1C-BFP]	2.76	2.60	2	1	1	1	577	64.9
187975847	conserved hypothetical protein [Pyrenophora tritici-repentis Pt-1C-BFP]	2.96	2.89	1	1	1	1	658	75.1
187973308	predicted protein [Pyrenophora tritici-repentis Pt-1C-BFP]	2.00	0.56	1	1	1	1	2856	304.6
164651506	predicted protein [Laccaria bicolor S238N-H82]	19.88	1.60	1	1	1	7	1002	114.1
164647316	hypothetical protein LACBIDRAFT_313890 [Laccaria bicolor S238N-H82]	2.06	1.60	1	1	1	1	562	62.8
256735415	hypothetical protein NECHADRAFT_65808 [Nectria haematococca mpVI 77-13-4]	2.55	1.85	1	1	1	1	1192	133.6
256734138	predicted protein [Nectria haematococca mpVI 77-13-4]	2.93	1.23	1	1	1	1	2035	228.0
238878703	conserved hypothetical protein [Candida albicans WO-1]	1.61	2.57	3	1	1	1	350	38.3
159123282	glucanase, putative [Aspergillus fumigatus A1163]	2.57	5.11	1	1	1	1	470	55.1
159129739	conserved hypothetical protein [Aspergillus fumigatus A1163]	3.22	1.79	1	1	1	1	839	94.4
149387820	predicted protein [Scheffersomyces stipitis CBS 6054]	2.81	1.23	1	1	1	1	1542	174.4
149387477	Suppressor of Sulfoxyde Ethionine resistance, partial [Scheffersomyces stipitis CBS 6054]	2.29	1.99	1	1	1	1	552	63.6
149387035	Acetyl-CoA hydrolase (Acetyl-CoA deacylase) (Acetyl-CoA acylase) [Scheffersomyces stipitis CBS 6054]	2.01	2.29	1	1	1	1	524	58.4
149385347	substrate-specific activator of APC-dependent proteolysis [Scheffersomyces stipitis CBS 6054]	2.60	5.24	1	1	1	1	592	65.3
395333478	hypothetical protein DICSQDRAFT_132050	2.43	1.87	1	1	1	1	588	66.1

	[Dichomitus squalens LYAD-421 SS1]								
340522547	calcium/calmodulin dependent protein kinase C [Trichoderma reesei QM6a]	2.09	2.02	1	1	1	1	643	72.2
340522073	predicted protein [Trichoderma reesei QM6a]	2.39	4.44	1	1	1	1	293	32.9
237904372	conserved hypothetical protein [Uncinocarpus reesii 1704]	2.14	2.30	1	1	1	1	435	48.1
237901318	predicted protein [Uncinocarpus reesii 1704]	4.82	2.85	1	1	1	2	526	59.0
237905049	hypothetical protein UREG_04296 [Uncinocarpus reesii 1704]	2.07	3.39	1	1	1	1	620	69.3
237903455	conserved hypothetical protein [Uncinocarpus reesii 1704]	2.63	1.62	1	1	1	1	987	111.0
154693593	hypothetical protein SS1G_09197 [Sclerotinia sclerotiorum 1980 UF-70]	2.21	8.74	1	1	1	1	183	19.6
119415495	RAD52 DNA repair protein RADC [Neosartorya fischeri NRRL 181]	2.05	2.83	1	1	1	1	565	60.8
119414334	hypothetical protein NFIA_038470 [Neosartorya fischeri NRRL 181]	2.71	6.83	1	1	1	1	322	36.6
119412832	sphingosine kinase (SphK), putative [Neosartorya fischeri NRRL 181]	2.05	2.88	2	1	1	1	486	52.5
119412820	calcium channel subunit Cch1 [Neosartorya fischeri NRRL 181]	2.14	0.42	1	1	1	1	2124	240.4
119406408	1,3-beta-glucanosyltransferase Gel2 [Neosartorya fischeri NRRL 181]	3.09	2.53	2	1	1	1	475	51.5
119404353	hypothetical protein ACLA_001400 [Aspergillus clavatus NRRL 1]	10.53	5.19	1	1	1	4	231	25.8
119413867	oxidoreductase [Neosartorya fischeri NRRL 181]	2.60	3.23	1	1	1	1	371	42.1
119411525	hypothetical protein NFIA_066320 [Neosartorya fischeri NRRL 181]	3.01	10.17	1	1	1	1	236	26.9
119407471	C6 zinc finger domain protein [Neosartorya fischeri NRRL 181]	2.80	12.99	1	1	1	1	177	20.4
119398060	ATP-dependent Clp protease, putative [Aspergillus clavatus NRRL 1]	2.88	2.61	1	1	1	1	613	66.8

