INTRODUCTION

1.0 Introduction

Hypertension or high blood pressure is a silent disease which can lead to many complications (e.g. cardiovascular diseases, stroke and kidney diseases) and death if it is not treated. Hypertension commonly refers to high blood pressure when the pressure of artery is too high. It is classified into two classes: essential hypertension and secondary hypertension. Essential hypertension is hypertension that is not caused by secondary conditions such as those that affect kidneys, arteries, heart or endocrine systems. On the other hand, secondary hypertension is hypertension that is caused by the secondary conditions. According to the National Health and Morbidity Survey II, the prevalence of this illness in Malaysia is 24% higher compared to cancer, cardiac related disease and diabetes mellitus. Based on Asia-Pacific Cohort Studies, Alexandra Martiniuk suggested that two out of three heart attacks and strokes occurred in Asia are caused by hypertension (Martiniuk *et al.*, 2007). Furthermore, at least 970 million people worldwide have elevated blood pressure; with about 330 million in developed countries and around 640 million in developing countries. The World Health Organization rates this disease as one of the important cause of premature death worldwide and this problem keeps on increasing through time. It is estimated about 1.56 billion adults will live with high blood pressure in 2025 (Kearney *et al.*, 2005).

Due to the rapid increase in the number of people with hypertension at present and prospected in the future; several preventive strategies are being carried out worldwide to control this disease. Hypertension is a complex disease which could originate via a simple mechanism or a combination of various mechanisms. Due to this reason, anti-hypertensive drugs that have been commercialized are normally designed for a specific mechanism such as diuretic drug, beta-blocker drug, calcium channels drug, Angiotensin Converting Enzyme (ACE) inhibitors and many more. Education in schools, rural places, hospitals and through multimedia is some of the ways taken by

certain countries to control this disease. Promotions on a healthy lifestyle help to control and prevent essential hypertension. Besides, intake of a balanced diet, supplements and herbs or natural products together with frequent exercise and a better lifestyle may help to reduce or control this disease.

Due to the emergence of several reported side-effects of synthetic drugs usage, patients start to seek for some alternative solutions to help them reduce or control their hypertension. In recent times, many researchers explore the advantages of using natural products for human consumptions and try to evaluate their effects on several diseases including diabetes mellitus, cancer as well as hypertension. Examples of natural products derived from foods, herbs and fruits that have been studied for the treatment of hypertension include onion, garlic, grape seed, hibiscus, European mistletoe (*Viscum album*), Indian snakeroot (*Rauwolfia serpentina*), olive leaf, reishi and other mushrooms, hawthorn leaf and flower extract, *Coleus forkoshlii*, ginsengs and many more. *Apocynum venetum* Luobuma leaves and *Lepidium sativum* L were reported to exhibit antihypertensive effects in Spontaneously Hypertensive Rats (SHR) upon daily administration (Kim *et al.*, 2000; Maghrani *et al.*, 2005). *Allium ursinum, Amelanchier ovalis, Cistus clusii* and *Lespedezae capitata* showed active potential as ACE inhibitor in an *in vitro* screening of ACE activity inhibition using HPLC (Wagner *et al.*, 1991). Interestingly, flavanoids, procyanidins and peptides were isolated in the study with anti – ACE potential (Elbl & Wagner, 1991; Wagner & Elbl, 1992).

Thus far, none of the studies on natural product medicinal plants reported the effect of *Ficus deltoidea* Jack towards hypertension. *F. deltoidea* is a houseplant or a shrub that belongs to family of Moraceae (Brickell & Zuk, 1997). The synonym is *Ficus diversifolia* Blume (Barley & Barley, 1976). In Malaysia, this plant is widely known as Mas Cotek or Secotek Emas that is referring to the golden spots on the upper surface of its leaves. According to traditional practitioners, this plant has been used to treat various

health conditions for example as treatment after childbirth and birth control. It is also used to improve blood circulation, reduce blood cholesterol, control high blood pressure and others. However, these claims still lack of scientific evidence to support them. Other than these claims, several local researchers have discovered the potential of this plant in lowering blood glucose level (Adam *et al.*, 2009; Aminudin *et al.*, 2007), as an antinociceptive agent (Sulaiman *et al*., 2008), as anti-oxidant (Abdullah *et al*., 2009; Hakiman & Maziah, 2009) and as anti-ulcer agent (Siti-Fatimah-Zahra *et al.*, 2009).

This present study was conducted to evaluate the effect of *F. deltoidea* aqueous extracts in reducing hypertension. Experiments were conducted using SHR rats. Rat sera were collected and analyzed using proteomics techniques. A preliminary study on ACE inhibitory activity was done to determine the potential of this plant in having anti-hypertensive property. Captopril (a synthetic ACE inhibitor) was used as the positive control.

Proteomics is a large scale proteins study particularly on its structures and functions (Anderson & Anderson, 1998; Blackstock & Weir, 1999). In this study, SDS-PAGE was used initially to monitor the changes in serum protein profile with reference to their molecular weights. Proteins that were altered (in terms of their expressions) following the treatment with *F. deltoidea* extracts were further separated using two-dimensional gel electrophoresis (2-DE) analysis. 2-DE separates serum proteins based on its isoelectric point (pI) and molecular weight (MW) (Klose, 1975; O'Farrell, 1975). Image Master Platinum 7.0 Software (GE Healthcare Biosciences), a bioinformatics tool was used to analyse the protein spots on 2-DE gels. This determined significant changes in expression of the serum protein(s). Proteins that exerted significant changes in their expression were identified using Matrix Assisted Laser Desorption / Ionisation – Time of Flight/ Time of Flight (MALDI-TOF/TOF), a high throughput mass spectrometry technology.

The complexity of serum protein with the presence of highly abundant proteins such as serum albumin and immunoglobin renders difficulties in detecting the differentially expressed protein of low abundance as well as those with low MW (Merrell *et al*., 2004). Thus, to monitor the low MW serum protein that maybe affected by *F. deltoidea* extracts, Surface Enhanced Laser Desorption / Ionisation- Time of Flight (SELDI-TOF) was used to complement the investigation (Hutchens & Yip, 1993; Papale *et al.*, 2008).

The use of proteomics tools in this study will help to achieve the aim of identifying the protein(s) expression affected by the administration of *F. deltoidea* extracts *in vivo*. This in turn will give further insight(s) of the possible mechanism(s) involved.

The objectives of this study are;

- To investigate the effects of *F. deltoidea* towards Angiotensin Converting Enzyme activity.
- To monitor the alterations of serum proteins expression in SHR rats following administration with *F. deltoidea* extracts.
- To identify serum proteins that may potentially involved in hypertension.
- To monitor the effects of *F. deltoidea* aqueous extracts on SHR rats' serum low molecular weight proteins expression.

LITERATURE REVIEW

2.0 Literature Review

2.1 *Ficus deltoidea*

Ficus deltoidea is a small plant derived from Moraceae family locally known as Mas Cotek among the Malays especially in Kelantan. This plant also known as Secotek Emas, Telinga Beruk, Telinga Gajah, Serapat Angin and other names based on its varieties. Detailed morphological studies to differentiate the varieties have not yet been reported. The name Mas Cotek refers to the presence of golden spots on the upper leaf surface. In the Malay language, golden spots can be directly translated as Mas or Emas (gold) and cotek (spot).

F. deltoidea can be classified into 'male' and 'female' varieties. However, this classification is not associated with the plants' gender but rather a classification based on morphological characteristics. The male variety has smaller leaves (SL) with red spots on the underside whereas the leaves of the female variety are bigger in size and have black spots. The female variety can be further divided based on the size of the leaves; plant with medium type leaves (ML) and large type leaves (BL). Fruits borne by the male variety are smaller (SF) compared to female (Figure 2.1). *F. deltoidea* can easily grow as epiphytes and can abundantly be found on the beaches of Kelantan and Terengganu. However, due to public awareness towards the extinction of natural product resources recently, this plant is now grown in many plantations in Malaysia. These plantations cater to the increasing demand by the consumer due to its reported benefits. *F. deltoidea* has been claimed to provide many health benefits. This could be due to the presence of phytochemicals such as phenols, proanthocyannins, triterpenoids, tannins and flavanoids.

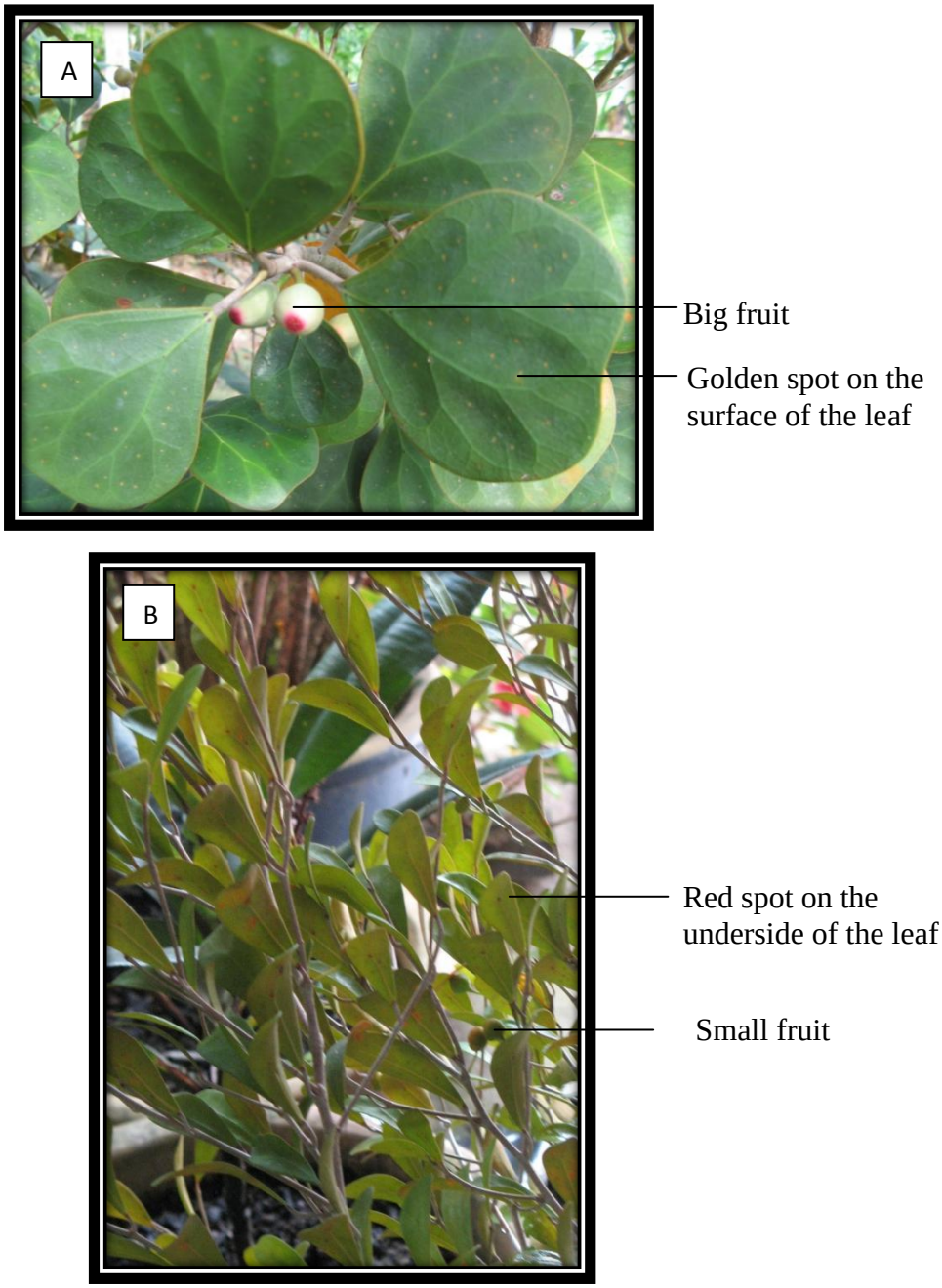


Figure 2.1: The characteristics of *F. deltoidea*.

(A) *F. deltoidea* plant from the big leaf variety depicting fruits and leaves. Golden spots can be seen on the upper surface of the leaves.

(B) *F. deltoidea* plant from small leaf variety showing the leaf and fruit. Note the red spot on the underside of the leaf.

2.1.1 Benefits of *F. deltoidea*

Traditionally, several types of herbal plants found in Malaysia have been used as remedy to treat various ailments. Examples include *Gynura procumbens* (Akar Sebiak) as an anti-hypertensive agent, *Pereskia sacharosa* (Pokok Jarum Tujuh Bilah) as an anti-cancer agent, *Eurycoma longifolia* (Tongkat Ali) as an aphrodisiac for men and *Labisia pumila* (Kacip Fatimah) for general health in women. According to traditional practices, *F. deltoidea* has been used as a decoction or tea to treat fever, increase and recover sexual desire, reduce cholesterol, remove toxins and improve blood circulation. Interestingly, *F. deltoidea* can be used by both sexes unlike *E. longifolia* which is used by men to increase virility or *L. pumila* which is used by women to facilitate childbirth.

Research on the benefits of this plant is generating great interest among scientists. This is in part due to positive results demonstrated by the consumers in the traditional use of this plant. Hakiman and Mahmood (2009) reported that the aqueous extract of *F. deltoidea* possesses positive non-enzymatic and enzymatic antioxidant activities based on 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, ferric reducing power assay, peroxidase activity, ascorbate oxidase activity and catalase activity. Sulaiman *et. al.* (2008) reported that *F. deltoidea* aqueous extract showed positive antinociceptive activity. Aminudin *et. al.* (2007) reported that both leaves and fruits extracts of *F. deltoidea* significantly reduced the external glucose load at a dosage of 50 mg/kg body weight in rats. According to Adam *et. al.* (2007), 1000 mg/kg of *F. deltoidea* aqueous extract exhibited hypoglycaemic activity in post-prandial mild diabetic rats. *F. deltoidea* aqueous extract have been shown to exhibit positive effects towards uterine contraction which could be a possible explanation for the utilization of this extract during post-partum or after childbirth (unpublished data).

There is a concern among consumers regarding the toxicity of herbal plants. Therefore, researches have been performed to investigate the toxicity effects of these plants. Fazliana *et. al.* (2008*)* demonstrated that *F. deltoidea var. Augustifolia* aqueous extract showed no toxic effect on bone marrow, liver and renal functions following its administration to rats at 100 and 300 mg/kg body weight daily for 90 days.

Our previous study showed that different varieties and plant parts of *F. deltoidea* produced good inhibitory response towards Angiotensin Converting Enzyme (ACE) (unpublished data). The study showed that the aqueous extracts of the fruit (from the small variety plant) and the leaves (from the big variety plant) of *F. deltoidea* could be potential candidates as anti-hypertensive agents.

2.2 Hypertension

Hypertension can be defined as sustained elevation of blood pressure in arteries that may cause organ damage and other adverse consequences. When blood pressure in arteries is increased, this will caused the heart to pump blood at higher pressure and forcing the blood to push against the vessels' wall. Such conditions may cause organ damage (heart or kidney failure) and diseases such as cardiovascular disease (CVD) (Carretero & Oparil, 2000). Organ damage is more severe with the development of high systolic blood pressure (SBP) compared to high diastolic blood pressure (DBP) (Cutler, 1996). Individuals with higher blood pressure have a greater tendency to develop heart attack, stroke and kidney disease. The relationship between CVD and blood pressure is continuous, consistent and independent (Chobanian *et al.*, 2003).

Hypertension is commonly divided into two groups; essential (primary) and secondary hypertension. Primary, essential or idiopathic hypertension is defined as hypertension with no identifiable cause. Secondary hypertension is hypertension caused by other medical problem such as kidney failure, hormones, diseases and tumour. Hypertension caused by the side effect from certain medication is also considered as secondary hypertension. About 90-95% of patients with high blood pressure belongs to the essential hypertension group (Carretero & Oparil, 2000). However, this theory is only partially accepted as little information is available on the genetic variation and intermediary phenotypes that may cause high blood pressure (Luft, 1998).

There are several environmental factors which could increase blood pressure that include obesity, insulin resistance, high alcohol intake, high salt intake (in salt-sensitive patients), ageing and possibly a sedentary lifestyle, stress, low potassium intake and low calcium intake (INTERSALT-Co-operative-research-group, 1988; Sever & Poulter, 1989). Based on these studies, ageing could also increase the risk of hypertension. Half

of individuals aged from 60 to 69 years old may develop hypertension whilst the majority of individuals with aged above 70 years old have higher probability to develop hypertension.

However, the interaction between environmental and genetic factors influence the intermediary phenotypes and hence, may contribute to higher blood pressure (Carretero & Oparil, 2000). Therefore, people with essential hypertension have high risk to develop hypertension-related diseases if not treated. Changes in lifestyle, including reducing body weight, fat and alcohol intake, increasing potassium and calcium intake (Appel *et al*., 1997) together with exercise may reduce or normalize blood pressure in patients (Krotkiewski *et al*., 1979; Petrella, 1998).

2.2.1 Classification of hypertension

In the 7th Report of the Joint National Committee (JNC 7) on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (2003) hypertension in adults aged 18 and above can be classified as shown in Table 2.1.

Table 2.1: Classification of hypertension in adults reported in JNC 7.

BP classification	SBP mm Hg	DBP mm Hg
Normal	< 120	and < 80
Pre-hypertension	120-139	or 80-89
Stage 1 hypertension	140-159	or 90-99
Stage 2 hypertension	≥ 160	or ≥ 100

Source: 7th Report of the Joint National Committee (JNC 7) as reported by The National High Blood Pressure Education Program.

Pre-hypertension is not considered as disease but it is a term referring to individuals with high risk of developing hypertension. Thus, both patient and clinicians will be alerted and encouraged preventive measures to be undertaken. This might help to avoid or delay the disease from developing. Individuals with pre-hypertension are not suitable candidates for drug therapy but are advised to practise lifestyle modification (Chobanian, *et al.*, 2003). However, for those with diabetes and kidney disease, they need to be put on an appropriate drug therapy if the lifestyle modification fails to reduce their BP to 130/80mmHg or less. As for the individuals with hypertension (stage 1 and 2), the treatment goal is to lower their BP to less than 140/90 mmHg.

2.2.2 Diagnosis of Hypertension

2.2.2.1 Blood pressure (BP) Measurement

Clinically, patients were diagnosed based on the systolic and diastolic blood pressure levels using a device called sphygmomanometer. Systolic blood pressure (SBP) reading is the maximum pressure of blood when the heart contracts. Diastolic blood pressure (DBP) on the other hand is the minimum pressure in artery when heart relaxes. Generally, the normal blood pressure is 120/80 mmHg by where 120 is the systolic and 80 is the diastolic. Any measurement between 120/80 mmHg to 139/89 mmHg is considered pre-hypertension. In this stage, a person has the potential to develop hypertension if BP is not controlled. Nevertheless, significant blood pressure measurement of more than 140/90 mmHg is considered hypertension (Table 2.1).

In hypertension, monitoring of SBP is more important compared to DBP since clinical trials have demonstrated that controlled SBP reduces total mortality, cardiovascular mortality, stroke and heart failure in older people (Staessen *et al.*, 1999; Kostis *et al.*, 1997).

2.2.2.2 Cortisol level

Monitoring of the cortisol level in serum or urine has been suggested to be useful in the screening of the potential to develop hypertension and CVD in normotensive person. Cortisol is usually released in the body due to fasting, food intake, exercising, awakening and physiological stressors (Wallerius *et al.*, 2003;Vicennati, 2002; Ely, 1995; McEwen, 1980).

Cortisol is a stress hormone by which its level in the body increases in response to stress. During normal stress response, the pituitary gland increases its release

of adrenocorticotropic hormone (ACTH). This hormone will then trigger the synthesis of cortisol from adrenal gland (adrenal cortex) to regulate blood pressure. Thus, in continuous stress, the cortisol will be synthesized higher than usual. This is normally observed once a person awake (Filipovsky, 1996) or when an individual is experiencing physical and emotional stress.

Study suggested that excess of cortisol is associated with the increase of BP and altered the intermediary metabolism, which results in obesity, insulin resistance and abnormal lipid metabolism (Connell *et al*., 1986). They have also reported that free cortisol excretion rates in urine are higher in hypertensive subject. Therefore, it is important to monitor the level of cortisol for patients with high cortisol level but normal BP as a precaution step to prevent or delay the occurrence of hypertension and / or CVD.

The following list represents the normal range cortisol level in blood and urine.

Blood

- adults (8 am) : 6-28 mg/dL
- adults (4 pm) : 2-12 mg/dL
- child one to six years (8 am) : 3-21 mg/dL
- child one to six years (4 pm) : 3-10 mg/dL
- newborn : 1-24 mg/dL.

Urine

- adult : 10-100 mg/24 hours
- adolescent : 5-55 mg/24 hours
- Child : 2-27 mg/24 hours.

2.2.3 Medication to treat hypertension

There are several types of medication available to lower high blood pressure and it may be necessary to take more than one type of medication to control the blood pressure effectively. However, the combination depends on the physiological condition of the patient. For this reason, it is important for the patient to take the medication properly as directed. Otherwise, the medicine may not work, or it could also cause side effects. Degree of the side effects varies, ranging from mild to severe.

Drugs used as high blood pressure medication can be divided into eight common groups which include:

- Diuretic
- Beta blockers
- ACE (Angiotensin Converting Enzyme) inhibitors
- Angiotensin II receptor blockers
- Calcium channel blockers
- Alpha blockers
- Vasodilators
- Central Acting Adregenic Agents / Central Alpha Agonists

Among those listed, diuretics, beta blockers and ACE inhibitors are the commonly used drugs in treating patients with hypertension.