114196282	predicted protein [Aspergillus terreus NIH2624]	2.58	2.76	1	1	1	1	762	83.7
114195918	predicted protein [Aspergillus terreus NIH2624]	2.23	3.22	1	1	1	1	435	48.8
114193610	conserved hypothetical protein [Aspergillus terreus NIH2624]	2.69	3.54	1	1	1	1	622	70.5
119397485	conserved hypothetical protein [Aspergillus clavatus NRRL 1]	2.65	3.12	1	1	1	1	577	65.4
119397053	cell wall proline rich protein, putative [Aspergillus clavatus NRRL 1]	3.23	2.49	1	1	1	1	923	99.4
114196349	conserved hypothetical protein [Aspergillus terreus NIH2624]	2.12	4.99	1	1	1	1	561	61.1
114190723	hypothetical protein ATEG_07039 [Aspergillus terreus NIH2624]	5.68	2.98	1	1	1	2	1075	120.5
114189921	predicted protein [Aspergillus terreus NIH2624]	2.12	0.73	1	1	1	1	2610	289.4
86196845	hypothetical protein MGCH7_ch7g890 [Magnaporthe oryzae 70-15]	1.64	2.89	2	1	1	1	381	43.1
86196797	hypothetical protein MGCH7_ch7g842 [Magnaporthe oryzae 70-15]	2.51	3.90	2	1	1	1	461	48.0
86196352	hypothetical protein MGCH7_ch7g397 [Magnaporthe oryzae 70-15]	2.16	1.03	2	1	1	1	1355	150.4
393234120	ARM repeat-containing protein [Auricularia delicata TFB-10046 SS5]	2.60	2.20	1	1	1	1	908	102.4
393229486	subtilisin-like protein [Auricularia delicata TFB-10046 SS5]	7.53	1.12	1	1	1	3	893	95.3
393221458	hypothetical protein FOMMEDRAFT_152281 [Fomitiporia mediterranea MF3/22]	2.55	5.84	1	1	1	1	291	33.1
393227786	alpha/beta-hydrolase [Auricularia delicata TFB-10046 SS5]	2.82	4.65	1	1	1	1	344	38.3
393227316	hypothetical protein AURDEDRAFT_188867 [Auricularia delicata TFB-10046 SS5]	2.07	2.27	1	1	1	1	1012	113.2
393223621	hypothetical protein AURDEDRAFT_178529 [Auricularia delicata TFB-10046 SS5]	2.61	8.76	1	1	1	1	217	25.1
393220819	HCP-like protein [Fomitiporia mediterranea	2.41	1.73	1	1	1	1	752	81.3

	MF3/22]								
351642517	hypothetical protein MGG_03268 [Magnaporthe oryzae 70-15]	2.10	6.04	1	1	1	1	265	28.4
351640818	chromodomain helicase DNA binding protein 1 [Magnaporthe oryzae 70-15]	2.10	0.91	1	1	1	1	1649	185.1
351647188	inner centromere protein [Magnaporthe oryzae 70-15]	2.88	1.38	1	1	1	1	1378	150.2
160705133	hypothetical protein SNOG_15358 [Phaeosphaeria nodorum SN15]	2.18	6.52	1	1	1	1	322	35.9
392864421	hypothetical protein CIMG_12902 [Coccidioides immitis RS]	2.23	6.95	1	1	1	1	187	20.6
392597957	hypothetical protein CONPUDRAFT_116467 [Coniophora puteana RWD-64-598 SS2]	2.64	6.52	1	1	1	1	368	43.2
392595538	cysteine desulfurase [Coniophora puteana RWD-64-598 SS2]	2.73	5.71	1	1	1	1	420	45.9
392593698	hypothetical protein CONPUDRAFT_52939 [Coniophora puteana RWD-64-598 SS2]	5.37	4.56	1	1	1	2	395	43.9
392590520	Aldo keto reductase [Coniophora puteana RWD-64-598 SS2]	4.77	4.75	1	1	1	2	337	37.3
392587469	hypothetical protein CONPUDRAFT_168555 [Coniophora puteana RWD-64-598 SS2]	2.05	2.20	1	1	1	1	500	56.6
392586661	NAD(P)-binding protein [Coniophora puteana RWD-64-598 SS2]	2.28	4.91	1	1	1	1	285	31.2
392597486	mRNA transport regulator [Coniophora puteana RWD-64-598 SS2]	2.16	1.39	1	1	1	1	932	102.9
392592858	tRNA modification GTPase TrmE [Coniophora puteana RWD-64-598 SS2]	3.20	4.35	1	1	1	1	552	60.4
392586797	hypothetical protein CONPUDRAFT_146671 [Coniophora puteana RWD-64-598 SS2]	2.06	0.88	1	1	1	1	1247	138.5
392578355	hypothetical protein TREMEDRAFT_60409 [Tremella mesenterica DSM 1558]	2.55	10.25	1	1	1	1	283	30.2
392567948	FAD/NAD-P-binding domain-containing protein	10.08	2.21	1	1	1	4	634	69.3

	[Trametes versicolor FP-101664 SS1]								
392567838	hypothetical protein TRAVEDRAFT_19515 [Trametes versicolor FP-101664 SS1]	2.66	3.56	1	1	1	1	477	51.7
49655010	DEHA2E00352p [Debaryomyces hansenii CBS767]	2.73	31.11	1	1	1	1	90	10.4
391869640	multidrug resistance-associated protein [Aspergillus oryzae 3.042]	2.51	1.66	2	1	1	1	1205	132.7
391868192	solute carrier protein [Aspergillus oryzae 3.042]	2.96	5.92	1	1	1	1	355	38.6
391870299	inositol polyphosphate multikinase [Aspergillus oryzae 3.042]	2.82	2.01	3	1	1	1	994	111.5
391863518	putative transporter [Aspergillus oryzae 3.042]	2.74	3.00	3	1	1	1	534	58.9
390597784	hypothetical protein PUNSTDRAFT_126991 [Punctularia strigosozonata HHB-11173 SS5]	2.12	3.33	1	1	1	1	450	50.1
390596503	WD40 repeat-like protein [Punctularia strigosozonata HHB-11173 SS5]	2.01	0.80	1	1	1	1	1756	192.5
390595452	glucoamylase [Punctularia strigosozonata HHB-11173 SS5]	2.55	3.79	1	1	1	1	581	61.2
390594179	RdRP-domain-containing protein [Punctularia strigosozonata HHB-11173 SS5]	2.30	2.06	1	1	1	1	973	110.5
390600647	Metallo-dependent phosphatase [Punctularia strigosozonata HHB-11173 SS5]	2.53	3.98	1	1	1	1	503	54.8
390599308	hypothetical protein PUNSTDRAFT_133889 [Punctularia strigosozonata HHB-11173 SS5]	2.78	2.97	1	1	1	1	1143	127.0
389739375	alpha/beta-hydrolase [Stereum hirsutum FP-91666 SS1]	2.04	1.82	1	1	1	1	1155	126.0
389738766	hypothetical protein STEHIDRAFT_163216 [Stereum hirsutum FP-91666 SS1]	2.74	17.50	1	1	1	1	120	12.5
389749352	ATP-dependent DNA helicase [Stereum hirsutum FP-91666 SS1]	4.72	2.24	1	1	1	2	669	74.7
389743707	hypothetical protein STEHIDRAFT_122829 [Stereum hirsutum FP-91666 SS1]	2.51	1.64	1	1	1	1	1279	138.1
388857684	uncharacterized protein [Ustilago hordei]	2.35	1.96	1	1	1	1	767	82.7

388856278	related to RRP9-protein associated with the U3 small nucleolar RNA [Ustilago hordei]	2.70	2.71	1	1	1	1	702	75.7
388858089	related to WD40 protein Ciao1 [Ustilago hordei]	2.70	3.50	1	1	1	1	457	49.4
388858000	uncharacterized protein [Ustilago hordei]	2.61	3.14	1	1	1	1	573	66.0
388856295	related to cyclin dependent kinase C [Ustilago hordei]	2.73	1.39	1	1	1	1	1148	123.6
388583656	hypothetical protein WALSEDRAFT_55860 [Wallemia sebi CBS 633.66]	8.86	2.00	1	1	1	3	649	72.9
388582418	SIR2-domain-containing protein [Wallemia sebi CBS 633.66]	2.08	2.15	1	1	1	1	651	72.3
388580829	hypothetical protein WALSEDRAFT_60595 [Wallemia sebi CBS 633.66]	2.65	1.15	1	1	1	1	1222	139.7
388583474	NAD(P)-binding protein [Wallemia sebi CBS 633.66]	2.80	4.70	1	1	1	1	362	40.4
388583340	kinase-like protein [Wallemia sebi CBS 633.66]	2.53	4.00	1	1	1	1	725	80.4
388580234	Dbl-like domain-containing protein [Wallemia sebi CBS 633.66]	2.75	1.67	1	1	1	1	1260	144.0
317460807	conserved hypothetical protein [Cryptococcus gattii WM276]	2.15	0.71	1	1	1	1	1826	199.9
317460494	Gamma-glutamyltranspeptidase 1 precursor, putative [Cryptococcus gattii WM276]	4.80	2.56	1	1	1	2	741	79.8