2.2.3.1 Diuretic medications for hypertension

Diuretics are drugs known as 'water pills' due to its effects in assisting the flushing out of excess water and sodium in the body through urination. In addition to high blood pressure, diuretics are often used to treat other illnesses including abnormal heart function, oedema and sometimes poisoning. The target organs of this drug are kidneys. Different diuretics work on different part of the kidney. There are three classes of diuretics used to treat hypertension: (1) thiazides, (2) loop-active agents and (3) potassium-sparing agents. These agents act as either mineralcorticoid antagonists or inhibitors of the epithelial sodium channel located at late distal renal tubule or collecting duct.

Thiazide diuretics such as hydrocholorothiazide and benzoflumethiazide could be used as monotheraphy or in combination with β-blockers, ACE inhibitors or angiotensin receptor blockers (Krakoff, 2005) . However, there are still arguments regarding the use of thiazide-type diuretics as the initial treatment for all hypertensive. Study from the Systolic Hypertension in the Elderly Program (SHEP) provided evidence that emphasizes a low-dose thiazide-type drug as initial therapy for isolated systolic hypertension in older patients (Kostis *et al.*, 2005). Moreover, the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT) strongly supports the use of this drug as the best choice for African-American hypertension (Wright *et al.*, 2005).

Loop-active agents, furosemide and its analogs (bumetanide or torsemide) interrupt re-absorption of sodium, calcium and potassium in the distal renal tubule at the ascending limb of the Loop of Henle at sites distinct from the thiazide-sensitive loci. These loop-active agents have a short duration of action and for treatment of hypertension, must be given twice daily (Krakoff, 2005).

Potassium-sparing diuretics are mineralcorticoid antagonists and sodium channel antagonists known as spironolactone, eplerenone, amiloride and triamterene. Spironolactone can be highly effective in many patients with refractory hypertension in combination with a thiazide-type diuretic and can correct hypokalemia as well. However, gynecomastia is a limiting adverse reaction for men treated with spironolactone because of the antiandrogenic effect of this drug. Premenopausal women treated with spironolactone may develop menstrual irregularities, so spironolactone is most likely to have sustained acceptance only by postmenopausal women. Eplerenone should be considered an alternative for those who have a good clinical response to spironolactone but develop unacceptable adverse reactions. Amiloride and triamterene are also customarily used drugs that inhibit the epithelial sodium transport channel of the collecting duct (Krakoff, 2005).

Usage of diuretics may provide side effects to the consumer. After prolonged use of diuretics, sudden withdrawal can reduce body's potassium level which leads to weakness, fatigue and leg cramps. The less common side effect that affect long term diuretics intake is gout. Besides, some of these hypertension drugs can increase blood glucose levels.

2.2.3.2 Beta blockers medication for hypertension

Beta blockers are drugs used to block the effects of the sympathetic nervous system on the heart. It will reduce body's requirement for blood and oxygen and thus reducing the workload of the heart. As a result, the heart doesn't have to work as hard, which in turn lowers BP. They help to control heart beat rate and are also used in the treatment of abnormal heart rhythms that may too fast or irregular.

Examples of beta blockers include:

- acebutolol
- atenolol
- betaxolol
- labetalol
- bisoprolol
- cardevilol
- esmolol
- metoprolol
- propanolol
- nevibolol

There are three known types of beta receptors, designated as β_1, β_2 and β_3. β_1-adrenergic receptors are located mainly in the heart and kidneys. β_2- adrenergic receptors are located mainly in the lungs, gastrointestinal tract, liver, uterus, vascular smooth muscle and skeletal muscle while β_3 receptors in fat cells. The use of β-blockers as first line therapy for hypertension has been widely conceptual for a long time and many debates have continued ever since.

Wink (2001) stated that a combination of β-blockers and diuretics is more efficient in preventing morbidity and mortality in elderly patients with hypertension. Kahn and McAlister (2006) concluded that in trials comparing other antihypertensive medication with β-blockers, all agents showed similar efficiency in younger patients, while in older patients; β-blockers were associated with a higher risk of both composite events and strokes. Therefore, patients over 60 years old with ischemic heart disease or heart failure should still be prescribed β-blockers for heart failure and angina. Also, in

older patients with hypertension who need multiple agents to control their blood pressure, a β-blocker could be added as a third or fourth agent in addition to a diuretic, ACE inhibitor, Angiotensin II receptor blocker or calcium-channel blocker. Metoprolol is a good choice, even though it is expensive but it has been proven to reduce mortality in patients with a history of myocardial infarction or heart failure.

The side effects of β-blocker consumption include insomnia, tiredness, extreme cold, depression and symptoms of asthma. Moreover, consumption of β-blocker drugs in a diabetic person undergoing insulin treatment should be monitored.

2.3.3.3 Angiotensin Converting Enzyme (ACE) Inhibitors for hypertension

ACE inhibitors trigger the production of vasodilators by inhibiting the formation of angiotensin II. This vasoconstrictor is formed by the proteolytic action of renin (released by the kidneys) acting on circulating angiotensinogen to form angiotensin I. Angiotensin I is then converted to angiotensin II by ACE. This system is called the renin-angiotensin system (Figure 2.2).

ACE also breaks down bradykinin (a vasodilator substance). Therefore, ACE inhibitors, by blocking the breakdown of bradykinin help to increase bradykinin levels. The increased bradikinin level can contribute to Nitric Oxide (NO) activation resulting in the relaxation of vascular smooth muscle (vasodilation) thus lowering BP. Increase in bradykinin is also believed to be responsible for a troublesome side effect of ACE inhibitors, namely, dry cough.

Angiotensin II constricts arteries and veins by binding to Angiotensin II receptor type 1 (AT_1) located on the smooth muscle, which are coupled to a Gq-protein and the inositol trisphosphate (IP_3) signal transduction pathway. Angiotensin II also facilitates

the release of norepinephrine from sympathetic adrenergic nerves and inhibits norepinephrine reuptake by these nerves. This effect of angiotensin II augments sympathetic activity on the heart and blood vessels.

The first ACE inhibitor marketed, captopril, is still in widespread use today. Although newer ACE inhibitors differ from captopril in terms of pharmacokinetics and metabolism, all the ACE inhibitors have similar overall effects on blocking the formation of angiotensin II. ACE inhibitors include benazepril, enalapril, fosinopril, moexipril, quinapril and ramipril.

ACE inhibitors actions normally will dilate the arteries and veins by blocking angiotensin II formation and inhibiting bradykinin metabolism. This vasodilation reduces arterial pressure, preload and afterload on the heart. Furthermore, it down regulates sympathetic adrenergic activity by blocking the facilitating effects of angiotensin II on sympathetic nerve release and reuptake of norepinephrine. ACE inhibitors also promote renal excretion of sodium and water (natriuretic and diuretic effects) by blocking the effects of angiotensin II in the kidney and also by blocking angiotensin II stimulation of aldosterone secretion. This reduces blood volume, venous pressure and arterial pressure. It also inhibits cardiac and vascular remodelling associated with chronic hypertension, heart failure and myocardial infarction.

Elevated plasma renin is not required for the actions of ACE inhibitors, although ACE inhibitors are more efficacious when circulating levels of renin are elevated. Renin-angiotensin system is distributed in many tissues, including heart, brain, vascular and renal tissues. Therefore, ACE inhibitors may act at these sites in addition to blocking the conversion of angiotensin in the circulating plasma. Cough and angiodema is the side effect of ACE inhibitor. If administered in the second or third trimester of pregnancy, ACE inhibitors can cause a number of fetal anomalies including

aligohydramnios, fetal calvarial hypoplasia, fetal pulmonary hypoplasia, fetal growth retardation, fetal death, neonatal anuria and neonatal death (DiBianco, 1986; Pryde *et al.*, 1993). For this reason, ACE inhibitors should be used with caution in women of child-bearing potential.

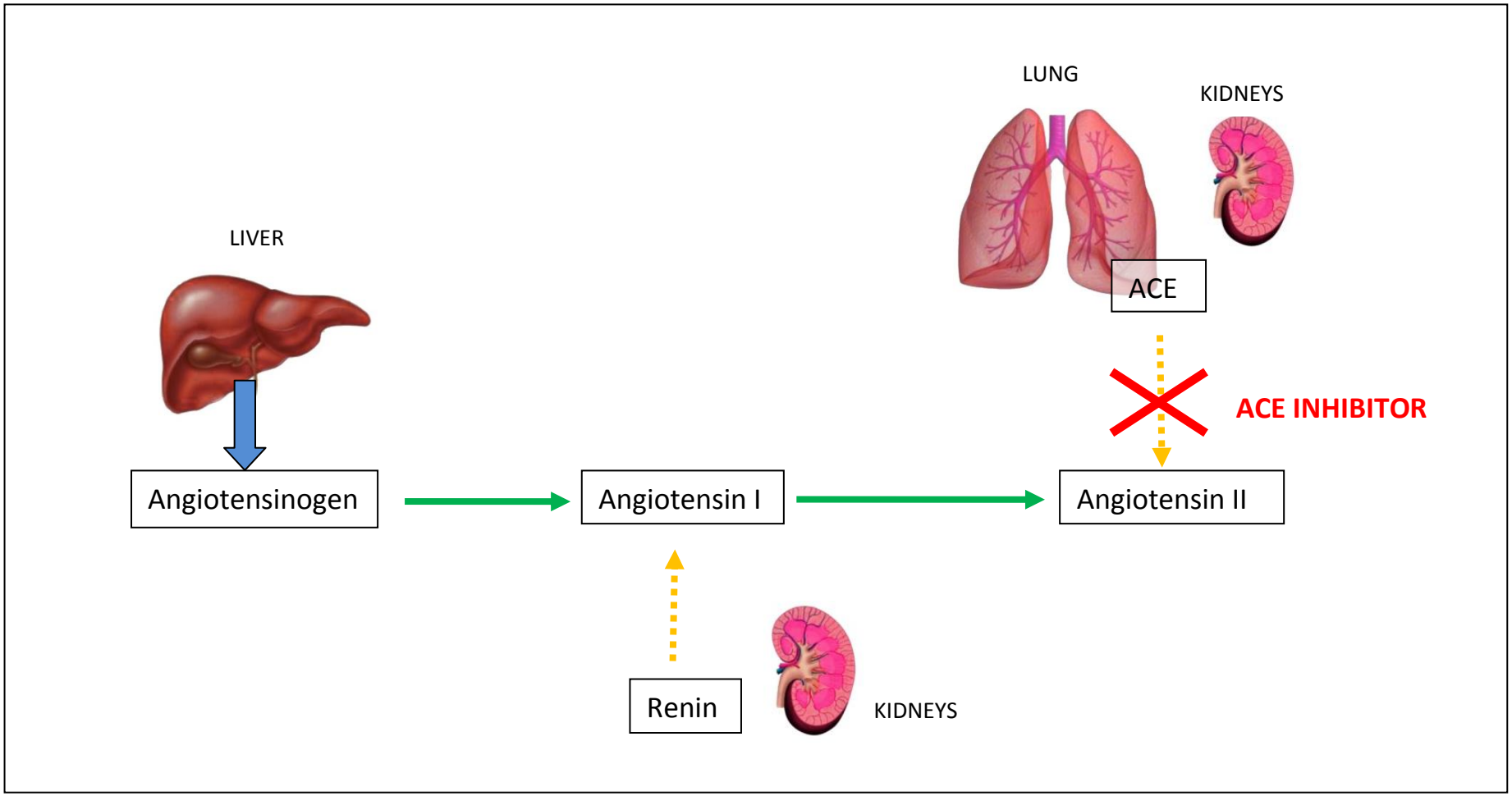


Figure 2.2: Renin-angiotensin system. This system involves various organs such as liver, pulmonary and kidneys in its circulation. ACE is released by pulmonary and kidneys as Angiotensin I produced from the synthesis of Angiotensinogen in the liver. The proteolysis of Angiotensinogen to Angiotensin I are activated by renin released by the kidneys.

2.3.3.4 Other anti-hypertensive drugs.

Table 2.2: Other types of anti-hypertensive drugs.

Type	Mechanism of action & Side effect(s)	Drugs Name
Angiotensin II receptor blockers	**Mechanism** Protect blood vessels from the angiotensin II hormone. Instead of lowering hormone levels, these drugs keep the hormone from interacting with blood vessels. As a result, the vessels become more dilated and blood pressure goes down. **Side effect** Occasional dizziness.	losartan, candesartan, irbesartan, eprosartan, valsartan, telmisartan
Calcium channel blockers (calcium antagonist)	**Mechanism** This type of anti-hypertensive drug keeps calcium from moving into the muscle cells and causing constriction of blood vessels. As a result, blood vessels relax and blood pressure is lower. **Side effect(s)** Swollen ankles, heart palpitations, constipation, or dizziness.	Nifedipine, bepridil, verapamil, diltiazem, amlodipine, nimodipine, felodipin, nisoldipine

Table 2.2 (continued): Other types of anti-hypertensive drugs.

Alpha blockers	**Mechanism** Alpha blockers reduce another sympathetic nerve impulse to blood vessels, which permits easier passage for blood, thus reducing pressure. **Side effect(s)** Quicker heart rate, dizziness, and a sudden drop in blood pressure when standing.	Doxazosin mesylate, Prazosin hydrochloride, Prazosin and polythiazide, Terazosin hydrochloride,
Vasodilators	**Mechanism** Vasodilators are an older class of drugs that work to open blood vessels by relaxing vessel walls, resulting in lower blood pressure. **Side effect(s)** Headaches, heart palpitations, swelling around the eyes, as well as achy joints. Loniten (minoxidil) can cause excess hair growth and weight gain.	Hydralazine hydrochloride, Minoxidil

Table 2.2 (continued): Other types of anti-hypertensive drugs

Central Acting Adrenergic Agents / Central Alpha Agonists	**Mechanism** Prevents the brain from sending signals to the heart and blood vessels, which slows the heart and prevents blood vessels from constricting. **Side effect(s)** Sudden drop in blood pressure especially when the patient stands or walks, which can lead to weakness or fainting. Other possible side effects are drowsiness, dry mouth, fever, constipation, and anaemia. Men might experience impotence.	clonidine hydrochloride, methyldopa and hydrochlorothiazide, guanabenz acetate, methyldopa, clonidine hydrochloride and chlorthalidone, methyldopa and chlorothiazide, guanfacine hydrochloride

A number of hypertension medications have been commercialised and prescribed to patients. However, most of the patients do not succeed in keeping their blood pressure under control. This is because the medications were prescribed with wrong combinations. Physicians often choose the drugs by trial and error and monitor which combination work best.

Three guidelines for drug prescription in hypertensive patients have been proposed. Firstly, it has been suggested that the effects of prescribed drugs are different in different ethnic groups (Gupta *et al*., 2010). Patient's age and race can also be used as a guideline for second line treatments. Other studies involved renin levels. Turner and his group (2010) found that the level of renin can be an effective method for selecting blood pressure medication. They also stated that patients with higher renin levels should not be prescribed with a diuretic because they responded better to beta-blockers. Alderman (2010) suggested that patients with low renin levels should be prescribed with diuretic drugs rather than anti-renin agents and vice versa.

2.2.4 Natural product medication

Due to the side effects of synthetic drug usage, many researches on foods, animals and plants are attempting to identify compounds that can be utilized in controlling hypertension. A common herb which is usually used in daily cooking that is known to have anti-hypertensive effect is garlic (*Allium sativum*).

It has been shown that garlic has the potential to treat mild or moderate hypertension at a dosage of 600-900 mg garlic powder per day for twelve weeks (Silagy & Neil, 1994a). This study was conducted on 415 patients and it was found that there was a typical drop of 7.7 mmHg systolic and 5 mmHg diastolic BP. However, higher doses of garlic may be more effective as McMahon and Vargas (1993) demonstrated that a dosage of 2400 mg caused a drop of 16 mmHg drop in DBP after only five hours of administration. Besides its effects on BP, garlic also have positive effect in the lowering of cholesterol level and prevention of atherosclerosis (Silagy & Neil, 1994b).

An example of natural product from animal origin that has potential to treat blood pressure is Sea Cucumber or known as *gamat* in Malaysia. Sea Cucumber is usually used by the Chinese to nourish blood, moisturize dryness-syndrome, regulate menstruation, help in recovery after child delivery, as well as a tonic for the kidney. It is also used in treating ulcers, dysentery, impotence, arthritis pain, weakness and recovery from illness. It also helps to regulate blood lipid and blood pressure, promote blood circulation as this animal has been claimed to contain Omega 3 and 6. However, no scientific experimental data is available.

The International Collaborative Study of Macronutrients and Blood Pressure (INTERMAP) reported that addition of vegetables in daily diets also helps in significantly reducing BP. In the report, they found that high BP of patients with higher consumption of vegetable amino acid together with animal amino acids (cysteine,

proline, phenylalanine and serine) has lower BP compared to others. This could be due to the presence of glutamic acid in vegetables.

Recently, many scientific studies on the effect of natural products have been done worldwide. The effect of natural products as an anti-hypertensive is commonly screened using preliminary *in vitro* ACE inhibitory activity assay. An example of natural product that has been evaluated using this assay is a tuber storage protein from yam (*Dioscorea alata* cv. Tainong No. 1) known as dioscorin. The IC_{50} of dioscorin using FAPGG as substrate was 6.404 µM dioscorin. (Hsu *et al.*, 2002). The ethyl acetate extract of *Rabdosia coetsa,* a medicinal plant from China also showed ACE inhibitory activity. Three isolated compounds from the extract, ethyl caffeate, rosmarinic acid and methyl rosmarinate exhibited 32.42%, 55.19% and 39.50% ACE inhibition respectively at the concentration of 10 µg/ml using HHL as the substrate (Li *et al*., 2008).

Other plants that have potential to inhibit ACE are Brazillian Strawberry (Pinto *et al*., 2008), rapeseed (Pedroche *et al*., 2004), *Undaria pinnatifida* or known as wakame or seaweed (Suetsuna *et al.*, 2004), milled whole grain (Nogata *et al.*, 2009) and green tea (Persson *et al.*, 2010) and many more.

Study on hypertension using SHR is widely used to monitor the anti-hypertensive effect *in vivo.* SBP observation of SHR supplemented with ethanol extracts of pink guava (*Psidium guajava*) within four weeks exhibited reduction of BP (Ayub *et al.*, 2010). Cinnamon extract also helps in reducing blood pressure in SHR which received diet containing starch and sucrose (Preuss *et al.*, 2006). In China, Xin Jiang red raspberry fruit ethyl acetate extract showed dose-dependent antihypertensive effect through nitric oxide activation and improved vascular endothelial dysfunction (Jia *et al.*, 2011).

2.3 Proteomics

Proteomics is a large-scale *in vivo* protein study which involves a variety of high-throughput techniques and new technology. The term was first coined by Peter James in 1997 (James, 1997). Proteomics is a study of the proteome. The word proteome was first introduced by Wilkins (Wasinger *et al.,* 1995) where the word proteome comes from proteins (prot) and genome (ome). Wilkins described proteome study as a study of the entire complement of proteins expressed by the genome, cells, tissue or organism. However, a study of the proteome is more complex than the genome. This is because it involves knowledge of protein structure and functional interaction between proteins instead of only looking at the sequences. The study of the proteome also includes the understanding of how the proteins are involved in pathways.

Anderson and Anderson (1998) stated in their article that the aim of proteomics is to explain "a comprehensive, quantitative description of protein expression and its changes under the influence of biological perturbations such as disease or drug treatment." Normally, the objectives of proteomics research are to discover the protein expressions, quantification, protein identification and proteins with related-function in a proteome (Abdullah-Soheimi *et al.,* 2010; Amacher *et al.,* 2005; Piubelli *et al.,* 2005).

A standard gel-based proteomics research workflow involves; (1) separating the proteins through two dimensional gel electrophoresis (2-DE), (2) visualisation using Coommasie blue staining or silver staining, (3) quantification of the protein spots using gel image analysis and (4) identification of the proteins using mass spectrometry (MS).

2.3.1 Sodium dodecyl sulphate polyacrilamide gel electrophoresis

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a protein fractionation technique first introduced by Laemmli (1970) using Tris-base buffer. Laemmli developed this technique by combining two established methods; development of PAGE (Davis, 1964; Ornstein, 1964) and the importance of SDS addition to PAGE (Shapiro *et al.*, 1967). SDS, a dissociating agent will denature the native proteins to individual polypeptides. Approximately 1.4g of SDS will wrap around 1 g of polypeptides backbone and once this mixture was heated at 100°C it will produce negatively charge polypeptides. The negatively charged polypeptides will move towards the anode in electrophoresis through the SDS polyacrylamide gel with the aid of tris-base buffer (PAGE). Beta-mercaptoethanol and dithiothreitol (DTT) were used as dissociating agents to disrupt disulphide bonds of native proteins. This technique have been popularly cited by many researcher as it can be used to determine the MW of separated proteins based on its relative mobility (Weber & Osborn, 1969). SDS-PAGE has been used in proteomics as the preliminary study to examine the range of protein MW and to determine the presence of interfering substances such as salt in extracts or sample before proceeding to isoelectric focusing (IEF) system (Cilia *et al.*, 2009).

2.3.2 Two dimensional gel electrophoresis (2-DE)

Two-dimensional gel electrophoresis (2-DE) was first introduced in 1975 by Patrick H O-Farrell (1975) and J. Klose (1975). It is a proteomics tool that is capable of resolving up to thousands of proteins in one biological sample. The term 'two dimension' comes from the separation of proteins on the basis of its isoelectric point (pI) by isoelectric focusing (IEF) and its MW by SDS-PAGE. Originally, the first dimension was performed in carrier ampholyte-containing polyacrylamide gels cast in narrow tubes. However, in the late 1980s, A. Görg and colleagues established a new technique in IEF whereby they introduced immobilised pH gradients (IPG) strips (Görg *et al.*, 2000; Görg *et al.*, 1987; Görg *et al.*, 1978).

In IEF, the protein sample was first solubilised and disaggregated in rehydration buffer containing urea or thiourea, zwitterionic detergent such as CHAPS, carrier such as IPG buffer, denaturing agent; DTT and a tracking dye. Prior to IEF, to avoid aggregation and intermolecular interaction protein samples must be ensured to be fully denatured and soluble. Urea acts as a denaturant to solubilise and unfold the proteins, and hence expose the ionisable group to the solution. Zwitterionic agent, CHAPS, is more effective compared to other agents (NP-40 or Triton X-100). It has similar function to urea but it prevents aggregation through hydrophobic interactions. Carrier ampholytes such as IPG buffer is used to increase the solubility of protein by reducing aggregation via charge to charge interactions. DTT, a denaturing agent, allows cleaving of the disulphide bond and maintains the reduced state of proteins. Reduced protein samples will be loaded onto IPG gel strips either by using in-gel or cup-loading methods.

In IEF or first dimension step, proteins are separated based on its pI either to the positive or negative ends of the IPG strips. Proteins with a positive net charge will move

towards the cathode until it reaches its pI. Negatively charged proteins will move towards the anode until it reaches its pI. This is the focusing part where all proteins are separated on the basis of its pIs and this allows separation of proteins even when the difference of charge is small. The focusing requires high voltage for better resolution.

Following the 1st dimension, the strips will be equilibrated. This step ensures all proteins are saturated with SDS for SDS-PAGE separation. SDS buffer containing Tris-base, urea, glycerol, reductant, SDS, and dye will maintain the solubility of the protein and improve protein transfer into the second dimension gel. Replacement of iodoacetamide with reductant in additional equilibration buffer prevents streaking and artifacts during electrophoresis. The concept of electrophoresis in second-dimension follows the principle of SDS-PAGE with the exception of a stacking gel. Equilibrated IPG strips on the second-dimension gel will be sealed with agarose to prevent the strip from floating during electrophoresis. This allows the focused proteins to penetrate into the second dimension gel.

Thousands of proteins will be separated in the second dimension gel. Staining procedure is required for visualisation of proteins. Depending to the ease of use, sensitivity and compatibility to mass spectrometry (MS), different procedures can be utilized.

Coomassie Brilliant Blue (CBB) is a common and traditional visualization technique. It was first introduced by Volker Neuhoff in 1985. The dye was named after an African City, Kumasi. This procedure has become a favourite in SDS-PAGE protein visualization due to the ease of use and its compatibility with MS analysis. There are two types of CBB; R-250 and G-250. The latter is less sensitive compared to the former. CBB binds to proteins through ionic interactions between sulfonic acid groups and positive protein amine groups through Van der Waals interaction. However, this

procedure has limited sensitivity where the minimum detection limit is in the range of 200 – 500 ng proteins (Wilson, 1979). However, there are several reasons why this method is still widely used; (1) Low cost- where only a few chemicals are required compared to other staining procedures. (2) CBB staining is visible with eyes when the entire protein-dye complexes are formed. (3) No specific scanner is required. (4) Allows quantitative analysis. (5) Easy handling and less time consumption. (6) Compatible with MS analysis without any modification in the staining procedure (Westermeier, 2006).

Silver staining is often preferred due to its high sensitivity (minimum detection limit of up to 1 ng protein) (Ocbs *et al.*, 1981; Shevchenko *et al.*, 1996). Originally, this procedure is not compatible with MS analysis due to the incorporation of aldehyde. Aldehyde groups modify the lysine residue which forms complex with silver which is the fundamental principle of this procedure. The modification that promotes silver-protein complex formation reduces the digestion of peptides by proteolytic enzyme and thus reduces the efficiency of peptide extraction. In order to overcome this problem, Shevchenko *et al.* (1996) suggested replacing aldehyde with sodium thiosulfate.

2.3.3 Protein identification by Matrix Assisted Laser Desorption/Ionisation - Time of Flight/ Time of Flight (MALDI-TOF/TOF)

Matrix Assisted Laser Desorption/Ionization (MALDI), a laser based soft ionisation technique introduced in 1988. It is a powerful technique used to examine large ionic molecules such as peptides and proteins through mass spectrometry (Tanaka *et al.*, 1988; Karas *et al.*, 1987). Time of Flight (TOF) mass spectrometry is commonly coupled with MALDI as the analyser to analyse ions. However, for better resolution and sensitivity in proteomics study, a next generation tandem mass spectrometry (MS/MS) technique has been developed for MALDI where the element of TOF/TOF is introduced. In TOF/TOF, two modes of MS operation are utilised. In the first MS mode, the sample is hit by a laser beam. Matrix will facilitate and accelerate vaporisation and ionization of the sample. Common matrices such as 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), α-cyano-4-hydroxycinnamic acid (alpha-cyano or alpha-matrix) and 2,5-dihydroxybenzoic acid (DHB) will co-crystallize with the protein sample and protect the sample from the laser beam. The resulting ions will be analyzed based on its mass to charge ratio, m/z. In the second MS mode, collision-induced dissociation (CID) is introduced where some of the ionised molecules is broken into smaller fragments and detected by the mass spectrometer.

MALDI-TOF/TOF is a proteomics technique used in identification of proteins called mass fingerprint or peptide mass mapping (Medzihradszky *et al*., 2000). MALDI mass fingerprint typically performed on 2-DE gel spots (e.g. 2DE-coomasie blue, 2DE-silver staining, 2DE-DIGE and etc). Due to complexity of some biological sample with numerous proteins, separation of proteins to individual spots is required (Blackstock & Weir, 1999). This is then followed by the excision of protein spot of interest from the gel. The particular protein spots were then cleaved into smaller piece of peptides using a proteolytic enzyme, trypsin (Quadroni & James, 1999). Peptides were then extracted

from the gel and contaminants were removed using chromatographic column such as C-18. This cleaning-up procedure helps to improve the spectra in mass spectrometry (Yates, 2000). The protein identification using mass spectrometry is achieved by comparing the mass of the protein sample with the mass fingerprint of peptides derived from the databases (MASCOT from Matrix-Science or ProFound from Proteometrics).

2.3.4 Surface-Enhanced Laser Desorption/Ionization-Time of Flight (SELDI-TOF)

Surface-Enhanced Laser Desorption/Ionization (SELDI) original concept was first described by Hutchens and Yip (1992). SELDI-TOF is a high-throughput technique particularly used to investigate low molecular weight proteins (<20 kDa) up to femtomole sensitivity (Papale *et al.*, 2008). This technique is one of the easiest techniques to handle due to its ability to examine native proteins with less laborious sample preparation required. Only a small quantity of sample (about 1-10 μg protein per spot) is required in running SELDI-TOF compared to 2-DE. Besides that, SELDI-TOF provides a better resolution for proteins with extreme low MW, hydrophobicity and pI which is often limited in other proteomics technique such as 2-DE (Seibert *et al.*, 2004).

The principle of SELDI-TOF that combines chromatography with mass spectrometry allows unwanted proteins to be separated from the sample mixture. This is the easier way to identify biomarker in complex biological specimen which depends on the targeted characteristics of the proteins. This technique allows the discovery of several biomarkers from biological sample and this will facilitate diagnosis of diseases (Li *et al.*, 2002; Jr *et al.*, 1999).

SELDI-TOF is a fully automated machine completes with robotic handling system such as Auto-Loader-equipped ProteinChip Reader, barcode-labelled arrays and ChiphergenExpress™ Data Manager Software. Therefore, multiple samples (up to 100) can be prepared and analysed in a short period. Results will be presented as spectra which exhibited signal intensity in correlation with protein concentration. Analysis of spectra using Expression Difference Mapping application can be directly performed once data has been obtained from the ProteinChip Reader. This machine also provides another software - Biomarker Pattern Software - to observe the expression of proteins and protein profiles. These informations can be used for biomarker discovery (Seibert, *et al.*, 2004).

The components of SELDI-TOF are (1) protein chip arrays (2) mass analyser and (3) data analysis software (Issaq *et al.*, 2003). Protein chip arrays are the important components that differentiate SELDI-TOF from other MS. Two types of chromatography are available i.e. chemical surface (hydrophobic, hydrophilic, ionic and immobilized metal affinity capture [IMAC]) and biochemical surface (antibody, DNA, enzyme, receptor and drug) (Figure 2.3). The common surfaces include CM10, H50, IMAC30 and Q10. CM10 is a protein array coated with weak anionic carboxylate group which functions as a weak cation exchanger. This array captures positively charged analytes such as lysine, arginine and histidine. Q10 functions in an opposite manner to CM10. It captures molecules with positive charge such as aspartate and glutamate due to the presence of cationic groups (quaternary ammonium group) on the array. In order to separate large proteins/peptides, H50 can be used. It allows the separation of proteins based on hydrophobicity. IMAC30 is used to analyse metal-binding proteins, phosphorylated proteins and polyhistidine-tagged proteins. This array is coated with the nitrilotriacetic acid (NTA) group which will chelates metal ions in the presence of histidine, tryptophan and cysteine. These components provide different

chemistry surfaces designated to retain proteins with specific characteristics. The entire bound proteins/peptides will then mix and crystallize with matrix such as alpha-cyano-4-hydroxycinnamic acid (CHCA) or sinapinic acid (SPA) to facilitate ionization of the sample.

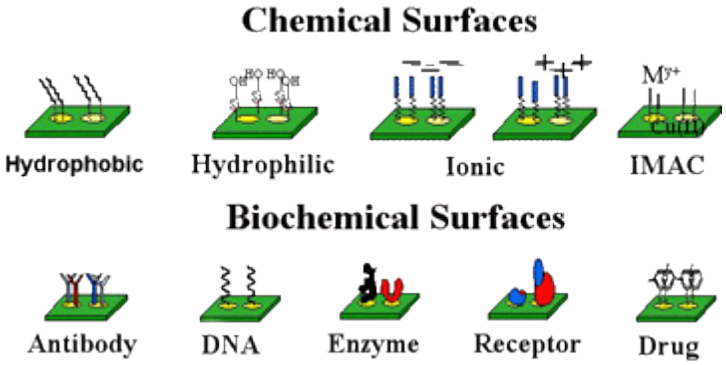


Figure 2.3: Protein arrays surfaces. Figure was extracted from Ciphergen.

The second component in SELDI-TOF, the mass analyser, shares the same principal with other mass spectrometry. In this step, the retained proteins on the protein array will be deionised or ionised by irradiation with a nitrogen laser. All the ionised proteins/peptides will then pass through a flight tube and detector. Results are expressed as mass to charge ratio (m/z) values and intensities of the proteins/peptides. The detector also presents the protein profile of the specific biological sample.

The third component, the analysis software - EDM - helps in detection of unique proteins upon comparison between multiple protein profiles. It also provides quantitative analysis by comparing different intensities of the same protein mass. Significance of differences in protein expression is determined during this analysis.

METHODOLOGY

3.0 Methodology

3.1 List of chemicals

Angiotensin Converting Enzyme (ACE) activity inhibitory assay

Angiotensin Converting Enzyme (Sigma: A 6778 -1UN; EC: 3.4.15.1), N-Hippuryl-His-Leu hydrate (Sigma: H1635-500mg), sodium hydroxide, boric acid, sodium chloride (Merck), dioxane, cyanuric chloride, hydrochloric acid, KH_2PO_4 and hippuric acid.

Animal studies

Diethyl ether, Captopril (Sigma)

SDS-PAGE

Acrylamide, N,N'- methylenebisacrylamide, Sodium dodecyl sulphate (SDS), Tris-Base, hydrochloric acid, ammonium persulfate (APS), N, N, N', N' – tetramethylethylenediamine (TEMED), Sodium hydroxide (NaOH), Bromophenol blue, Tris HCL, glycerol, β-mercaptoethanol (Merck), broad range prestained protein marker (Bio-Rad Laboratories Inc.), acetic acid glacial, methanol, Commasie Briliant Blue R250. *

Protein Content Estimation

Pierce BCA Protein Kit (Thermo Scientific)

Two Dimensional Electrophoresis (2-DE)

Urea, CHAPS, IPG buffer, bromophenol blue, DTT, Tris Base,Glycerol, SDS, Glycine, Acrylamide, Bis-acrylamide, APS, Agarose, n-butanol or t-butanol, absolute ethanol, 25% glutaryldehyde, sodium thiosulphate, sodium acetate trihydrate, silver nitrate, formaldehyde, sodium carbonate, EDTA-Na_2H_2O, drystrip cover fluid and paper wicks (GE Healthcare). *

MALDI-TOF/TOF

Ammonium bicarbonate, potassium fericyanide, sodium thiosulphate, DTT, iodoacetamide, acetonitrile (ACN), ammonium bicarbonate, Trypsin Gold (PROMEGA, EC: 3.4.21.4), α-cyano-4-hydroxycinnamic acid (CHCA), triflouroacetic acid (TFA) and formic acid. *

SELDI-TOF

CM low-stringency buffer, pH 4.0 or sodium acetate buffer (Bio-Rad Laboratories Inc.) and matrix CHCA. *

*All chemicals were purchased from SIGMA unless otherwise stated.

*All solvents were purchased from SIGMA unless otherwise stated. Solvent for proteomics analysis were of HPLC grade.

3.2 Animal subjects

Spontaneously Hypertensive Rats were purchased from Animal House, Faculty of Medicine, University of Malaya, Kuala Lumpur.

3.3 Experimental plant materials

Raw materials of *F. deltoidea* were purchased from Delto Medicama (M) Sdn Bhd. The plant samples was identified and stored in the Herbarium, Rimba Ilmu, University of Malaya with the voucher specimens numbers: KLU 046470 – Big type leaves, KLU 046467 – Small type fruit.

3.4 List of equipments and apparatus

Heater (Thermolyne)

Stirer (Thermolyne)

Electronic Dry Cabinet (WEIFO®)

Microplate reader (ASYS UMV 340)

Centrifuge (Amersham Biosciences)

Spectrophotometer (Model: Ultraspec 2100 Pro UV/Visible spectrophotometer)

Dual vertical mini gel unit (Model: MGC-206, C.B.S Scientific Co.)

Power supply (Model:PSU 400/200)

Microcentrifuge (Model: Micro 20 Hettich Zentrifugen)

Immobiline^{TM} Drystrip Reswelling Tray (GE Heathcare Biosciences)

1D Ettan IPG phor 3 (GE Healthcare Biosciences)

2D EttanDalt six gel caster (GE Healthcare Biosciences)

2D EttanDalt six electrophoresis (GE Healthcare Biosciences)

Image Scanner III (GE Healthcare Biosciences)

Temperature controller (Model: 631-Protech)

Electrophoresis Power Supply (Model: EPS 601)

Speed Vacuum Concentrator (Thermoscientific)

C 18-ZipTips (Eppendorf)

Dual filter tips (100-1000 µl, 20-200 µl, 0.5-10 µl) (Eppendorf)

MALDI plate (Applied Biosystems)

MALDI-TOF/TOF mass spectrometer (Model: AB4800 plus, Applied Biosystems)

ProteinChip CM-10 array (Bio-Rad Laboratories, Inc.)

Bioprocessor reservoir (ProteinChip cassette and reservoir) (Bio-Rad Laboratories, Inc.)

ProteinChip SELDI-TOF system (PSC 4000) (Bio-Rad Laboratories, Inc.)

Pierce® BCA™ Protein Assay Kit (Thermo Scientific)

3.5 Preparation of *F. deltoidea* extracts

3.5.1 Preparation of fruit aqueous extract (SF)

Dried ground fruits of *F. deltoidea*, 100 g, was immersed in 500 ml of distilled water (ratio 1:5) and boiled for two hours. After two hours, another 500 ml of distilled water was added and boiling was continued for another two hours. The extract was filtered using sieve cloth once it was cooled to room temperature. The filtrate was then concentrated until it reaches at least 800 ml by heating at lower temperature (50-60°C). Centrifugation was performed at 4,500 x g, 10 minutes to remove sediments. Following that, the solution was poured into several freeze drying flask and stored in -80°C freezer. The frozen extract was then freeze-dried to yield fine dark brown powder. The plant extract was kept in capped container in WEIFO® Electronic Dry Cabinet with humidity below 30%.

3.5.2 Preparation of leaves aqueous extracts (BL)

Dried ground fruits of *F. deltoidea*, 100 g, was immersed in 1000 ml of distilled water (ratio 1:10) and boiled for two hours. After two hours, another 1000 ml of distilled water was added and boiling was continued for another two hours. Subsequent extraction procedures were conducted as describe in Section 3.5.1.

3.6 Angiotensin Converting Enzyme (ACE) activity inhibitory assay

ACE activity inhibitory assay by Paul L. Hurst and Chris J. Lovell-Smith (1981) was conducted in this study with some modifications. Synthetic substrate, Hippuryl-L-histidine-L-leucine (HHL) was used to measure the ACE activity by which ACE will hydrolyzes this HHL to hippurate. Cyanuric chloride in 1, 4-dioxane was used as the reagent, where it reacted towards hippurate in the presence of phosphate buffer.

3.6.1 Preparation of reagents / solutions

ACE, 0.04 U/ml

ACE from rabbit lung (SIGMA: A 6778-1UN) was dissolved in 5 ml of distilled water to reach a final concentration of 0.2 U/ml. This stock solution was kept in microcentrifuge tubes as aliquot and stored at -20°C until used. Prior to the experiment, 300 µl of 0.2 U/ml of the aliquot was diluted five times with 1200 µl of distilled water. This enzyme solution was maintained in ice bath during the experiment.

N-Hippuryl-His-Leu hydrate (HHL) substrate solution, 20 mM

The substrate N-Hyppuryl-His-Leu-hydrate, 500 mg, (SIGMA: H1635) was dissolved in 58.2 ml of 0.02 M NaOH. Substrate solution was kept at -20°C. In reagent blank tubes, the substrate solution was replaced with 0.02 M NaOH instead of incubation buffer.

Note: NaOH was used to dissolve the HHL due to prevent appearance of precipitates that may affect the final concentration of substrate.

Hippurate standard solutions

Hippurate standard was prepared by diluting the stock solution (2500 µmol) into 1250 µmol and 250 µmol with distilled water. All the three concentrations were used as standard in this experiment. The stock solution was kept in -20°C.

Incubation buffer (Borate buffer) pH 8.3

Boric acid (H_3BO_3), 0.99 g, and NaCl, 9.35 g, were dissolved in 150 ml of distilled water. The pH was adjusted to 8.3 using 5 M NaOH and topped up to 200 ml with distilled water. Solution was stored at room temperature**.**

Terminating solution, 1 M Hydrochloric Acid (HCl)

Hydrochloric acid, 1 M, was prepared by diluting 85.1 ml of 36% HCl and topped up to one litre with distilled water. Solution was stored at room temperature.

Neutralizing solution, 1 M NaOH

NaOH, 40 g, was dissolved in one litre of distilled water. Solution was stored at room temperature.

Diluent buffer or 0.2 M phosphate buffer pH 8.3

KH_2PO_4, 27.22 g, was dissolved in 900 ml of distilled water. The pH was adjusted to 8.3 using 5 M NaOH and topped up to one litre with distilled water. Solution was stored at room temperature.

Colouring reagent

Cyanuric chloride, 7.5 g, was dissolved in 250 ml of 1, 4-dioxane. Solution was stored at room temperature in a dark-brown glass bottle.

0.02 M NaOH

NaOH, 0.16 g, was dissolved in 200 ml of distilled water. Solution was stored at room temperature.

5 M NaOH

NaOH, 40 g, was dissolved in 200 ml of distilled water. Solution was stored at room temperature.

3.6.2 Preparation of sample

Both extracts (fruits, SF and big leaves type, BL of *F. deltoidea*) were prepared by dissolving the extracts with distilled water until it reaches 4 mg/ml. Samples were then diluted ten times and serial dilution was done to reach final concentration between 0 - 0.4mg/ml.

3.6.3 ACE Activity Inhibitory Assay

Three sets of centrifuge tubes were prepared in replicates. One set will be used for sample and another two sets were used for sample blank and hippurate standard. For sample and sample blank sets, 50 µl of sample (0.05, 0.1 and 0.2 mg/ml of SF and BL extracts), 100 µl of incubation buffer and 50 µl of 0.04 U/ml ACE solution were added together. However, for the control, 50 µl of distilled water was added to replace the sample. For the hippurate standard, 50 µl of hippurate standard solution (250 µmol, 1250 µmol and 2500 µmol) and 150 µl of incubation buffer were added into the tubes. For the reagent blank (0 µmol of hippurate), 50 µl of distilled water and 150 µl of 0.02 M NaOH were added into the tubes. All the tubes were vortex and pre-incubated at 37°C for five minutes. At the same time, the ACE substrate solution was equilibrated for five minutes at 37°C. 50 µl of 0.02 M HHL and 0.02 M NaOH was then added into the sample set and the hippurate standard set respectively to start the reaction for 15 minutes at 37°C. Then, 250 µl of terminating solution was added into all tubes to stop the reaction and 30 seconds later, the mixture was neutralized by the addition of 250 µl of 1 M NaOH. After the neutralization, 1 ml of diluent buffer was added to all tubes followed by 750 µl of colouring reagent. This mixture was vortex-mixed vigorously and allowed to stand for five minutes before it was vortex-mixed again. Then, all tubes were centrifuged at 3000 rpm for five minutes to remove all the denatured protein and excess cyanuric chloride. For sample blanks, terminating and neutralizing solutions were added before the addition of substrate. Absorbances of the supernatants were measured at 382 nm using microplate reader versus the reagent blank.

3.7 Animal studies

Eight weeks old male Spraque Dawly Rat (SD) and Spontaneously Hypertensive Rat (SHR) were purchased from the Animal House of Faculty of Medicine, University of Malaya, Kuala Lumpur. Animal study was conducted in the Experimental Block, Faculty of Medicine. Animal were caged individually, maintained on a 12 hour light, 12 hour dark cycle at constant temperature and given gold coin palette and tap water ad libitium. Rats were divided into five groups with four rats in each group based on the type of treatment as shown in Table 3.1.