240134244	vacuolar protein sorting-associated protein 21 [Candida tropicalis MYA-3404]	2.95	10.73	1	1	1	1	205	22.3
240132724	conserved hypothetical protein [Candida tropicalis MYA-3404]	2.71	12.05	1	1	1	1	249	26.4
220699788	spindle pole body component, putative [Aspergillus flavus NRRL3357]	2.55	3.43	2	1	1	1	903	97.5
220699492	conserved hypothetical protein [Aspergillus flavus NRRL3357]	2.91	3.24	3	1	1	1	740	83.3
212003447	isoleucyl-tRNA synthetase [Schizosaccharomyces japonicus yFS275]	4.32	1.33	1	1	1	2	981	111.4
211999487	meiosis protein mei2 [Schizosaccharomyces	14.10	3.02	1	1	1	5	729	79.4

	japonicus yFS275]								
190408541	conserved hypothetical protein [Saccharomyces cerevisiae RM11-1a]	2.32	6.30	4	1	1	1	349	38.2
384498570	hypothetical protein RO3G_13772 [Rhizopus delemar RA 99-880]	2.59	1.45	1	1	1	1	1383	157.0
238851525	hypothetical protein CLUG_05116 [Clavispora lusitaniae ATCC 42720]	2.52	6.85	1	1	1	1	219	25.5
225561598	peroxin 11C [Ajellomyces capsulatus G186AR]	2.26	6.41	1	1	1	1	359	39.5
225558752	conserved hypothetical protein [Ajellomyces capsulatus G186AR]	5.38	8.63	1	1	1	2	197	21.6
150412647	peptide methionine sulfoxide reductase msrA [Ajellomyces capsulatus NAm1]	2.05	4.89	1	1	1	1	225	25.5
146452879	predicted protein [Lodderomyces elongisporus NRRL YB-4239]	2.41	1.15	1	1	1	1	1304	149.7
146451281	hypothetical protein LELG_03716 [Lodderomyces elongisporus NRRL YB-4239]	2.68	3.11	1	1	1	1	578	66.5
387514838	hypothetical protein TBLA_0G03370 [Tetrapisispora blattae CBS 6284]	2.57	2.69	1	1	1	1	1039	119.0
343771189	hypothetical protein NDAI_0J02790 [Naumovozyma dairenensis CBS 421]	2.05	2.76	1	1	1	1	471	52.9
342301567	hypothetical protein NCAS_0C03470 [Naumovozyma castellii CBS 4309]	2.67	3.37	1	1	1	1	563	62.3
199424882	YALI0A13233p [Yarrowia lipolytica CLIB122]	2.15	0.80	1	1	1	1	1372	142.0
385305724	spindle-like protein [Dekkera bruxellensis AWRI1499]	2.74	5.94	1	1	1	1	320	36.5
385302581	hypothetical protein AWRI1499_3418 [Dekkera bruxellensis AWRI1499]	2.59	5.40	1	1	1	1	315	35.5
385301814	hypothetical protein AWRI1499_4148 [Dekkera bruxellensis AWRI1499]	2.79	11.64	1	1	1	1	146	16.7
385301153	actin-related protein [Dekkera bruxellensis AWRI1499]	2.91	8.14	1	1	1	1	381	42.7
385303582	atp-binding cassette sub-family f member 2	2.70	4.50	1	1	1	1	556	62.6

	[Dekkera bruxellensis AWRI1499]								
385303251	hypothetical protein AWRI1499_2764 [Dekkera bruxellensis AWRI1499]	5.89	3.22	1	1	1	3	435	47.4
49649804	YALI0E14234p [Yarrowia lipolytica CLIB122]	2.52	0.92	1	1	1	1	1637	181.1
49647384	YALI0C06446p [Yarrowia lipolytica CLIB122]	2.11	2.77	1	1	1	1	541	59.8
49644037	KLLA0E21055p [Kluyveromyces lactis]	3.13	13.03	1	1	1	1	261	25.7
49641801	KLLA0C10505p [Kluyveromyces lactis]	2.69	3.78	1	1	1	1	582	64.3
199425291	YALI0C16709p [Yarrowia lipolytica CLIB122]	2.29	0.95	1	1	1	1	1364	152.0
49646473	YALI0B07513p [Yarrowia lipolytica CLIB122]	2.58	3.10	1	1	1	1	807	91.0
49646362	YALI0B04466p [Yarrowia lipolytica CLIB122]	2.25	7.35	1	1	1	1	204	23.0
49644282	KLLA0F01463p [Kluyveromyces lactis]	2.13	2.43	1	1	1	1	781	89.2
49643658	KLLA0E12717p [Kluyveromyces lactis]	2.63	2.24	1	1	1	1	446	51.3
380483371	mRNA-capping enzyme subunit beta [Colletotrichum higginsianum]	2.55	3.00	1	1	1	1	600	67.2
380480593	Pro-kumamolisin, partial [Colletotrichum higginsianum]	3.48	3.25	1	1	1	1	615	66.9
380493677	cytochrome P450 [Colletotrichum higginsianum]	2.69	4.16	1	1	1	1	529	59.3
380491345	nucleolar GTP-binding protein 1 [Colletotrichum higginsianum]	2.56	2.74	1	1	1	1	658	75.3
380487753	F-box protein pof6 [Colletotrichum higginsianum]	2.87	9.97	1	1	1	1	291	32.9
380483501	hypothetical protein CH063_02385 [Colletotrichum higginsianum]	7.32	2.55	1	1	1	3	549	62.5
380473153	hypothetical protein CH063_15178 [Colletotrichum higginsianum]	2.54	14.61	1	1	1	1	89	9.7
380091455	unnamed protein product [Sordaria macrospora k-hell]	2.05	10.91	1	1	1	1	165	17.0
202953213	DEHA2F12100p [Debaryomyces hansenii CBS767]	2.13	4.38	1	1	1	1	320	36.8
238935857	KLTH0F06182p [Lachancea thermotolerans CBS 6340]	17.61	4.38	1	1	1	7	320	35.2
378756463	hypothetical protein NERG_00127 [Nematocida	2.62	22.22	1	1	1	1	99	11.8

	sp. 1 ERTm2]								
378754744	hypothetical protein NERG_02176 [Nematocida sp. 1 ERTm2]	21.91	1.03	1	1	2	8	2925	339.8
378754490	hypothetical protein NERG_02331 [Nematocida sp. 1 ERTm2]	8.08	2.30	1	1	1	4	740	82.1
378756376	hypothetical protein NERG_00040 [Nematocida sp. 1 ERTm2]	2.82	5.06	1	1	1	1	474	53.1
358376728	ankyrin repeat protein [Aspergillus kawachii IFO 4308]	2.86	1.82	3	1	1	1	713	81.1
358368841	cell polarity protein [Aspergillus kawachii IFO 4308]	2.90	1.97	1	1	1	1	915	101.4
359384527	Piso0_000675 [Millerozyma farinosa CBS 7064]	34.12	1.33	1	1	1	13	903	102.9
353242732	hypothetical protein PIIN_08302 [Piriformospora indica DSM 11827]	25.80	2.46	1	1	1	13	488	52.3
353239774	related to pyridoxine 4-dehydrogenase [Piriformospora indica DSM 11827]	9.08	3.87	1	1	1	4	284	32.0
353237027	hypothetical protein PIIN_02868 [Piriformospora indica DSM 11827]	2.01	2.78	1	1	1	1	324	34.7
353235165	probable SAC1-recessive suppressor of secretory defect [Piriformospora indica DSM 11827]	5.45	1.97	1	1	1	2	661	75.1
353242228	hypothetical protein PIIN_07842 [Piriformospora indica DSM 11827]	2.54	2.93	1	1	1	1	615	66.2
353241116	related to alpha-1,3-mannosyltransferase [Piriformospora indica DSM 11827]	2.43	1.52	1	1	1	1	527	60.3
353227370	hypothetical protein PIIN_00525 [Piriformospora indica DSM 11827]	2.72	2.51	1	1	1	1	1197	134.1
372124511	Tsr1 [Penicillium citrinum]	2.87	11.27	6	1	1	1	204	22.7
372121198	RNA polymerase II largest subunit [Penicillium italicum]	2.51	4.72	1	1	1	1	318	35.0
284799168	RNA polymerase II second largest subunit, partial [Saccharata proteae]	2.57	3.85	1	1	1	1	390	44.0
367017822	hypothetical protein TDEL_0H03390	2.46	4.44	1	1	1	1	383	44.6

	[Torulaspora delbrueckii]								
367017944	hypothetical protein TDEL_0H04000 [Torulaspora delbrueckii]	2.04	0.72	3	1	1	1	1665	186.2
367011343	hypothetical protein TDEL_0C00720 [Torulaspora delbrueckii]	2.97	2.03	1	1	1	2	1037	118.2
367002516	hypothetical protein TPHA_0F00720 [Tetrapisispora phaffii CBS 4417]	4.46	0.86	1	1	1	2	1283	148.0
361127473	hypothetical protein M7I_4740 [Glarea lozoyensis 74030]	8.34	8.61	1	1	1	3	302	32.7
361127404	putative Phthiocerol synthesis polyketide synthase type I PpsC [Glarea lozoyensis 74030]	2.02	0.75	1	1	1	1	2136	232.7