Table 3.1: Rats grouping based on treatment received.

Group	Treatment
Group 1	Normal rats or normotensive rat (NR) : SD : distilled water
Group 2	Diseased: SHR rats : distilled water
Group 3	Treated disease: SHR treated with fruit aqueous extract of *F. deltoidea*
Group 4	Treated disease: SHR treated with big type leaf aqueous extract of *F. deltoidea*
Group 5	Treated disease: SHR treated with captopril

3.7.1 Treatment with aqueous extracts

For the treatment with aqueous extract solutions (group 3 and group 4), the dosage given to each rat was 75 mg/kg body weight. Based on the weight of SHR rat, (approximately 300 g), 22.5 mg of extract will be given to each rat. In this study, the plant extracts were given as drinking water to prevent experimental stress towards the SHR rats. The drinking water solution was prepared by dissolving 1.125 g of each extract in 2.5 litre of distilled water and was kept in 4°C. 30 ml of the extract solution were given daily for each individual rat, for ten days.

3.7.2 Treatment with inhibitor, Captopril

In this study, Captopril was used as the positive control. Dosage given to SHR rat (Group 5) was 60 mg/kg body weight. Each rat was treated with 0.125 mg of Captopril. One tablet of Captopril, approximately 25 mg, was dissolved in 100 ml of distilled water. For each rat, 30 ml of this solution was given daily for ten days.

3.7.3 Serum extraction

On the 11^{th} day, each one of the rat was brought to unconsciousness using diethyl ether saturated glass cage for a few seconds. Subsequently, the rat was laid down on the tray and blood was collected directly via cardiac puncture. The blood was then transferred into chilled micro centrifuge tube and spun for five minutes at 5,000 rpm to obtain the serum. Serum was then transferred into another labelled tube and stored in -80°C freezer.

3.8 Estimation of serum protein content using BCA™ Protein Assay Kit

The protein content in each serum was estimated using Pierce BCA™ Protein Assay Kit. Two µl of serum was diluted 100 times and subjected to protein content estimation using test tube procedure. Bovine serum albumin (BSA) was used as the standard (5-250 µg/ml). All the experimental procedure was done according to the protocol as mentioned in the kit (Appendix D).

3.9 Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.9.1 Preparation of reagents/solutions

Solution A: 30% (w/v) Acrylamide and 0.8% (w/v) N, N'-methylenebisacrylamide

Acrylamide, 60 g, and N, N'- methylenebisacrylamide, 1.6 g, were dissolved in 200 ml of double distilled water. The solution was then deionised using Amberlite MB-1. Deionised solution were stirred for one hour and later filtered. The filtrate was then stored in a dark bottle and kept at 4^0C.

Solution B: 1.5 M Tris-HCl pH 8.8

Tris-Base, 36.35 g, was dissolved completely in 200 ml of double distilled water. The pH of the solution was then adjusted to 8.8 using concentrated hydrochloric acid (HCL). This solution was kept at 4^0C.

Solution C: 10% (w/v) SDS

SDS, 10 g, was dissolved completely in 100 ml of double distilled water and was kept at room temperature.

Solution D: 10% (w/v) Ammonium persulfate (APS)

APS, 0.1 g, was dissolved completely in one ml of double distilled water. The solution was freshly prepared before use or kept at -20^0C.

Solution E: N, N, N', N' – tetramethylethylenediamine (TEMED)

TEMED solution was taken directly from the bottle and kept in micro tube.

Solution F: 0.5 M Tris-HCl , pH 6.8

Tris Base, 12.11 g, was dissolved in 200 ml of double-distilled water. The pH of this solution was adjusted to 6.8 using concentrated HCl. The solution was kept at 4^0C.

SDS-PAGE sample buffer: 1.0 % (w/v) Bromophenol blue, 62.5 mM Tris-HCl pH 6.8 buffer (Solution F), 20% (w/v) glycerol, 10 % (w/v), β-mercaptoethanol

Solution F, 2.0 ml, glycerol, 1.6 ml, 10% SDS, 3.2 ml, β-mercaptoethanol, 0.8 ml and 1.0% bromophenol blue, 0.4 ml, were mixed together. The solution was kept in -80°C.

Cathode or trailing buffer: 2.5 mM Tris, 198 mM Glycine, 0.1% (w/v) SDS, pH 8.3

Tris-Base, 3.30 g, glycine, 14.41 g, and SDS, 1.0 g, were mixed and made up to one liter with doubled-distilled water. pH was adjusted to 8.3 using concentrated HCL.

Prestained Protein Marker (Molecular Weight: 6 – 175 kDa)

The Prestained Broad Range Protein Marker (Bio-Rad Laboratories Inc.) was vortex well and aliquoted into separate tube. The marker was then heated to 95°C for five minutes. A quick 5 µl microcentrifuge spin was applied before loading it onto the gel. The remainder were kept in -20^0C.

Coomassie Blue staining solutions

Fixing solution: 10% acetic acid and 40 % methanol

Acetic acid glacial, 10 ml, and concentrated methanol, 40 ml, were mixed and topped up to 100 ml of distilled water.

Staining Solution: 0.1% Coomassie Brilliant Blue R- 250 in 10% of acetic acid.

Coomassie Brilliant Blue R- 250, 1 g, and acetic acid, 100 ml, were mixed and dissolved in one litre of distilled water. The solution was then filtered and stored in dark bottle.

Destaining solution: 10 % acetic acid

Acetic acid glacial, 100 ml, was dissolved in distilled water to make up one litre and mixed thoroughly.

3.9.2 SDS-PAGE gel preparation

Gel caster for Dual vertical mini-gel unit model # MGV-200 SDS-PAGE (C. B. S. Scientific CO.) was used to cast the SDS-PAGE gel with the thickness of 0.5 mm.

3.9.2.1 Preparation of 12.5 % Separating Gel

Solution for separating gel was prepared by mixing all the solutions stated in Table 3.2. APS and TEMED were added last and the solution was stirred slowly for a few second to mix it well. Then the solution was immediately layered into the gel caster using a dropper. The gel was overlaid slowly with distilled water and allowed to polymerize for a minimum of one hour.

3.9.2.2 Preparation of Stacking Gel

Stacking gel solution was prepared as shown in the Table 3.2 by finally adding APS and TEMED. Before preparing the stacking gel solution, the overlaid distilled water was removed and the surface of separating gel was dried using filter paper. Immediately, following the addition of TEMED and mixed properly, the stacking gel solution was layered onto the separating gel using a dropper and a comb was then inserted before the stacking gel starts to polymerize.

Table 3.2: Preparation of Separating Gel (12.5%) and Stacking Gel

Solution	Separating Gel (12.5%)	Stacking Gel
Solution A	8.34 ml	0.65 ml
Solution B	5.00 ml	-
Solution C	0.20 ml	50.00 µl
Double distilled water	6.36 ml	3.05 ml
Solution F	-	1.25 ml
Solution D	100.00 µl	25.00 µl
Solution E	6.60 µl	5.00 µl

3.9.3 Preparation of serum sample for serum protein profile in SDS-PAGE

All serum (normotensive rat, SHR treated with SF [SHR-SF] and BL extract [SHR-BL], SHR treated with Captopril [SHR-Cp] and nontreated SHR) collected from rats were used in the analysis. 5 µl of serums were mixed with 5 µl of sample buffer (ratio 1:1) in individual microcentrifuge tube. Then, 3.5 µl of the solution were heated about three minutes in boiling water and were loaded into different wells. Frozen Prestained protein standard marker was heated in 40°C about five minutes and 5-10 µl was loaded onto the gel. Separation of sample via SDS-PAGE was performed in triplicate.

3.9.4 Running SDS-PAGE

The glass plates containing polymerised gels were assembled into the electrophoresis tank with the short plate facing on the inside. The comb was removed and electrophoresis buffer (cathode buffer) was poured between the gel cassettes until all wells were filled with the buffer.

3.5 µl of sample solution (refer method 3.9.3) was loaded using gel loading tip into respective wells. Lane 1 was loaded with normotensive rat serum; Lane 2 with SHR serum; Lane 3 and 4 with SHR-BL and SHR-SF serum and Lane 5 was loaded with SHR-Cp serum. Lastly, 10 µl of standard protein marker was loaded into the last well (lane 6).

The tank was then assembled appropriately and connected to the power supply correctly. The samples were allowed to run at 60 V for the first 15 minutes and eventually changed to 100 V when the dye reaches the separating gel. The voltage supply was terminated once the dye reaches 1 cm from the bottom of the gel.

3.9.5 Staining and destaining procedure

Once the SDS-PAGE electrophoresis was completed, the gel was carefully taken out from the gel caster and immersed in fixing solution for 30 minutes with continuous shaking. Following the fixing solution was removed slowly by holding the end side of the gel. The fixing solution was then replaced with Coomassie Brilliant Blue R-250 and left with continuous shaking for 30 minutes. Then, the staining solution was replaced with de-staining solution and continuously shaked until the background of the gel was clear and the protein bands revealed. This de-staining procedure may took about 3 hours.

3.9.6 Gel calibration and determination of unknown serum protein molecular weight (MW)

The molecular weight of proteins separateded in the gel was determined by comparing the distance travelled relative to the marker called relative mobility (R_m). The MW of the proteins was determined from the calibration curve of the protein standards' MW versus its R_m (Table 3.3). Calibration curve was prepared by plotting the $\log_{10}$ MW of the standards proteins versus relative mobility, R_m. The R_m was determined by the following formula:

$$R_m = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}$$

Table 3.3: Molecular weight of protein standard.

Protein Standard	Molecular Weight (kDa)
Myosin	210
B-galactosidase	125
Bovine serum albumin	101
Ovalbumin	55.2
Carbonic anhydrase	35.8
Soybean trypsin inhibitor	29
Lysozyme	21
Aprotinin	6.9

3.10 2-D Electrophoresis

3.10.1 Preparation of reagents

Urea rehydration stock solution

(8 M urea, 2% CHAPS, 0.5% IPG buffer, 0.002% bromophenol blue)

Urea, 12 g, CHAPS, 0.5 g, IPG buffer, 125 µl and 1% Bromophenol blue stock solution, 50 µl, were mixed and double-distilled water (ddH_2O) was added up to 25 ml. This solution was stored in 1ml aliquots at -20^0C.

Urea rehydration buffer

(8 M urea, 2% CHAPS, 0.5% IPG buffer, 0.002% bromophenol blue, 0.0028% DTT)

DTT, 0.1 g, was added into 1ml of urea rehydration stock to obtain 10% DTT in the solution. 28 µl of this solution was then added to the other 1ml urea rehydration stock solution prior to use. This urea rehydration buffer was used in sample rehydration (Section 3.10.2).

SDS equilibration buffer solution

(6 M urea, 75 mM Tris-HCl pH 8.8, 29.3 % glycerol, 2% SDS, 0.002% Bromophenol blue)

Urea, 72.1 g, Tris-HCl pH 8.8, 10ml, 87% (w/w) glycerol, 69 ml, and 1% Bromophenol blue stock solution, 400 µl, were mixed and double-distilled water was added to make up 200 ml. SDS equilibration buffer A was prepared by adding 0.1 g of DTT in 10 ml of SDS equilibration buffer solution. SDS equilibration buffer B was prepared by adding 0.25 g of iodoacetamide in 10 ml of SDS equilibration buffer solution. 10 ml of solution was prepared per IPG strip.

10X Laemmli SDS electrophoresis buffer

(250 mM Tris-base, 1.92 M Glycine, 1% SDS)

Tris-base, 30 g, glycine, 144.1 g, and 10 g of SDS were mixed and double-distilled water was added up to one litre. This buffer was diluted by one time for 2-DE.

Monomer stock solution

(30% Acrylamide, 0.8% N,N'-methylenebisacrylamide)

Acrylamide, 300 g and N, N'-methylenebisacrylamide, 8 g, were mixed and double distilled water was added to make up one litre. This solution was kept in dark bottle at 4°C.

4 X Resolving gel buffer solution (1.5 M Tris-base, pH 8.8)

Tris-base, 181.7 g, was diluted in 750 ml of double-distilled water and the pH of the solution was adjusted to 8.8 using concentrated HCl. After that, double-distilled water was added to make up to one litre. This solution was stored at 4°C.

Bromophenol blue stock solution

(1% Bromophenol blue, 0.6% Tris-base)

Bromophenol blue, 0.1 g, and Tris-base, 0.06 g, were mixed and double-distilled water was added to make up to 10 ml. This solution was kept in aliquots at 4°C.

10% w/v SDS solution

SDS, 5 g, was dissolved in 50 ml of double-distilled water. Solution may be stored at room temperature.

10 % APS solution

APS, 0.1 g, was dissolved in 1 ml of double-distilled water. This solution was prepared prior to use.

Agarose sealing solution

(25 mM Tris base, 192 mM glycine, 0.1% SDS, 0.5% agarose, 0.002% bromophenol blue)

Laemmli SDS electrophoresis buffer, 100ml, agarose, 0.5 g, and 200 µl of 1% bromophenol blue stock solution were mixed together and heated in the microwave. After all the agarose had dissolved, the solution was stirred until it is cooled and later stored. During the equilibration step, the agarose sealing solution can be reheated three to five minutes before use.

Overlaying solution (water saturated-butanol)

n- butanol or t- butanol, 50 ml, was mixed with 5 ml of double-distilled water.

Gel storage buffer

4 X resolving buffer solution, 250 ml, and 10ml of 10% SDS was mixed and double-distilled water was then added up to one litre. This solution is sufficient to store six sets of glass plates containing the polymerized gels. The gels were immersed with this buffer and kept in 4°C until used. Usually, this solution was replenished after third times of usage.

3.10.2 Rehydration of IPG Drystrip

Protein concentrations of sera samples were done by using BCA™ Protein Assay Kit (as mentioned in section 3.8). A specified volume of serum sample that contain 200 µg/ml proteins, was mixed with the urea rehydration buffer containing 10% DTT up to 900µl. Only 450 µl of the sample solution (which contains approximately 100 µg/ml protein) were used in the rehydration process. 450 µl of the solution were taken out from the tube and pipetted in the well of the reswelling tray (GE Healthcare Bioscience). Then, the immobiline dry strip (pH 4-7, 24 cm) were laided on the solution with the gel surface facing down the well surface. After all the solution covered the entire strip and all the bubbles were removed, the IPG Drystrip was overlaid with PlusOne Drystrip Cover Fluid to prevent crystallization of the urea. The rehydration process was left for 20 hours to ensure the entire IPG Drystrip was fully rehydrated.

3.10.3 First Dimension Gel Electrophoresis (isoelectric Focusing)

After rehydration, the IPG Drystrip was subjected to 1st dimension electrophoresis using IPGphor III (GE Healthcare Bioscience). The strip was taken out from the reswelling tray carefully using a forcep and the excessive oil was removed by gently dabbing the strips on a damp filter or tissue paper. The strip was then transferred on the silica plate assembled in the IPGphor III with the gel surface facing up. The positive side of the strip positioned on the positive side of the IPGphor III and vice versa. The damp paper wicks were placed on both ends of the strip and followed by the assembly of electrodes. In this process, up to 12 strips can be run at the same time.

First gel dimension electrophoresis was run for 18 hours with the total voltage of 75 kV/Hrs. The specific process of 1st dimension electrophoresis was performed as follows:

S1:	Step and Hold	500V	1:00 Hr
S2:	Gradient	1000V	7:00 Hrs
S3:	Gradient	8000V	3:00 Hrs
S4:	Step and Hold	8000V	7:10 Hrs or Until reach 75 kV/Hrs

After the iso-electric Focusing (IEF) process was completed, the strips were rinsed with milli-Q water to remove the PlusOne drystrip cover fluid. The strip was stored in an equilibration tube with the support film facing towards the wall of the tube. The strip can be transferred directly to the 2nd dimension electrophoresis by performing equilibration step or can be stored in -80^{0}C freezer.

3.10.4 IPG strip equilibration

The equilibration step is required to saturate the IPG strip with the SDS buffer for protein separation in second dimension electrophoresis (2-DE). Equilibration procedure involves two equilibration steps. The first equilibration step requires 100 mg DTT per 10 ml SDS equilibration buffer (buffer A) and the second equilibration step requires 250 mg iodoacetamide per 10 ml SDS equilibration buffer (buffer B). These solutions were prepared prior to use. IPG strips were placed in individual tubes with the support film facing towards the wall. Then, 10 ml of buffer A was added to each tube. Tubes were capped and placed on the shaker. The strips were equilibrated for 15 minutes. After the first equilibration, buffer A was removed and buffer B was then added subsequently. The second equilibration step was continued for 15 minutes and the solution was then removed.

3.10.5 Second Dimension Gel Electrophoresis (2-DE)

3.10.5.1 Preparation of 11% second dimension gel

Eleven percent of second dimension gel was prepared according to Table 3.4. All the solutions were added in a beaker and were mixed by slow stirring. 10% APS solution was freshly prepared and added just prior to use and TEMED was added last. Usually, the 11% second dimension gel was prepared a day before the electrophoresis. Once the solution is ready, the solution was poured slowly on the top of gel caster channel until it reached approximately 0.5 cm from the top of the gel plate. Then, water saturated butanol was laid slowly on the solution using a dropper. The solution was allowed to polymerize for a minimum of one hour.

After all the gels have polymerised, the gel plate was disassembled from the gel caster. Each gel plates were rinsed with milliQ water to remove all the water saturated butanol. Then, the gels were kept in the storage solution at 4°C.

Table 3.4: The preparation of 2-DE gel.

Solution	Volume (ml)
Monomer solution (30% Acrylamide, 0.8% N,N'-methylenebisacrylamide)	37.0
4X resolving gel buffer (1.5 M Tris Base, pH 8.8)	25.0
Double distilled water	36.5
10 % SDS	0.5
10 % ammonium persulfate	1.0
TEMED	0.033

Note: This preparation is sufficient for six 26 X 28 cm 2-DE gels.

3.10.5.2 Second Dimension Electrophoresis

In second-dimension electrophoresis step, firstly, equilibrated strips were rinsed with SDS electrophoresis buffer to lubricate it. Then, the strips were placed onto the surface of second-dimension gel and any formation of bubbles in between the strip and the second-dimension gel were removed for a perfect migration. After all the bubbles were removed, the strip was sealed with agarose sealing solution. Electrophoresis (Ettan Daltsix electrophoresis unit [GE Healthcare Bioscience]) was performed at a constant power in two steps using Electrophoresis Power Supply – EPS 601 (GE Healthcare Bioscience). The electrophoresis conditions were set as follows, at the temperature 25^0C:

Step	µA/gel	Voltage	Time (Hr:Min)	Walt/gel
S1	10	80	1:0	1
S2	40	500	4:30 – 6:30	13

The electrophoresis was terminated when the dye front reached approximately 1 cm from the bottom of the gel. After electrophoresis, gels were removed from their cassettes in order to prepare for staining.

The cassette was immersed in milliQ water and the cassette was opened at the end of cassette slowly. After the upper cassette was released from the gel, the strip was taken out and placed in a container filled with fixing solution. Then, small upper end of positive side of the gel was marked by a slant cutting. Next, the gel were taken out gently by holding both upper side of the gel and transferred into the same container as its strip.

3.10.6 Silver staining

The gel was immersed in the fixing solution individually. The recipe and protocol used for each silver staining step were done as follows. Approximately 500 ml of solution was required for each gel.

During the staining procedure, the removal of solution was done similarly as in section 3.9.5. However, in this procedure, more precaution is required due to its larger size of gel.

Table 3.5: Silver staining procedure

Step	Solutions	Amount	Time
Fixation	Ethanol Acetic acid, glacial ddH_2O make up to 500 ml	200 ml 50 ml	Overnight
Sensitizing	Ethanol 25% w/v Glutardialdehyde Sodium thiosulphate Sodium acetate trihydrate ddH_2O to make up 500 ml	150 ml 2.5 ml 1.0 g 34.0 g	30 minutes
Washing	Double distilled water		3 X 5 minutes
Silver reaction	Silver nitrate solution 37% w/v Formaldehyde ddH_2O to make up 500 ml	1.25 g 0.2 ml	20 minutes
Washing	Double distilled water		2 x 1 minute
Developing	Sodium carbonate 37% w/v Formaldehyde 5% w/v Sodium thiosulphate ddH_2O to make up 500 ml	12.5 g 0.2 ml 14 µl	4 minutes
Stop	EDTA-Na_2 H_2O ddH_2O make up to 500 ml	7.3 g	10 minutes
Washing	Double distilled water		3 X 10 minutes

Note: The fixation steps may be prolonged up to three days if desired for convenience.

The use of glutarylaldehyde in sensitizing step and formaldehyde in silver reaction solution were omitted as well as preserving solution if the gel was to be subjected to MS analysis.

3.10.7 2-DE gels protein spots analysis

The 2-DE gels were scanned using ImageScanner III (GE Healthcare Bioscience) and analyzed using Image Master Platinum 7.0 Software (GE Healthcare Bioscience). The spots were then finally analyzed based on the Matched Number and the significant value with ANOVA ($p < 0.05$). Significant value obtained from the software was normalized using Excell ANOVA ($p < 0.05$). The expression dynamic of protein spots for nontreated SHR, treated SHR and normotensive rats were calculated and compared based on respective percentage of volume (% volume) obtained from the software. Significant spots were selected for identification using MALDI-TOF/TOF. After the protein ID was obtained, the expression dynamic of the identified spot was observed to find the possible relationship with hypertension by comparing with several reported journals.

3.11 MALDI-TOF/TOF mass spectrometry

3.11.1 Reagents preparation

100 mM Ammonium bicarbonate

Ammonium bicarbonate, 0.791 g, was dissolved in double distilled water (ddH_2O) to make up 100 ml.