361126897	putative Oxidoreductase ucpA [Glarea lozoyensis 74030]	10.68	5.00	1	1	1	4	300	32.7
361125628	putative Glycerophosphodiester phosphodiesterase GDE1 [Glarea lozoyensis 74030]	2.53	1.71	2	1	1	1	997	110.4
358377880	hypothetical protein TRIVIDRAFT_211134 [Trichoderma virens Gv29-8]	2.82	2.95	1	1	1	1	576	63.1
358057047	hypothetical protein E5Q_03628 [Mixia osmundae IAM 14324]	2.63	7.02	1	1	1	1	299	31.2
358030862	translation elongation factor 2, partial [Gonapodya sp. JEL183]	40.82	2.68	1	1	1	14	597	66.5
354544733	hypothetical protein CPAR2_800100 [Candida parapsilosis]	2.67	2.98	2	1	1	1	738	82.2
325071333	nonreducing polyketide synthase, partial [Talaromyces pinophilus]	2.78	11.49	3	1	1	1	235	25.2
350634963	hypothetical protein ASPNIDRAFT_37334 [Aspergillus niger ATCC 1015]	2.60	4.21	2	1	1	1	570	63.5
347841073	hypothetical protein [Botryotinia fuckeliana]	2.90	3.95	1	1	1	1	557	62.9
347831388	similar to PAP2 domain-containing protein [Botryotinia fuckeliana]	2.09	7.54	1	1	1	1	305	34.3
347829875	hypothetical protein [Botryotinia fuckeliana]	2.51	5.48	2	1	1	1	383	42.6

345567919	hypothetical protein AOL_s00054g907 [Arthrobotrys oligospora ATCC 24927]	2.55	6.11	1	1	1	1	229	24.8
345567135	hypothetical protein AOL_s00076g422 [Arthrobotrys oligospora ATCC 24927]	2.81	3.70	1	1	1	1	432	49.0
343428734	related to long chain fatty alcohol oxidase [Sporisorium reilianum SRZ2]	2.52	4.67	1	1	1	1	771	82.6
343426524	related to Glioma tumor suppressor candidate region gene 2 protein [Sporisorium reilianum SRZ2]	2.81	3.33	1	1	1	1	451	50.4
343425088	probable gamma-glutamylcysteine synthetase [Sporisorium reilianum SRZ2]	2.91	3.25	1	1	1	1	707	79.4
342874503	hypothetical protein FOXB_12957 [Fusarium oxysporum Fo5176]	5.07	2.65	1	1	1	2	680	74.8
328350784	putative secreted protein [Komagataella pastoris CBS 7435]	2.65	22.67	1	1	1	1	150	17.4
328352264	SWI5-dependent HO expression protein 4 [Komagataella pastoris CBS 7435]	2.00	1.92	1	1	1	1	729	82.9
328351308	Topoisomerase 1-associated factor 1 [Komagataella pastoris CBS 7435]	2.55	1.69	1	1	1	1	1185	136.0
328351147	Uncharacterized protein yetA [Komagataella pastoris CBS 7435]	2.62	1.37	1	1	1	1	873	98.7
328350103	Transcription factor tau 138 kDa subunit [Komagataella pastoris CBS 7435]	2.04	0.90	1	1	1	1	1225	139.5
126212874	NAD-dependent malate dehydrogenase [Scheffersomyces stipitis CBS 6054]	3.24	7.12	1	1	1	1	337	35.9
317144458	tubulin-specific chaperone [Aspergillus oryzae RIB40]	2.72	3.12	3	1	1	1	609	68.2
317143045	DNA repair protein (Rad57) [Aspergillus oryzae RIB40]	2.81	3.67	2	1	1	1	600	65.1
317137884	hypothetical protein AOR_1_1938194 [Aspergillus oryzae RIB40]	2.52	3.14	1	1	1	1	477	52.2
145250995	glutamate decarboxylase 1 [Aspergillus niger	2.73	3.34	2	1	1	1	509	57.7

	CBS 513.88]								
145237550	hypothetical protein ANI_1_420064 [Aspergillus niger CBS 513.88]	11.13	2.86	1	1	1	4	560	61.0
134055132	unnamed protein product [Aspergillus niger]	25.30	5.41	1	1	1	8	314	34.6
323346802	Sla2p [Saccharomyces cerevisiae Lalvin QA23]	2.52	2.18	1	1	1	1	780	88.3
323355226	YFL042C-like protein [Saccharomyces cerevisiae VL3]	2.21	2.41	11	1	1	1	582	66.3
323347766	Mbr1p [Saccharomyces cerevisiae Lalvin QA23]	2.77	14.19	1	1	1	1	155	17.0
323335977	Cla4p [Saccharomyces cerevisiae Vin13]	2.60	2.44	1	1	1	1	697	78.0
317033144	hypothetical protein ANI_1_2502094 [Aspergillus niger CBS 513.88]	2.79	6.40	3	1	1	2	297	31.4
259481223	TPA: alpha/beta hydrolase, putative (AFU_orthologue; AFUA_6G11570) [Aspergillus nidulans FGSC A4]	2.81	4.04	2	1	1	1	545	60.6
320581742	Putative benzil reductase [Ogataea parapolymorpha DL-1]	2.78	9.52	1	1	1	1	252	27.3
320580900	GABA-specific high-affinity permease [Ogataea parapolymorpha DL-1]	2.87	2.74	1	1	1	1	584	63.3
320580712	heat shock transcription factor, putative [Ogataea parapolymorpha DL-1]	2.50	4.22	1	1	1	1	474	53.2
62632793	PRP8 protein precursor [Emericella nidulans]	2.69	4.85	3	1	1	1	618	69.4
153917328	pro-pol protein [Lentinula edodes]	2.14	1.10	1	1	1	1	1274	146.4
46135973	hypothetical protein FG09502.1 [Gibberella zeae PH-1]	2.01	2.24	2	1	1	1	402	44.3
303309969	SNF2 family N-terminal domain containing protein [Coccidioides posadasii C735 delta SOWgp]	2.20	1.13	2	1	1	1	1061	120.0
303320941	outer membrane protein, OMP85 family protein [Coccidioides posadasii C735 delta SOWgp]	2.65	2.71	3	1	1	1	516	56.0
302664294	CorA family metal ion transporter, putative [Trichophyton verrucosum HKI 0517]	2.60	3.04	4	1	1	1	658	73.3
302659557	conserved hypothetical protein [Trichophyton	5.96	4.68	6	1	1	2	278	32.4

	verrucosum HKI 0517]								
302656199	hypothetical protein TRV_06053 [Trichophyton verrucosum HKI 0517]	2.55	3.46	2	1	1	1	405	44.7
302662135	hypothetical protein TRV_03149 [Trichophyton verrucosum HKI 0517]	2.24	1.91	8	1	1	1	680	73.9
302658633	filamentation protein (Rhf1), putative [Trichophyton verrucosum HKI 0517]	2.60	0.87	1	1	1	1	1733	191.8
302500236	hypothetical protein ARB_01620 [Arthroderma benhamiae CBS 112371]	2.04	4.48	4	1	1	1	335	37.1
302503871	hypothetical protein ARB_08007 [Arthroderma benhamiae CBS 112371]	2.06	1.30	4	1	1	1	770	86.3
302497848	hypothetical protein ARB_02820 [Arthroderma benhamiae CBS 112371]	2.07	1.39	5	1	1	1	935	102.9
50288073	hypothetical protein [Candida glabrata CBS 138]	2.58	2.27	1	1	1	1	883	100.2
146422469	hypothetical protein PGUG_00549 [Meyerozyma guilliermondii ATCC 6260]	2.93	4.43	2	1	1	1	497	57.0
146419574	hypothetical protein PGUG_01419 [Meyerozyma guilliermondii ATCC 6260]	2.81	1.75	2	1	1	1	1145	126.9
299754782	hypothetical protein CC1G_02771 [Coprinopsis cinerea okayama7#130]	2.58	3.69	1	1	1	1	406	46.4
299754218	alginate lyase [Coprinopsis cinerea okayama7#130]	2.06	5.73	1	1	1	1	506	52.7
299753991	DNA mismatch repair protein MSH3 [Coprinopsis cinerea okayama7#130]	2.84	1.28	1	1	1	1	1096	121.7
299747178	GTPase [Coprinopsis cinerea okayama7#130]	2.20	1.24	1	1	1	1	1211	132.0
299746509	hypothetical protein CC1G_07524 [Coprinopsis cinerea okayama7#130]	2.01	1.41	4	1	1	1	922	101.9
170941313	unnamed protein product [Podospora anserina S mat+]	2.60	1.17	1	1	1	1	1363	148.5
170940443	unnamed protein product [Podospora anserina S mat+]	2.60	8.64	1	1	1	1	405	44.7
170937555	unnamed protein product [Podospora anserina S	2.97	8.71	1	1	1	1	264	29.5

	mat+]								
25294143	Fum19p [Gibberella moniliformis]	2.42	1.01	1	1	1	1	1489	165.0