Destaining solution (15 mM Potassium fericyanide; 50mM Sodium thiosulphate)

Potassium fericyanide, 0.1 g, and 0.25 g of sodium thiosulphate was dissolved in ddH_2O to make up 20 ml.

Reducing solution (10 mM DTT in 100 mM ammonium bicarbonate)

DTT, 0.0308 g, was dissolved in 100 mM ammonium bicarbonate to make up 20 ml. This solution was prepared prior to use.

Alkylating solution (55 mM iodoacetamide in 100 mM ammonium bicarbonate)

Iodoacetamide, 0.2036 g, was dissolved in 100 mM ammonium to make up 20 ml. This solution was prepared prior to use.

Washing solution (50% acetonitrile [ACN] in 100 mM ammonium bicarbonate)

Absolute ACN, 20 ml, was diluted with 100 mM ammonium bicarbonate to make up 40 ml.

Dehydrating solution

100% or absolute ACN was directly used in this step.

Digestion solution

(7 ng/ml of trypsin in 40 mM ammonium bicarbonate in 10% ACN)

1 µg/ml trypsin, 5 µl, was dissolved with 709 ml of 40 mM ammonium bicarbonate in 10% ACN.

40 mM ammonium bicarbonate in 10% ACN

Ammonium bicarbonate, 0.316 g, was dissolved in 100 ml of 10% ACN.

10% ACN

ACN 10 ml was diluted to make up 100 ml.

Solutions for Zip-Tip (desalting) procedure:

Wetting solution

100% ACN was directly used in this step.

Equilibration solution (0.1% Formic acid in ddH_2O)

Formic acid (98%-100%), 20 µl, was diluted with 19.98 ml of ddH_2O.

Elution solution (0.1 % Formic acid in 50% ACN)

Formic acid (98%-100%), 20 µl, was diluted with 19.98 ml of 50% ACN.

Matrix solution (10 mg/ml of α-cyano-4-hydroxy-cinnamic acid)

α-cyano-4-hydroxy-cinnamic acid, 10 mg, was dissolved with 40% of buffer A and 60% of buffer B.

a) **Buffer A (2% ACN; 0.1% TFA)**

ACN, 2 ml, and 0.1 ml of TFA were mixed with 100 ml of milliQ water.

b) **Buffer B (98% ACN ; 0.1% TFA)**

ACN, 98ml, and TFA, 0.1 ml, were mixed with 100 ml of milliQ water.

3.11.2 MALDI-TOF/TOF analysis - digestion procedure

Protein selected from image master analysis based on the dynamic expression and significance was picked from preparative gel. This gel was previously loaded with 300 µg/ml of protein. Three gels were prepared for the MALDI-TOF/TOF. The protein spots of interest were picked by using clean eppendorf tips (size 5000 µl). Each gel plug was individually stored in labelled eppendorf microcentrifuge tubes and kept in 4^0C freezer until use. Clean handling is required in this step to avoid contamination from human keratin. Procedure for proteolytic digestion using trypsin is shown in Table 3.5.

Gel plug in microcentrifuge tubes were immersed with destaining solution for 30 minutes. Then, the destaining solution was carefully removed and replaced with reducing solution. The next steps were conducted according to Table 3.5. In digestion step, 7 ng/ml of trypsin solution was used to immerse the gel plug for one night. On the next day, the trypsin solution (containing digested protein in fragments) was transferred to a new and clean tube. The gel plug was then extracted twice and the extraction solutions were dispensed into the same tube. The solutions in the tube were dehydrated in speed-vacuum for one hour in low heat condition. After the solutions were fully dehydrated and produced white powder, the equilibration solution was added and shook continuously for 15 minutes. Along the process, non-powdered gloves were used and wiped with 70% ethanol to remove keratin and other contaminant. The extracted peptide was then desalted using ZipTip® procedure (Appendix E).

Note: During staining procedure for MALDI-TOF/TOF analysis, glutaraldehyde in sensitizing step and formaldehyde in silver reaction were omitted.

Table 3.6: Tryptic digestion procedure prior to mass spectrometry analysis.

Procedure / Steps	Duration
Destaining step 50 µl of 15 mM Potassium ferricyanide in 50 mM sodium thiosulphate	30 minutes
Reducing step 150 µl of 10 mM Dithiothreitol in 100 mM ammonium bicarbonate	30 minutes
Alkylating step 150 µl of 55 mM iodoacetamide in 100 mM ammonium bicarbonate	20 minutes
Washing step 500 µl of 50% ACN in 100 mM ammonium bicarbonate	3 X 20 minutes (shaking)
Dehydrating step 50 µl absolute ACN	15 minutes (dry in speedvac)
Digesting step 30 µl of 7 ng/µl Trypsin in 40 mM ammonium bicarbonate in 10% ACN	Overnight, 37°C
Extraction I 50 µl of 50% ACN	15 minutes
Extraction II 50 µl of 100% ACN	15minutes

Methods was cited from Shevchenko *et al.* (2007) with some modifications

3.11.3 MALDI-TOF/TOF mass spectrometry analysis and database searching

Extracted peptides were desalted using ZipTip C18 (Millipore, USA) according to protocol described by the manufacturer. The final elution volume following ZipTip cleanup was 1.5µl. The peptides samples were then mixed (1:1) with a matrix consisting of a saturated solution of CHCA (α-cyano-4-hydroxycinnamic acid, Sigma) prepared in 50% ACN/0.1% TFA. Aliquots of samples (0.7µl) were spotted onto stainless-steel sample target plates. Peptide mass spectra were obtained by MALDI-TOF/TOF mass spectrometer (AB4800 plus, Applied Biosystems) in the positive ion reflector mode. For precursor ion selection, all fractions were measured in single MS before MS/MS was performed. For MS/MS spectra, the peaks were calibrated by default. The 20 most abundant precursor ions per sample were selected for subsequent fragmentation by high-energy CID. The collision energy was set to 1 keV and air was used as collision gas. The criterion for precursor selection was a minimum S/N of 5. The mass accuracy was within 50 ppm for the mass measurement and within 0.1 Da for CID experiments. The other parameters settings for search were; trypsin, 1 missed cleavage, variable modification of carbamidomethyl, oxidation of methionine, peptide charge of 1+, and monoisotopic. For database searches, known contamination peaks such as keratin omitted and analyzed by the Global Protein Server Explorer 3.6 Software (Applied Biosystems). This uses an internal MASCOT (Matrix Science, UK) program for matching MS and MS/MS data against database information. The data obtained were screened against Rodentia databases downloaded from the Swiss-Prot/TrEMBL homepage (http://www.expasy.ch/sport). All the MALDI-TOF/TOF analysis was performed at least twice to confirm the identified protein.

3.12 SELDI-TOF mass spectrometry

3.12.1 Preparation of reagents / solutions

ProteinChip CM low stringency buffer (0.1 M Sodium acetate, pH 4.0)

ProteinChip CM low stringency buffer was purchased from Bio-Rad Laboratories. This buffer was used throughout SELDI-TOF sample preparation procedure. Buffer was stored at 11°C.

Matrix solvent (50% (v/v) ACN, 0.5% (v/v) TFA)

ACN, 200 µl and TFA, 2 µl was dissolved in 198 µl of water. This solvent was freshly prepared.

Matrix [α-Cyano-4-hydroxycinnamic acid (CHCA)]

α-Cyano-4-hydroxycinnamic acid (CHCA), 5 mg, was dissolved in 200 µl of matrix solvent and vortex for two minutes. The matrix was spun at 1,000 x g for one minute. Supernatant was transferred into new microcentrifuge tube and kept in the dark.

3.12.2 Sample preparation and analysis

ProteinChip CM-10 was used in this experiment. ProteinChip arrays were inserted into array cassette and placed onto the bioprocessor in the correct orientation. Spots were equilibrated with the addition of 150 µl of buffer in each well for five minutes with vigorous shaking. Buffer was then removed from the wells. Pre-washing step was then continued with 150 µl of buffer for five minutes. This step was repeated twice. The pre-washing solution was removed and replaced with 100 µl of sample (10 times dilution). The sample was incubated for 30 minutes in a humidified chamber with constant shaking. During the sample application step, spots were not allowed to air-dry and any bubbles on the spot were removed. Diluted sample was then removed and each spot was then washed with 150 µl of buffer with constant shaking for five minutes. Washing step was repeated three times. 200 µl of milliQ water was immediately added and removed to rinse the spot twice. ProteinChips were allowed to semi-dry in room temperature before the addition of 2 µl of matrix. The arrays were analyzed using the ProteinChip system using the following protocol setting (Table 3.6).

Table 3.7: Protocol setting for SELDI-TOF analysis

Acquisition setting	
Mass range	0 – 40,000 Da
Focus mass	10, 000 Da
Matrix attenuation	500 Da
Sampling rate	800 MHz
Laser energy	960 nJ
Shots kept	795

3.12.3 SELDI spectra analysis

3.12.3.1 Univariate analysis

Statistical analysis was performed using the ProteinChip Data Manager 3.5 (Bio-Rad Laboratories, Inc). Peaks with m/z values < 2.5 kDa were excluded from the analysis as these peaks were mainly ion noise from the matrix. For Expression Difference Map (EDM) analysis, spectra were compiled and qualified peak mass signal peaks with mass-to-charge ratios (m/z) between 2.5-40 kDa were auto-detected. Peak clusters were completed using second-pass peak selection with S/N > 2, within 0.1% of mass window and allowing estimated peaks to be added. *p*-values were determined using mean peak intensities from triplicate samples with $p<0.05$ considered as significant.

3.12.3.2 Multivariate analysis

For determination of possible biomarker, Biomarker Pattern Software™ (Bio-Rad Laboratories Inc.) was used. Different groups were used in this analysis for comparison between different treatments.

RESULTS

4.0 Results

4.1 Angiotensin Converting Enzyme (ACE) Inhibitory Assay

ACE inhibitory activity was determined by measuring the product; hippurate, produced in the experiment. The formation of hippurate-cyanuric chloride complex was calculated by comparing the absorbance value at 382 nm against the hippurate standard curve shown in Figure 4.1.

From the study (section 3.6.3), both fruit (SF) and big leaves type (BL) of *F. deltoidea* extracts showed significant inhibitory effect towards ACE activity as the *p* - values obtained from one way ANOVA were 2.51×10^{-7} and 4.23×10^{-6} respectively. Figure 4.2 and 4.3, showed the inhibition of SF and BL were increased in parallel to the increase of extracts concentrations. Results from this assay indicated the half maximal inhibitory concentrations (IC_{50}) of SF and BL extracts obtained are 0.13 ± 0.023 mg/ml and 0.18 ± 0.023 mg/ml as shown in Table 4.1. The two way ANOVA statistical analysis revealed no significant difference between the two treatments. Therefore, both extracts were equally good ACE inhibitors with no significant difference in terms of their effectiveness.

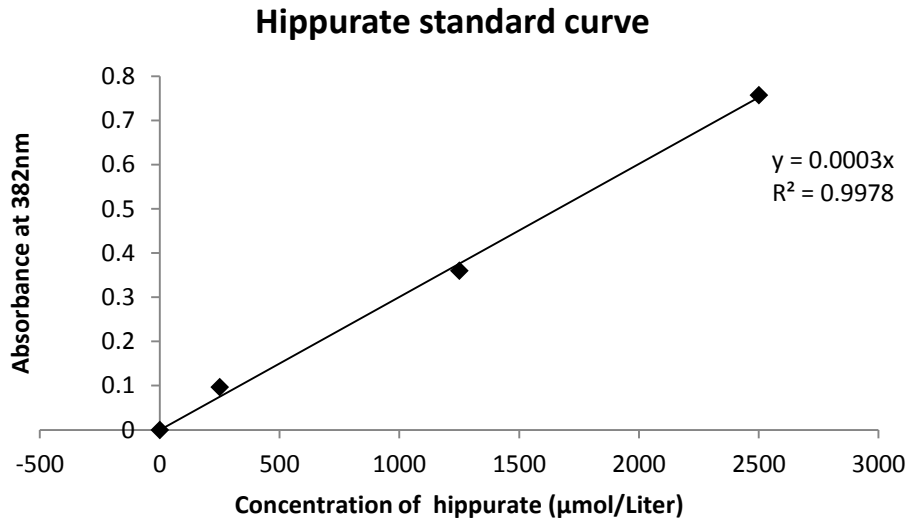


Figure 4.1: Hippurate standard curve obtained from ACE assay. The *p*-value obtained from ANOVA analysis is $1.84X10^{-18}$.

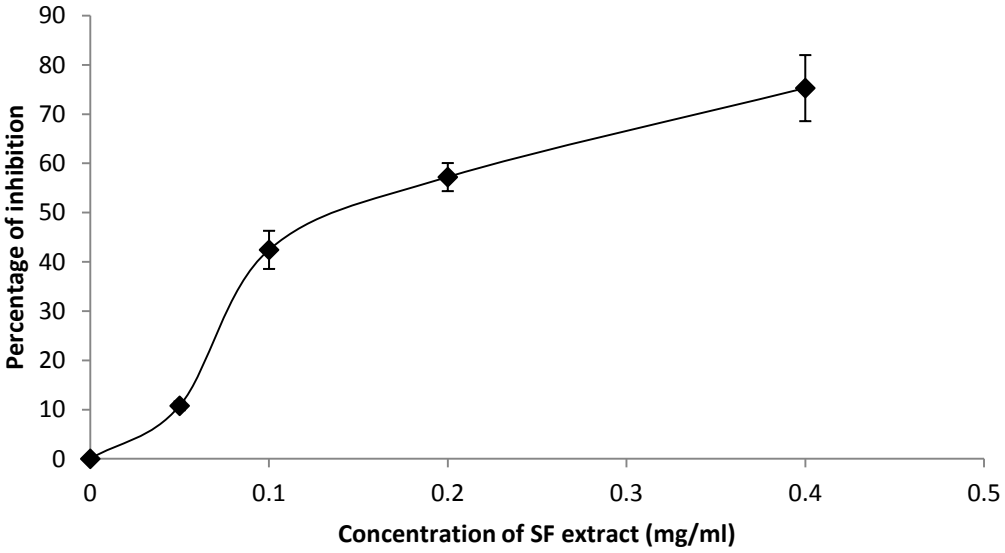


Figure 4.2: Percentage of ACE inhibition with SF extract. From the ANOVA statistical analysis, the *p*-value = $2.51X10^{-7}$.

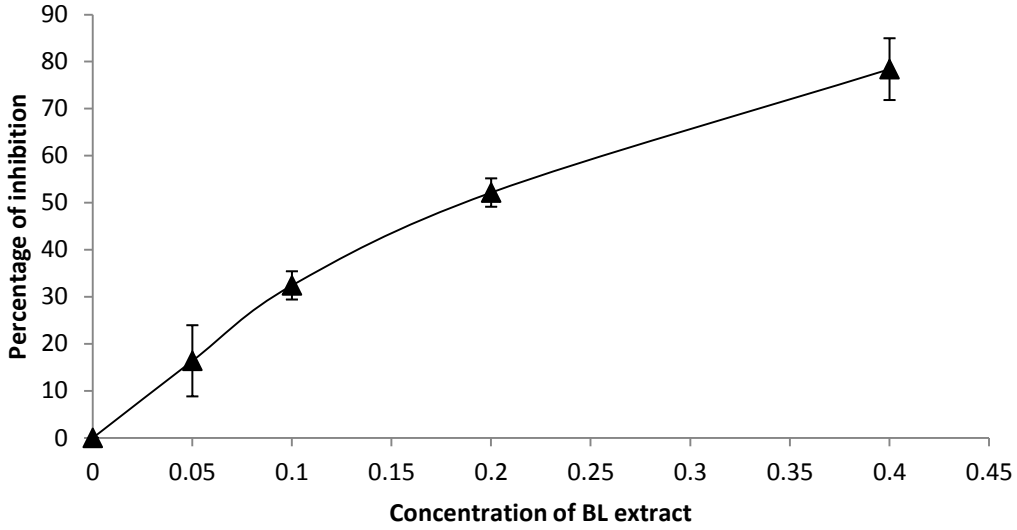


Figure 4.3: Percentage of ACE inhibition with BL extracts treatment. From the ANOVA statistical analysis, the *p*-value = $4.23X10^{-6}$.

Table 4.1: The half maximal inhibitory concentration (IC_{50}) of SF and BL extracts with S.E.M. From two-way ANOVA statistical analysis, $p = 0.401427$.

Sample	IC_{50} (mg/ml)
Small fruit extract (SF)	0.13 ± 0.023
Big Leave extract (BL)	0.18 ± 0.023

4.2 SDS-PAGE

SDS-PAGE serum protein profile is shown in Figure 4.4. The changes in serum protein profile could be observed in three molecular weight (MW) regions as shown in Figure 4.5a-c.

From the SDS-PAGE analysis, treatment with *F. deltoidea* extracts caused differential expression of serum protein bands in three regions, 6.9 kDa, 29 kDa and 35.8 kDa (Table 4.2). Several protein bands exhibited differences in their expression as compared with other treatments.

In MW region 35.8 kDa (Figure 4.5a), the protein expression profile from SHR treated (SF, BL and captopril) exhibited a reversed pattern compared to nontreated SHR. The pattern demonstrated changes towards a similar profile as normotensive. However, protein profile from the serum obtained from SHR treated with captopril did not show much difference from the serum protein profile obtained from the non-treated SHR.

In MW region 29 kDa (Figure 4.5b), similar protein profiles were found in all treatments and control, normotensive rats group. Differences were only observed in terms of their intensities.

In MW region 6.9 kDa (Figure 4.5c), only the serum profiles of SHR-SF and SHR-Cp exhibited similar profile with normotensive rats. SHR treated with BL however; did not express reversed pattern but showed rather similar pattern as in nontreated SHR.

Table 4.2: Differentially expressed protein bands found in SDS-PAGE in three MW regions.

MW region 35.8 kDa	MW region 29 kDa	MW region 6.9 kDa
52.60 ± 1.57 kDa	30.20 ± 0.41 kDa	8.67 ± 0.40 kDa
44.05 ± 1.10 kDa	23.70 ± 0.40 kDa	8.60 ± 0.68 kDa
		8.06 ± 0.32 kDa
		7.23 ± 0.28 kDa

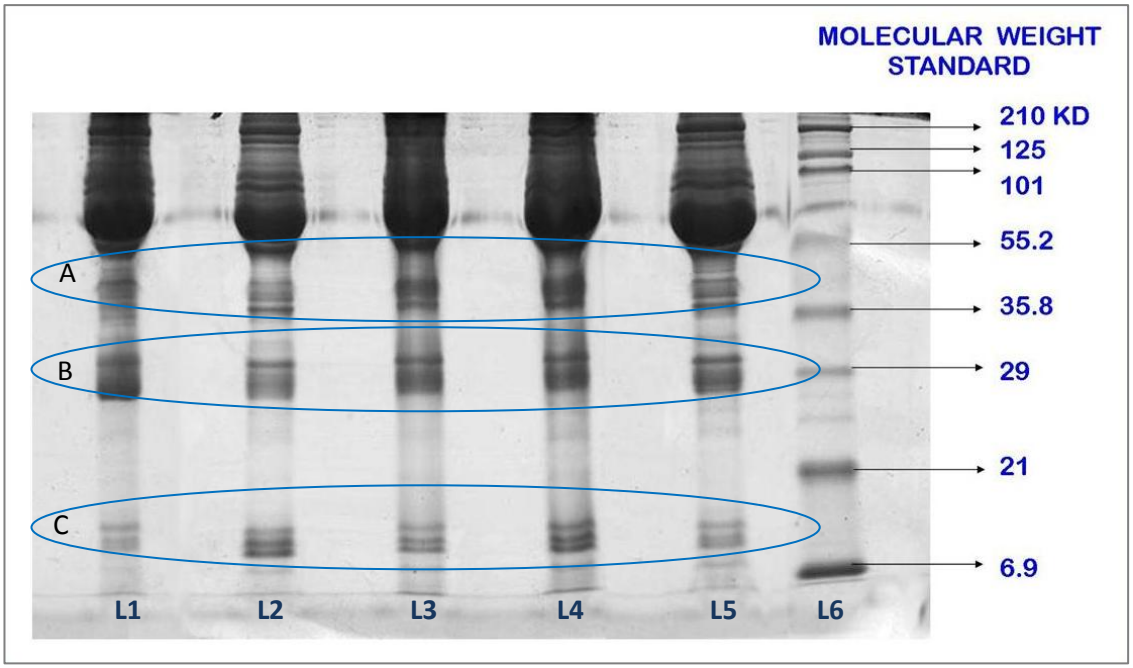


Figure 4.4: Serum protein profile separated in12.5% acrylamide gel.

Blue circles indicated the MW regions where the differences of protein profiles in different treatments were observed. (A) MW region for 35.8 kDa, (B) 29 kDa and (C) 6.9 kDa respectively.

L1 - serum from normotensive rat; L2 - nontreated SHR (SHR); L3 - SHR treated with SF (SHR-SF); L4 - SHR treated with BL (SHR-BL); L5 - SHR treated with captopril (SHR-Cp); L5 - pre-stained MW standards with specific molecular weight from 6.9-210 kDa.

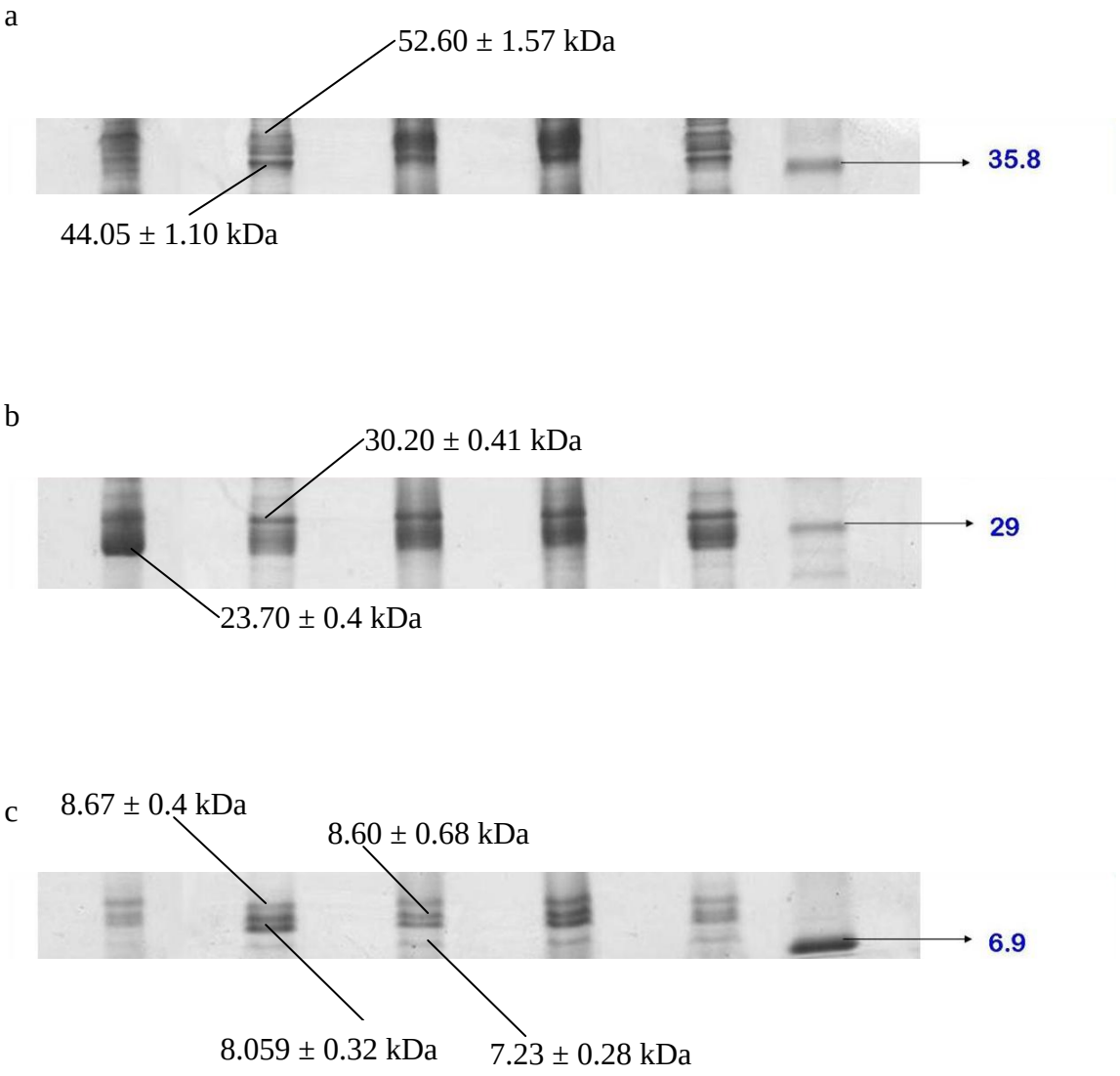


Figure 4.5 a-c: Serum protein profiles in different MW regions exhibiting the protein bands affected by the different treatments.

Different administration of both SF and BL of *F. deltoidea* exhibited protein alterations at various MW regions; 35.8 kDa (a), 29 kDa (b) and 6.9 kDa (c). Data showed the MW of the protein bands with its standard deviations.

4.3 2-DE Gel Electrophoresis

Serum protein profile of *F. deltoidea* (SF and BL extracts) treated with SHR were compared between 8-10 weeks old normotensive rats, nontreated SHR and SHR treated with SF and BL after ten days of treatment. SHR treated with captopril was used as the positive control. Normotensive rats represent normal healthy rats. Representative rat serum proteome profiles from 2-DE are shown in Figure 4.6.

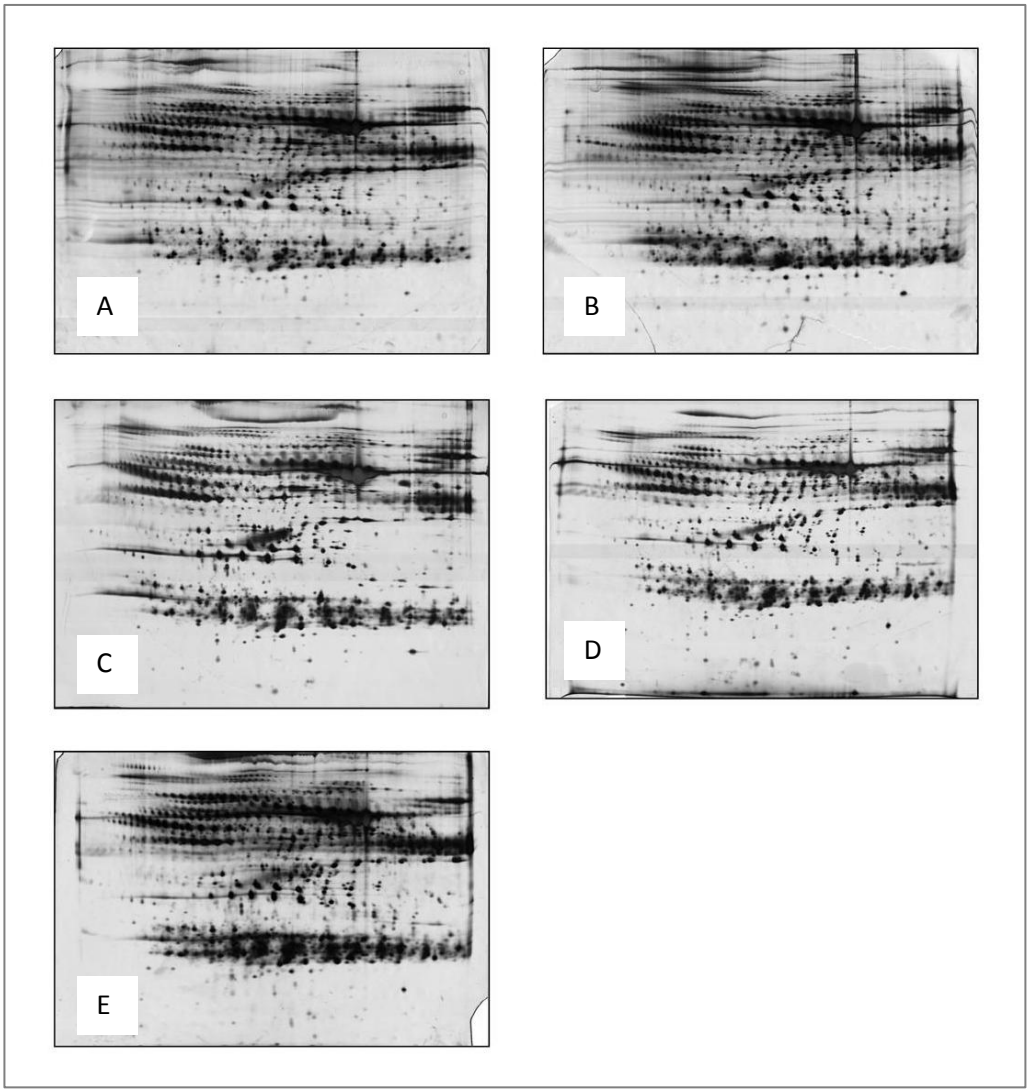


Figure 4.6: Serum proteome profiles on IPGstrips pH 4-7 separated in 11% of 2-DE gels. 100 µg/ml proteins were loaded onto the gels. The figure shows representative gels for normotensive rats (A), SHR/ disease (B), SHR-BL (C), SHR-SF (D) and SHR-Cp (E).

4.3.1 Proteome Analysis Using Image Master Platinum 7 software

Protein spots visualized by silver staining in normotensive rat, nontreated SHR, SHR-SF, SHR-BL and SHR-C gels were analysed using Image Master Platinum 7 software. The software facilitated identification of significantly differentially expressed proteins based on percentage volume of individual protein spots. Individual spots in all groups were then matched with each other to observe the protein expression dynamics. The statistical analysis of percentage of volume of individual spots obtained from the software was normalised using Excel ANOVA. Only spots with ANOVA $p < 0.05$ were deemed as significantly different and were selected for protein identification using MALDI-TOF/TOF. However, to further improve the power of testing while maintaining control over a meaningful measure of error, we have calculated the false discovery rate (FDR) by using the Benjamin and Hochberg method (Benjamini & Hochberg, 1995). From Image Master analysis, approximately 1000 protein spots were detected in each sample - normotensive rats (1238 ± 110), nontreated SHR (909 ± 63), SHR-SF (1077 ± 36), SHR-BL (1020 ± 42) and SHR-Cp (1010 ± 92). From the matched gels, 1430 matching spots were found in those groups (Normotensive rats vs nontreated SHR vs SHR-SF vs SHR-BL vs SHR-Cp). Statistical analysis from ANOVA confirmed that 12 matched spots were found to be significantly different in their expression (Figure 4.7). Based on the Benjamin and Hochberg procedure, at this level, only seven spots were significant.

In our study, the expression dynamic of protein spots in treated SHR rats (SHR-SF and SHR-BL) were compared with the non-treated SHR rats (SHR) to better understand the effects of treatment on protein expression in serum. The percentage of volume for the significantly different spots in different groups are shown in Table 4.3 and the calculated fold changes of expression dynamic are shown in Table 4.4.

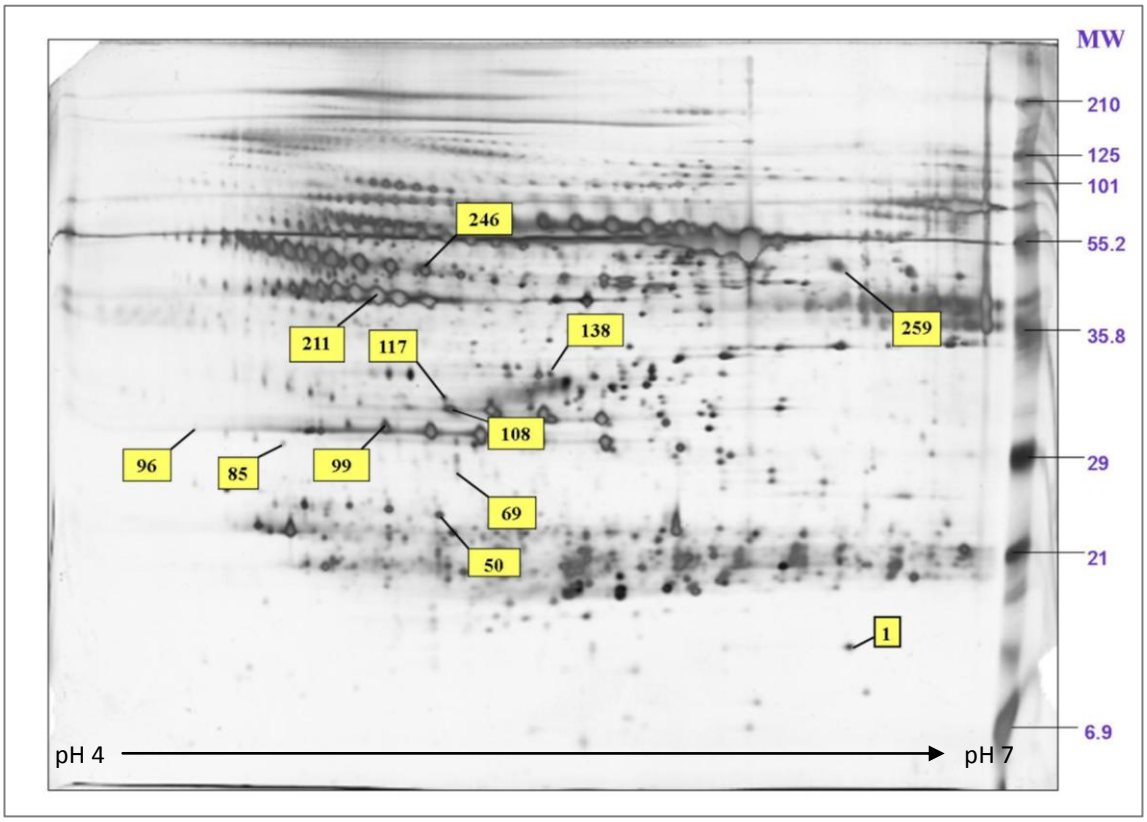


Figure 4.7: A **representative of silver-stained 2-DE gel image of rat serum proteome profile.**

Gel was loaded with 100 µg protein and were separated using IPG strip pH 4-7. Identified protein spots that showed significant difference in their expression compared to normal and nontreated SHR rats are numbered. All the numbering is obtained from gel analysis using Image Master Platinum 7.0 Software.

Table 4.3: Percentage volume of protein spots that are significantly different in expression.

Spot No	Normotensive	SHR	SHR-SF	SHR-BL	SHR-Cp	*p* - value
1	0.166963	0.068768	0.127979	0.099398	0.087419	0.020760
50	0.072497	0.123245	0.113435	0.168048	0.120118	0.007999**ϒ
69	0.124189	0.056224	0.044818	0.028093	0.028137	0.002392
85	0.008403	0.029586	0.026316	0.051608	0.025363	6.27×10^{-4}** ϒ
96	0.009116	0.043230	0.087596	0.028094	0.024504	0.008829**ϒ
99	0.179683	0.243203	0.301891	0.469338	0.293398	0.001826** ϒ
108	0.246078	0.230608	0.279863	0.377688	0.287745	0.046979** ϒ
117	0.080883	0.032488	0.058628	0.102981	0.064328	3.26×10^{-5}** ϒ
138	0.087800	0.046345	0.043946	0.094449	0.055478	0.021036
211	0.348065	0.927473	0.535445	0.568058	0.173290	0.034403
246	0.348065	0.335413	0.423150	0.448758	0.173290	0.001991 ϒ
259	0.256395	0.192732	0.212440	0.315238	0.244894	0.040552

Percentage of spot volume obtained from the Image Master Platinum 7.0 Software was used in the comparison of protein expression. SHR-SF: SHR treated with SF extract, SHR-BL: SHR treated with BL extract, SHR Cp: SHR treated with captopril, SD: Normotensive rat, SHR: nontreated SHR rats.

** spots with ANOVA *p*-value <0.01. ϒ exhibited significance in FDR analysis.

Table 4.4: **The fold changes of the expression dynamic in different treatments compared to the diseased group.**

Spot No.	Normotensive	SHR-SF	SHR-BL	SHR-Cp
1	+2.43	+1.86	+1.44	+1.27
50	-1.70	-1.09	+1.36	-1.03
69	+0.22	-1.25	-2.00	-2.00
85	-3.52	-1.12	+1.74	-1.17
96	-6.32	+2.03	-1.54	-1.77
99	-1.35	+1.24	+1.93	+1.21
108	+1.07	+1.21	+1.64	+1.25
117	+2.49	+1.80	+3.17	+1.98
138	+1.89	-1.05	+2.04	+1.20
211	-2.66	-1.73	-1.63	-5.35
246	+1.11	+1.26	+1.34	-8.35
259	+1.13	+1.10	+1.64	+1.27

(+) indicates up-regulation of the protein, (-) indicates down-regulation of the protein.

4.4 MALDI-TOF/TOF mass spectrometry

Significant differentially expressed protein spots were then processed for identification by mass spectrometry (MALDI-TOF/TOF). All identified spots are shown in Table 4.5. From 12 spots, only one protein spot (Spot 96) was not identified. The remaining spots were identified as plasma retinol binding protein (RETBP), serum albumin precursor (ALBU), clusterin precursor (CLUS), complement c3 precursor (CO3), haptoglobin (HPT), alpha 1 macroglobulin (A1M), actin (ACTB), alpha 1 antiproteinase (A1AT), alpha 2H glycoprotein (FETUA) and Apolipoprotein H (APOH). Spots 108 and 117 were both identified as A1M. Protein ID was accepted when the MOWSE protein score was more than the significance score suggested by the software (Figure 4.8).

Table 4.5: The protein ID obtained from MALDI-TOF-TOF. Shows the data acquired in the mass spectrometric analysis.

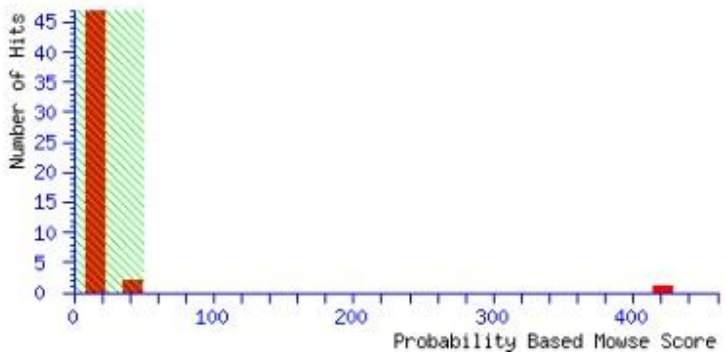
Matched protein identity	Spot	Protein Abbreviation	SwissProt Accession Number	Theorotical (kDa/pI)	Experimental (kDa/pI)	MOWSE Protein score	Sequence Coverage	Peptide match
Plasma retinol binding protein	1	RETBP	P04916	23.2/ 5.69	10.8/6.51	596	45%	7
Serum albumin precursor	50	ALBU	P02770	68.7/6.09	19.9/5.21	82	16%	3
Clusterin precursor (Sulfated glycoprotein 2)	69	CLUS	P05371	51.3/5.47	24.9/5.26	81	3%	1
Complement C3	85	CO3	P01026	186.4/ 6.12	28.6/4.69	200	6%	5
Haptoglobin precursor	99	HPT	P06866	38.5/6.10	30.5/5.02	421	35%	6
Alpha 1 macroglobulin precursor	108, 117	A1M	P 01023	167.0/6.46	33.6, 36.2/ 5.22,5.21	547	10%	8
Actin, cytoplasmic 1 (beta-actin)	138	ACTB	P60711	41.7/5.29	36.2/5.56	280	23%	4
Alpha 1 antiproteinase precursor	211	A1AT	P 17475	46.1/ 5.70	58.2/5.00	609	20%	9
Alpha 2HS glycoprotein precursor	246	FETUA	P 24090	37.9/ 6.05	65.2/5.15	237	14%	3
Apolipoprotein H precursor	259	APOH	P 26644	33.1/ 8.59	68.4/6.46	311	28%	9

A

User :
Email :
Search title : SampleSetID: 902, AnalysisID: 1971, MaldiWellID: 71953, SpectrumID: 71367, Path=\SAK2\011009\atiq
Database : Sprot 080307 (261513 sequences; 95638062 residues)
Taxonomy : Rattus (5877 sequences)
Timestamp : 2 Oct 2009 at 03:03:09 GMT
Warning : A Peptide summary report will usually give a much clearer picture of MS/MS search results.
Top Score : 421 for HPT_RAT, Haptoglobin precursor (Liver regeneration-related protein LRRG173) [Contains: Haptoglobin alpha cha

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 50 are significant (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



B

MATRIX SCIENCE **Mascot Search Results**

Protein View

Match to: HPT_RAT Score: 421 Expect: 4.7e-039
Haptoglobin precursor (Liver regeneration-related protein LRRG173) [Con

Nominal mass (M_r): 38539; Calculated pI value: 6.10
NCBI BLAST search of HPT_RAT against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Rattus norvegicus

Variable modifications: Carbamidomethyl (C),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: 35%

Matched peptides shown in Bold Red

```
     1 MRALGAVVTL LLWGQLFAVE LGNDATDIED DSCPKPPEIA NGYVEHLVRY
    51 RCRQFYKLQT EGDGIYTLNS EKQWVNPAAG DKLPKCEAVC GKPKHPVDQV
   101 QRIIGGSMDA KGSFPWQAKM ISRHGLITGA TLISDQWLLT TAQNLFLNHS
   151 ENATAKDIAP TLTLYVGKNQ LVEIEKVVLH PERSVVDIGL IKLKQKVLVT
   201 EKVMPICLPS KDYVAPGRMG YVSGWGRNVN FRFTERLKYV MLPVADQEKC
   251 ELHYEKSTVP EKKGAVSPVG VQPILNKHTF CAGLTKYEED TCYGDAGSAF
   301 AVHDTEEDTW YAAGILSFDK SCAVAEYGVY VRATDLKDWV QETMAKN
```

Figure 4.8: The representative data (haptoglobin; spot 99) obtained from MALDI-TOF/TOF (A). The significant MOWSE score was determined by the software, circled in blue. Matched peptides of the peptide sequence are shown in bold red at the right of the figure (B).

Table 4.6: MASCOT search results indicating the matched peptide sequence and the MOWSE score.

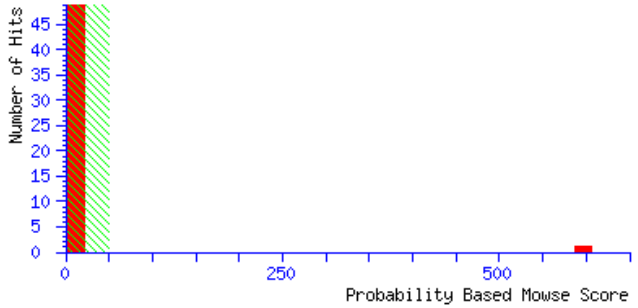
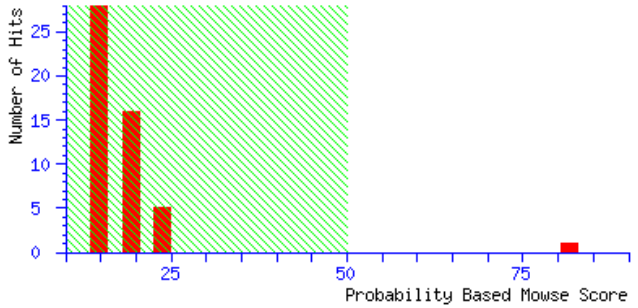
Peptide sequence	Ion score	Amino acid	MOWSE score
RETBP spot			
R.QEELCLER.Q + Carbamidomethyl (C)	22	174-181	
R.FSGLWYAIAK.K	75	38-47	
K.YWGVASFLQR.G	61	108-117	
R.QRQEELCLER.Q + Carbamidomethyl (C	24	172-181	
R.LQNLDGTCADSYSFVFSR.D + Carbamidomethyl (C)	147	140-157	
K.KDPEGLFLQDNIIAEFSVDEK.G	78	48-68	
R.LLSNWEVCADMVGTFTDTEDPAK.F + Carbamidomethyl (C)	98	81-108	
ALBU spot			
K.LVQEVTDFAK.T	20	66-75	
K.DVFLGTFLYEYSR.R	39	348-360	
K.APQVSTPTLVEAAR.N	10	439-452	

Table 4.6 (continued): MASCOT search results indicating the matched peptide sequence and the MOWSE score.