29421288	kinesin [Gibberella moniliformis]	2.09	1.20	4	1	1	1	1087	120.8
238941410	ZYRO0G12144p [Zygosaccharomyces rouxii]	2.55	3.95	1	1	1	1	456	47.8
223643511	pre-rRNA processing protein, putative [Candida dubliniensis CD36]	2.05	3.37	1	1	1	1	326	36.7
223641306	fumarylacetoacetate (FAA) hydrolase, putative [Candida dubliniensis CD36]	2.14	4.76	3	1	1	1	231	25.7
223643592	DNA topoisomerase 2, putative [Candida dubliniensis CD36]	2.87	1.44	3	1	1	1	1461	165.3
223642655	carnitine acetyltransferase, putative [Candida dubliniensis CD36]	2.95	1.35	1	1	1	1	887	100.9
223641952	quinone oxidoreductase, putative [Candida dubliniensis CD36]	2.60	6.57	2	1	1	1	335	37.0
223641634	protein she4, putative [Candida dubliniensis CD36]	2.53	3.10	1	1	1	1	775	88.5
223640627	(FAD-dependent) oxidoreductase, putative [Candida dubliniensis CD36]	2.07	4.25	1	1	1	1	400	44.5
270120970	unnamed protein product [Penicillium chrysogenum]	2.53	6.67	1	1	1	1	525	58.9
269860857	ABC-type multidrug transport system, ATPase and permease component [Enterocytozoon bieneusi H348]	2.10	1.63	1	1	1	1	552	63.6
269860302	hypothetical protein EBI_26083 [Enterocytozoon bieneusi H348]	2.42	6.72	1	1	1	1	119	14.3
269860003	hypothetical protein EBI_22774 [Enterocytozoon bieneusi H348]	2.63	12.28	1	1	1	1	171	19.9
255955041	Pc21g12430 [Penicillium chrysogenum Wisconsin 54-1255]	2.01	2.12	1	1	1	1	709	79.4
255951838	Pc24g00250 [Penicillium chrysogenum Wisconsin 54-1255]	2.88	5.93	2	1	1	1	236	25.9
255934802	Pc12g16300 [Penicillium chrysogenum	2.53	9.56	1	1	1	1	272	28.9

	Wisconsin 54-1255]								
254567069	DNA repair protein RAD50 [Komagataella pastoris GS115]	5.56	1.36	2	1	1	2	1323	152.0
254566629	hypothetical protein [Komagataella pastoris GS115]	2.52	14.29	2	1	1	1	189	21.5
68488704	hypothetical LPF family protein 1 [Candida albicans SC5314]	2.53	15.84	1	1	1	1	101	12.0
169806415	protein with WD40 repeat [Enterocytozoon bieneusi H348]	2.82	2.52	1	1	1	1	834	96.3
164655417	hypothetical protein MGL_4005 [Malassezia globosa CBS 7966]	2.80	1.76	1	1	1	1	1248	138.7
154288987	ubiquitin carboxyl-terminal hydrolase [Botryotinia fuckeliana B05.10]	2.01	4.84	1	1	1	1	310	34.0
85099370	hypothetical protein NCU01260 [Neurospora crassa OR74A]	2.52	0.61	1	1	1	1	2307	257.2
170674512	hypothetical secreted protein [Epichloe festucae]	8.79	4.71	1	1	1	3	531	59.8
134116156	hypothetical protein CNBJ0280 [Cryptococcus neoformans var. neoformans B-3501A]	2.67	7.63	1	1	1	1	367	41.3
154316360	predicted protein [Botryotinia fuckeliana B05.10]	2.09	4.46	2	1	1	1	404	42.1
154293357	hypothetical protein BC1G_13701 [Botryotinia fuckeliana B05.10]	2.61	3.83	2	1	1	1	575	64.9
599968	Ssm4p [Saccharomyces cerevisiae]	2.11	2.81	12	1	1	1	392	44.9
20086301	subunit of RNA polymerase II transcription factor TFIIH [Candida glabrata]	2.85	7.69	2	1	1	1	234	27.9
85690967	hypothetical protein ECU01_0490 [Encephalitozoon cuniculi GB-M1]	2.67	7.20	1	1	1	1	236	26.4
71003686	hypothetical protein UM00362.1 [Ustilago maydis 521]	2.64	4.97	1	1	1	1	463	51.6
32693853	ED24 elicitor protein [Cercospora zeae-maydis]	2.58	4.80	1	1	1	1	271	29.7
2130983	acetyl CoA carboxylase [Schizosaccharomyces pombe]	2.08	11.88	1	1	1	1	101	11.6