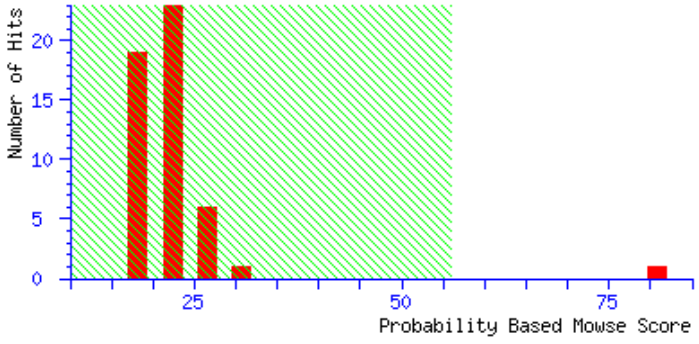
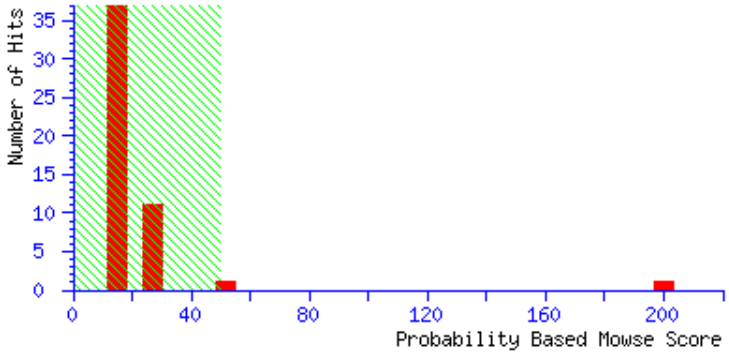
Peptide sequence	Ion score	Amino acid	MOWSE score
CLUS spot			
R.QQSQVLDAMQDSFTR.A	76	167-181	
CO3 spot			
K.RQEALELIK.K	15	1042-1050	
K.KGYTQQLAFK.Q	16	1051-1060	
K.GYTQQLAFK.Q	47	1052-1060	
R.DICEGQVNSLPGSINK.A + Carbamidomethyl (C)	72	1156-1171	
K.DFDSVPPVVR.W	33	1245-1254	

Table 4.6 (continued): MASCOT search results indicating the matched peptide sequence and the MOWSE score.

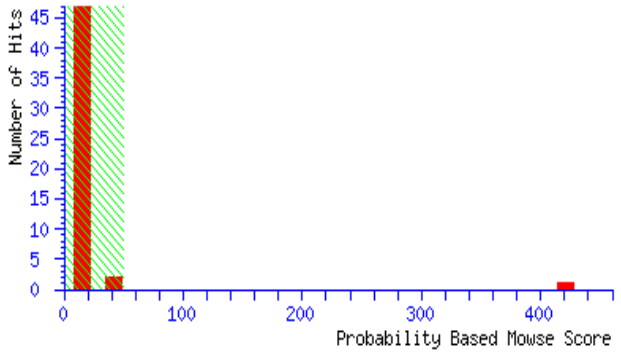
Peptide sequence	Ion score	Amino acid	MOWSE score
HPT spot			
K.VMPICLPSKDYVAPGR.M + Carbamidomethyl (C)	37	203-218	
R.MGYVSGWGR.N	65	219-227	
K.YVMLPVADQEK.C + Oxidation (M)	51	239-249	
K.HTFCAGLTK.Y + Carbamidomethyl (C)	45	278-286	
K.SCAVAEYGVYVR.A + Carbamidomethyl (C)	69	321-332	
R.ATDLKDWVQETMAK.N	98	333-346	

Table 4.6 (continued): MASCOT search results indicating the matched peptide sequence and the MOWSE score.

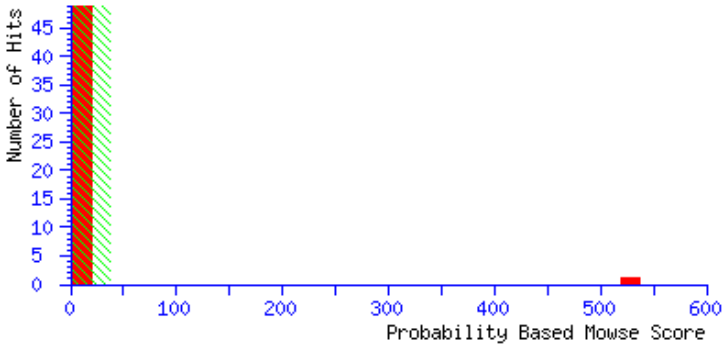
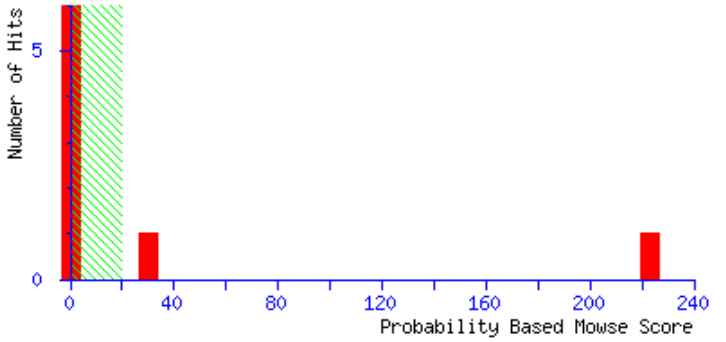
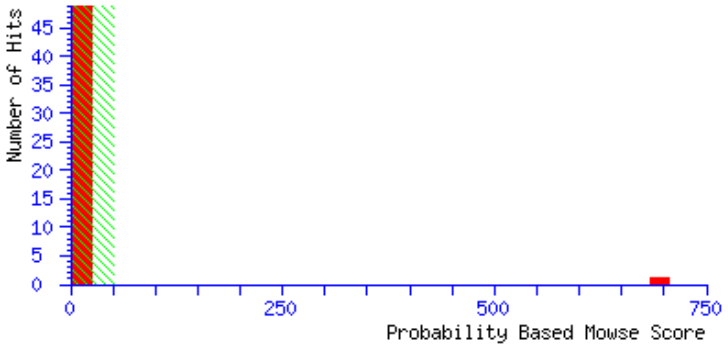
Peptide sequence	Ion score	Amino acid	MOWSE score
A1M spot			
R.LADLPGNYITK.V	65	1328-1338	
K.VNTLPLNFDK.A	45	1369-1378	
K.VSGSGCVYLQTSLK.Y + Carbamidomethyl (C)	68	1339-1352	
K.YNILPEAEGEAPFTLK.V	38	1353-1368	
K.MVSGFIPVKPSVK.K	89	1409-1421	
K.KLQDQSNIQR.T	60	1422-1431	
K.LQDQSNIQR.T	51	1423-1431	
R.TEVNTNHVLIYIEK.L	111	1432-1445	
ACTB spot			
R.AVFPSIVGRPR.H	36	29-39	
R.VAPEEHPVLLTEAPLNPK.A	69	96- 13	
R.GYSFTTTAER.E	33	197-206	
K.QEYDESGPSIVHR.K	85	360-372	

Table 4.6 (continued): MASCOT search results indicating the matched peptide sequence and the MOWSE score.

Peptide sequence	Ion score	Amino acid	MOWSE score
A1AT spot			
K.ISSNLADFAFSLYR.E	88	44 – 57	
K.NNYHSEAFSVNFADSEEAK.K	27	154 – 172	
K.NNYHSEAFSVNFADSEEAKK.V	103	154 – 173	
K.KVINDYVEK.G	33	173 - 181	
K.WKRPFNPEHTR.D	27	212 - 222	
K.RPFNPEHTR.D	34	214 – 222	
K.MQHLEQTLTK.D	50	278 – 287	
K.LSISGTYNLK.T	80	309 – 318	
R.VFNNDADLSGITEDAPLK.L	166	329 – 346	
FETUA spot			
R.LGGEEVSVACK.L + Carbamidomethyl (C)	74	238 - 248	
R.HAFSPVASVESASGEVLHSPK.V	93	306 - 326	
K.VGQPGDAGAAGPVAPLCPGR.V	49	327 - 346	



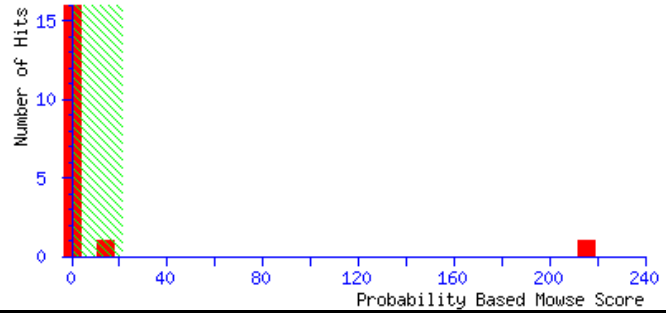
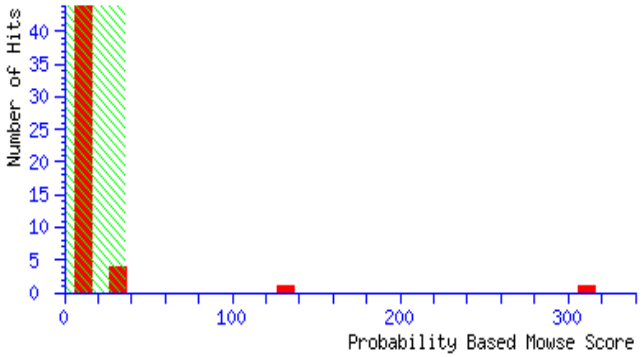


Table 4.6 (continued): MASCOT search results indicating the matched peptide sequence and the MOWSE score.

Peptide sequence	Ion score	Amino acid	MOWSE score
APOH spot			
K.CTEEGKWSPELPVCAR.I + 2 Carbamidomethyl (C)	9	76 - 91	
K.WSPELPVCAR.I + Carbamidomethyl (C)	24	82 – 91	
R.ITCPPPPIPK.F + Carbamidomethyl (C)	48	92 - 101	
K.AVFGCHETYKLDGPEEVECTK.T + 2Carbamidomethyl (C)	5	182 – 202	
K.LDGPEEVECTK.T + Carbamidomethyl (C)	48	192 – 202	
K.KATVLYQGQR.V	47	222 – 231	
K.ATVLYQGQR.V	33	223 – 231	
K.KCSYTEEAQCIDGTIEIPK.C + 2Carbamidomethyl (C)	29	258 – 276	
K.EHSSLAFWK.T	67	280 - 288	



4.5 Identified proteins related to hypertension

Seven proteins were found to be related to hypertension i.e. complement C3 precursor (CO3), haptoglobin (HPT), alpha 1 macroglobulin precursor (A1M), alpha 1 antiproteanase or alpha 1 antitrypsin (A1AT), Apolipoprotein (APOH), serum albumin precursor (ALBU) and retinol binding protein (RETBP).

The 3D view image and expression dynamic of representative spots related to hypertensive protein are shown in Figures 4.9 – 4.15. The related spots are circled in green indicating the area measured in the analysis. The percentage volume for each spot was averaged and used to evaluate the expressions of the protein in different treatments.

In this study, expression dynamic was calculated based on the ratio of percentage volume of the protein spots from 2-DE profiles of treated and non-treated SHR rats to monitor the effect of the extracts on hypertension.

4.5.1 Complement C3 (CO3)

The expression of this protein in normotensive rats is down-regulated by three folds compared to SHRs. Treatment with SF caused normalization of the aberrant expression of CO3 in SHR. CO3 expression in SHR-SF showed reversed pattern after the treatment as shown in Table 4.4 and Figure 4.9. However, treatment with BL extracts caused proportional increase of CO3.

Protein identification (ID) in MALDI-TOF/TOF exhibited significant score with a MOWSE score of 200 with 6% matched peptides. The matched peptides were observed between positions 1042 to 1254 of the protein sequence (Table 4.6).

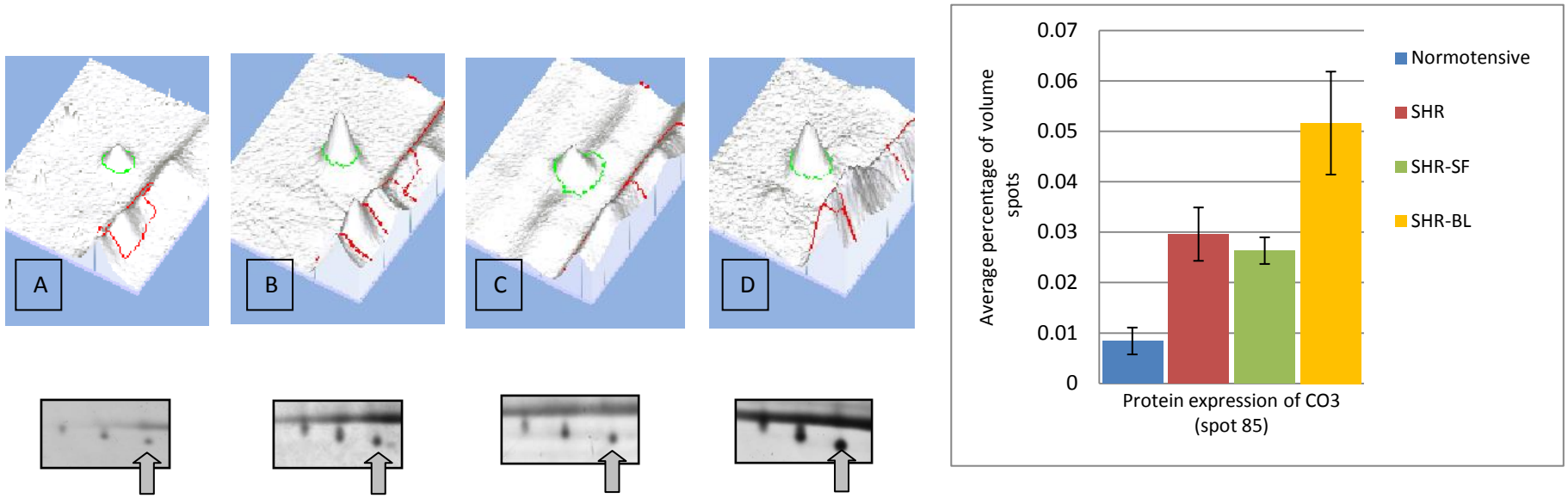


Figure 4.9: The 3D view of protein expression obtained from Image Master Platinum 7.0 Software for CO3.

(A) Representative spot 3D view for normotensive rat, (B) nontreated SHR rat, (C) SHR-treated with SF extract, (D) SHR treated with BL extract. Histogram shows the the average of spot percentage of volume obtained from the quantitaive analysis using the software.

4.5.2 Haptoglobin (HPT)

The expression of HPT in normotensive rats was down-regulated 1.35 fold as compared to SHRs. Treatments with these extract (SF and BL) caused significant increase of the expression of haptoglobin in SHR. The protein expression of HPT in treated rats increased 1.24 and 1.92 fold as compared to SHRs as shown in Table 4.4 and Figure 4.10.

Protein identification (ID) in MALDI-TOF-TOF exhibited significant score with a MOWSE score of 421 with 35% matched peptides. Matched peptides were observed between positions 37 to 98 of the protein sequence (Table 4.6).

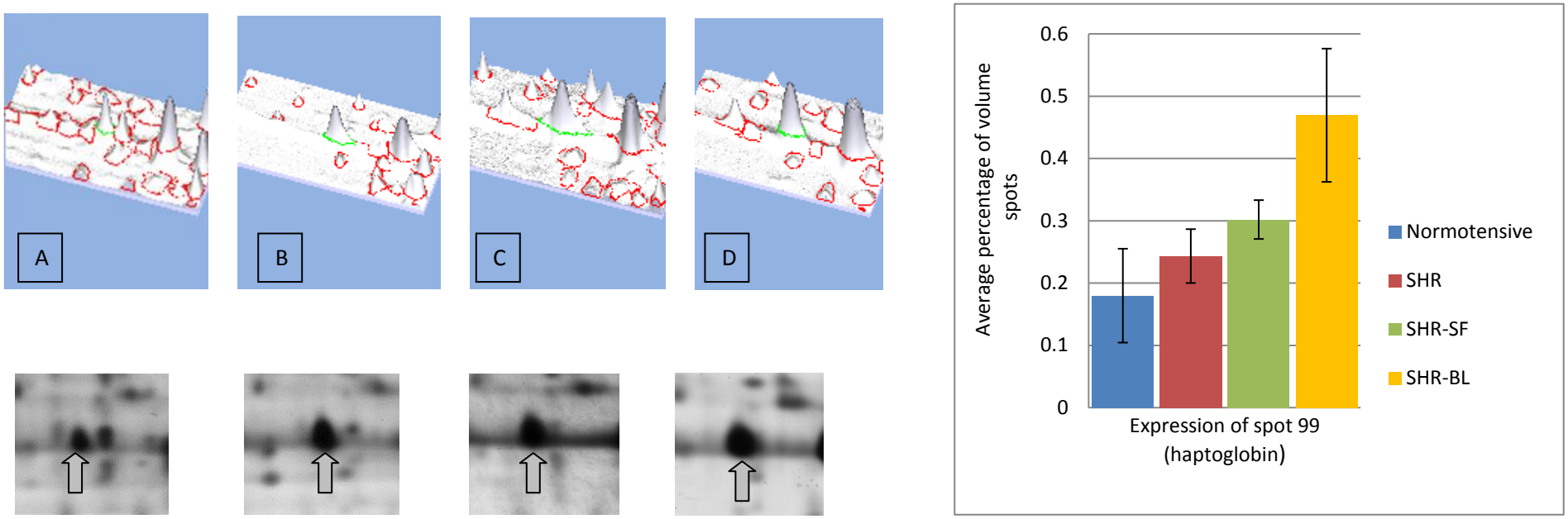


Figure 4.10: The 3D view of protein expression obtained from Image Master Platinum 7.0 Software for HPT.

(A) Representative spot 3D view for normotensive rat, (B) nontreated SHR rat, (C) SHR-treated with SF extract, (D) SHR treated with BL extract. Histogram shows the the average of spot percentage of volume obtained from the quantitaive analysis using the software.

4.5.3 Alpha 1 macroglobulin (A1M)

Two protein spots were identified as A1M; spots 108 and 117. The fold changes of normotensive and SHR-BL compared to SHRs was up-regulated 1.12 and 2.04 fold respectively. However, SHR-SF was down-regulated 1.13 fold compared to SHRs.

Along the treatment, all the treatments showed up-regulation of protein expression in normotensive rats, SHR-SF and SHR-BL with fold changes of 2.49, 1.80 and 3.17. The expression of protein spot 117 (alpha 1 macroglobulin) can be observed in Table 4.4 and Figure 4.11.

Protein identification (ID) by MALDI-TOF-TOF exhibited significant score with 541 MOWSE score and 10% matched peptides. The peptides matched were observed between position 1328 to 1445 of the protein sequence (Table 4.6).

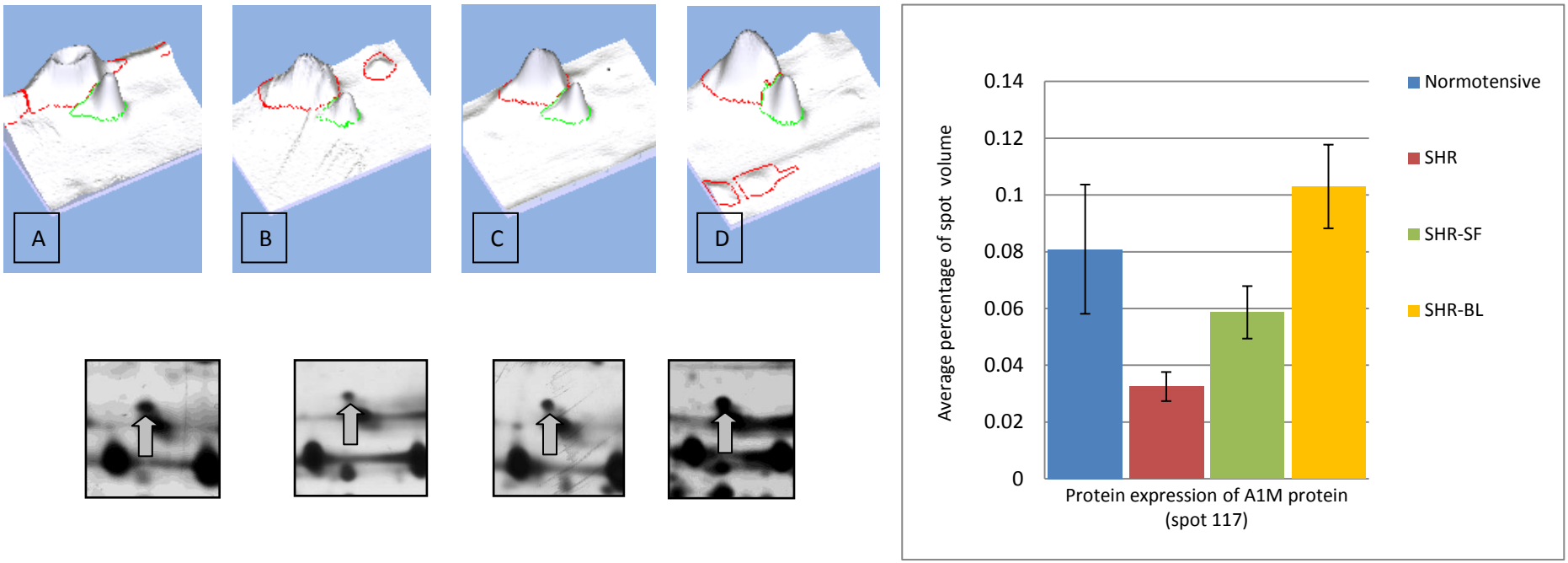


Figure 4.11: The 3D view of protein expression obtained from Image Master Platinum 7.0 Software for A1M.

(A) Representative spot 3D view for normotensive rat, (B) nontreated SHR rat, (C) SHR-treated with SF extract, (D) SHR treated with BL extract. Histogram shows the the average of spot percentage of volume obtained from the quantitaive analysis using the software.

4.5.4 Serum albumin (ALBU)

The expression of ALBU in SHR-SF and normotensive rats was down-regulated by 1.08 and 1.70 fold compared to SHRs. However, SHR-BL exhibited a contrary effect. Treatment with SF caused normalization of the expression of ALBU in SHR as shown in Table 4.4 and Figure 4.12. However, treatment with BL extracts caused a proportional increase of ALBU.

Protein identification by MALDI-TOF/TOF exhibited significant score with a MOWSE score of 82 and 16% matched peptides. The matched peptides were observed at positions 66-75, 348-360 and 439-452 of the protein sequence (Table 4.6).

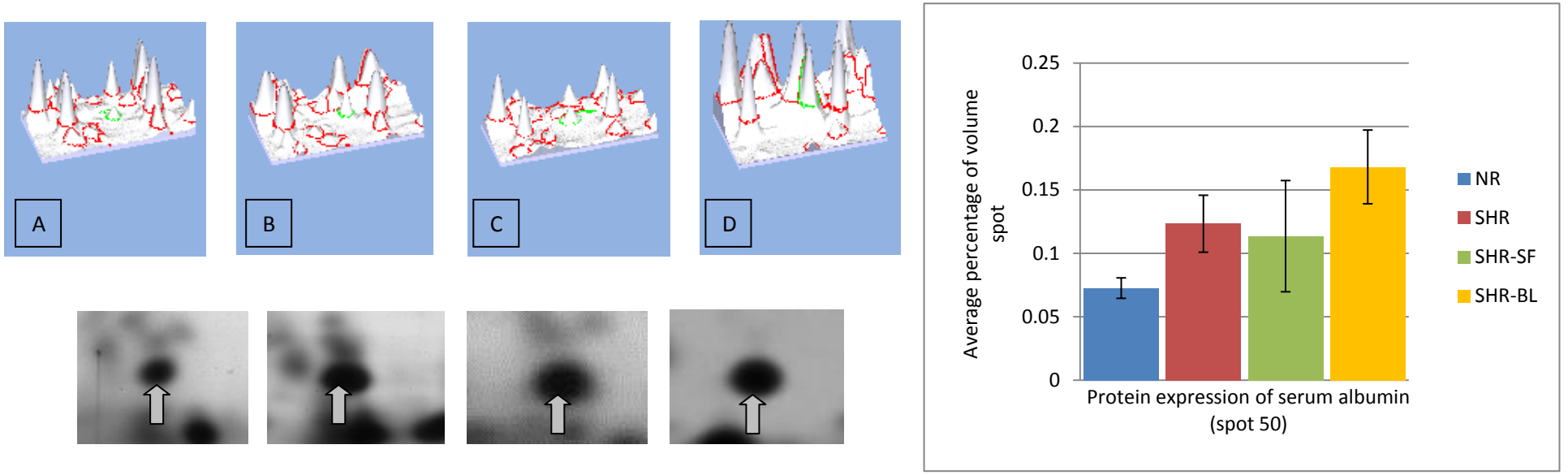


Figure 4.12: The 3D view of protein expression obtained from Image Master Platinum 7.0 Software for ALBU fragment.

(A) Representative spot 3D view for normotensive rat, (B) nontreated SHR rat, (C) SHR-treated with SF extract, (D) SHR treated with BL extract. Histogram shows the the average of spot percentage of volume obtained from the quantitaive analysis using the software.

4.5.5 Alpha 1 antiproteinase or Alpha 1 antitrypsin (A1AT)

The expression of A1AT in normotensive rats was down-regulated 2.66 fold compared to SHRs. Treatment with SF and BL caused normalization of the aberrant expression of A1AT in SHR. The expression of this protein was decreased by 1.73 and 1.63 folds in SHR-SF and SHR-BL respectively (Table 4.4 and Figure 4.13).

Protein identification by MALDI-TOF-TOF exhibited significant score with a MOWSE score of 609 and 20% matched peptides. The matched peptides were observed between positions 44 to 346 of the protein sequence (Table 4.6).

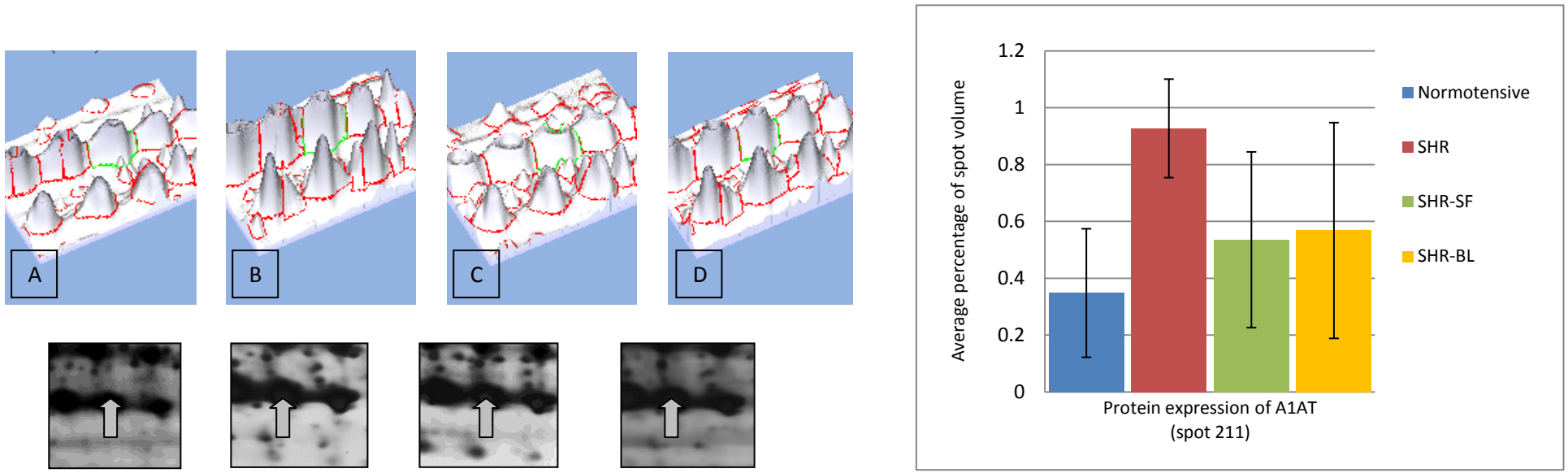


Figure 4.13: The 3D view of protein expression obtained from Image Master Platinum 7.0 Software for A1AT.

(A) Representative spot 3D view for normotensive rat, (B) nontreated SHR rat, (C) SHR-treated with SF extract, (D) SHR treated with BL extract. Histogram shows the the average of spot percentage of volume obtained from the quantitaive analysis using the software.

4.5.6 Apolipoprotein H (APOH)

The expression of APOH in normotensive rat, SHR-SF and SHR-BL was up-regulated by 1.13, 1.10 and 1.64 fold compared to SHRs. Treatment with SF and BL caused normalization of the aberrant expression of APOH in SHR as shown in Table 4.4 and Figure 4.14.

Protein identification by MALDI-TOF-TOF exhibited significant score with a MOWSE score of 311 and 28% matched peptides. The matched peptides were observed between positions 76 to 288 of the protein sequence (Table 4.6).

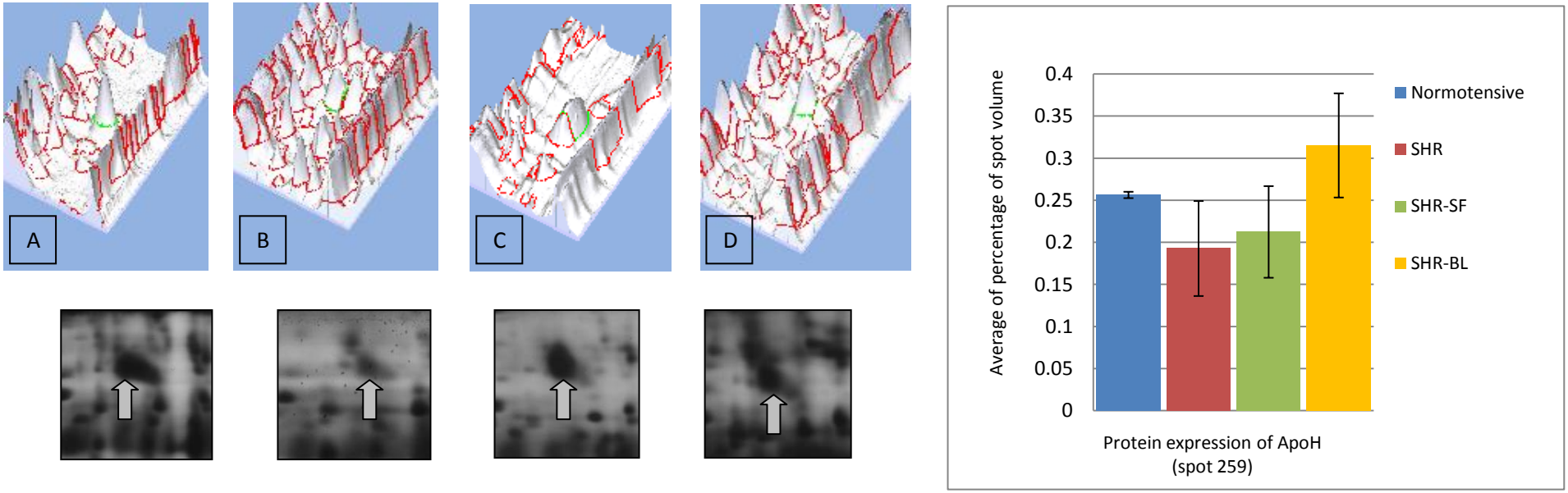


Figure 4.14: The 3D view of protein expression obtained from Image Master Platinum 7.0 Software for APOH.

(A) Representative spot 3D view for normotensive rat, (B) nontreated SHR rat, (C) SHR-treated with SF extract, (D) SHR treated with BL extract. Histogram shows the the average of spot percentage of volume obtained from the quantitaive analysis using the software.

4.5.7 Plasma retinol binding protein (RETBP)

The expression RETBP in normotensive rat, SHR-SF and SHR-BL was up-regulated by 2.43, 1.86 and 1.45 fold compared to the SHRs. The expression of RETBP in SHR-SF and SHR-BL was increased after the treatment as compared to SHRs. Treatment with SF and BL caused normalization of the expression of RETBP in SHR as shown in Table 4.4 and Figure 4.15.

Protein identification by MALDI-TOF-TOF exhibited significant score with a MOWSE score of 596 and 45% matched peptides. The matched peptides were observed between positions 38 to 181 of the protein sequence (Table 4.6).

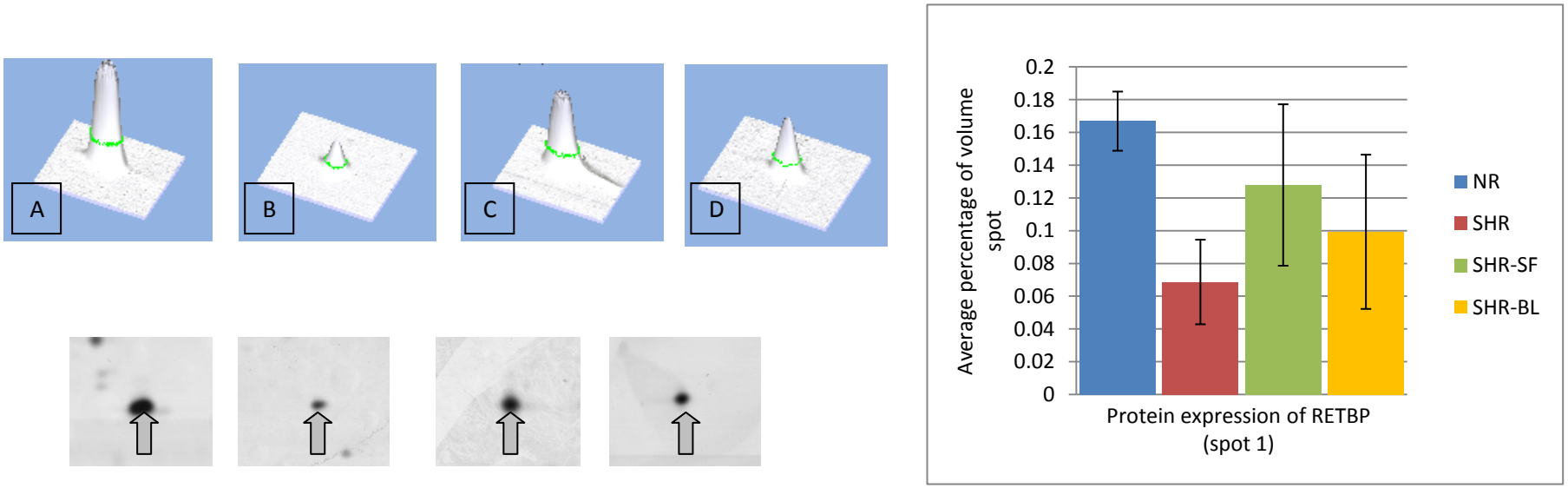


Figure 4.15: The 3D view of protein expression obtained from Image Master Platinum 7.0 Software for RETBP.

A) Representative spot 3D view for normotensive rat, (B) nontreated SHR rat, (C) SHR-treated with SF extract, (D) SHR treated with BL extract. Histogram shows the the average of spot percentage of volume obtained from the quantitaive analysis using the software.

4.6 SELDI-TOF mass spectrometry

Sera from rat treated with *F. deltoidea* aqueous extracts were examined using CM-10 arrays to monitor alterations of any native proteins in the serum proteome. SELDI analysis produced spectra with peaks representing low MW serum proteins. Peaks labeling was performed by the Chipergen Express Data Manager in the Ciphergen Protein Software 5.0. The amount of individual serum proteins were estimated based on the peak intensity of the mass spectral signal. The mass/charge ratio (m/z) obtained from the analysis is equivalent to the molecular weight of each protein. Two analyses were performed in this study i.e. univariate analysis and multivariate analysis. Univariate analysis is an analysis that is used to identify the significance of protein expression by using EDM. The intensity of labelled peaks were then transfered to Biomarker Pattern Software in multivariate analysis to correctly classify the protein peaks and generate regression tree models. This model will identify potential biomarker candidates depending on the percentage of cases based on the intensity of the peaks. The alterations of protein expressions were first monitored in normotensive rats and SHR, followed by observations after the administration of extracts.

4.6.1 Univariate analysis

Using EDM analysis, significant differentially expressed proteins ($p < 0.05$) were identified in protein peaks ranging from 2,500 to 40,000 Da. In the comparison between normotensive rats and SHR, all significant peaks from SHR showed down regulation as compared to normotensive rats (expression dynamic range 1.8 to 32). In SELDI analysis, the relative expression of proteins are represented in the form of intensity.

The down-regulated expression of protein peaks with m/z 3493.2, 22874 and 33086 were two times lower. Other peaks were two times higher than respective peak in normotensive rat (NR) with m/z 2317.6 showing the highest down-regulation with 32.34 fold change.

After the administration of SF extract, seven protein peaks were found to be significantly altered. Those peaks were the ones with m/z 3493.2, 8303.3, 9445.0, 16531.0, 18986, 22044 and 33086 as shown in Figures 4.16-4.22. However, these alterations did not show any extreme normalizing effetcs. Only peaks with m/z 8303.3 and 9445.0 were slightly up-regulated after 10 days of treatment.

In the case of BL extract only three peaks were found to be significantly altered after the treatment. The altered proteins were those with m/z 2317.6, 4180.6 and 8303.3. All these proteins were found to be significantly increased as compared to SHR. Typical spectra are shown in Figures 4.23-4.25.

Table 4.7: SELDI-TOF analysis demonstating the differences in protein profile of sera from normotensive and SHR rats.

m/z	Peak Intensity		Fold Changes	*p*-value
	Normotensive (NR)	SHR		
3 003.4	10.25	3.3	3.11	0.039
11 029.0	21.58	7.08	3.61	0.039
22 044.0	59.47	25.32	2.35	<< 0.01
33 086.0	49.72	25.35	1.96	< 0.01
4 180.6	26.83	11.67	2.30	0.014
13 230.0	43.99	14.01	3.14	0.028
8 303.3	37.62	8.27	4.55	<< 0.01
2 317.6	25.64	0.79	32.34	0.014
9 445.0	9.36	2.99	3.13	<<0.01
3 493.2	94.32	47.76	1.97	0.014
16 531.0	60.87	22.52	2.70	0.014
1 617.5	30.31	4.13	7.35	0.028
1 705.7	67.15	25.27	2.66	0.039
1 794.3	45.27	16.79	2.70	0.028
22 874.0	6.55	3.59	1.82	0.028
18 986.0	2.63	1.27	2.06	0.014

Data shows the down-regulation of protein peaks belongs to the non-treated SHR as compared to the same protein peaks belongs to normotensive rats. *p*-value was obtained from the EDM.

Modification of individual protein by administration SF extract

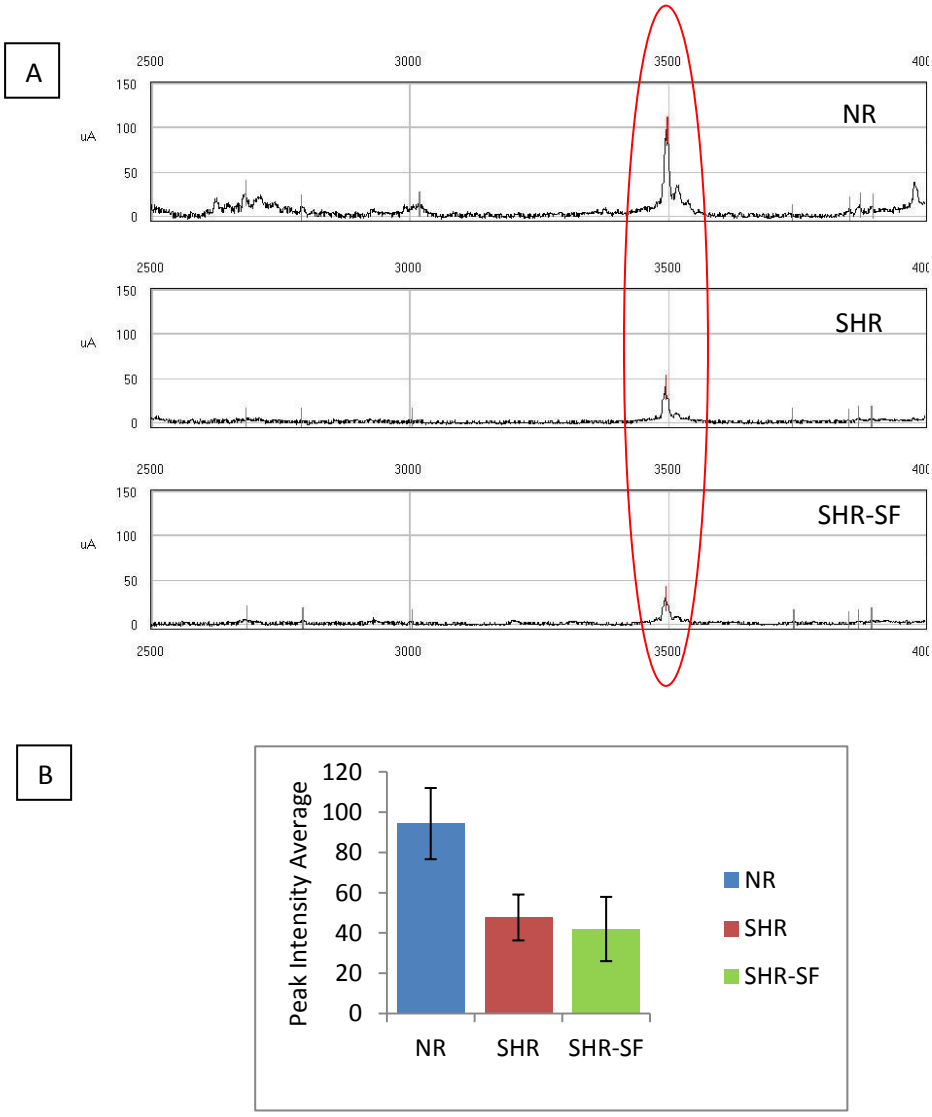


Figure 4.16: Changes of serum protein profiles (m/z 3493.2) upon administration of SF *F. deltoidea*. (A) Signal spectra of relative peak intensities at m/z 3493.2 in three different groups. Relative peak were exposed as mean, averaged m/z and intensity (n=4). (B) Histogram illustrating peak intensity average.

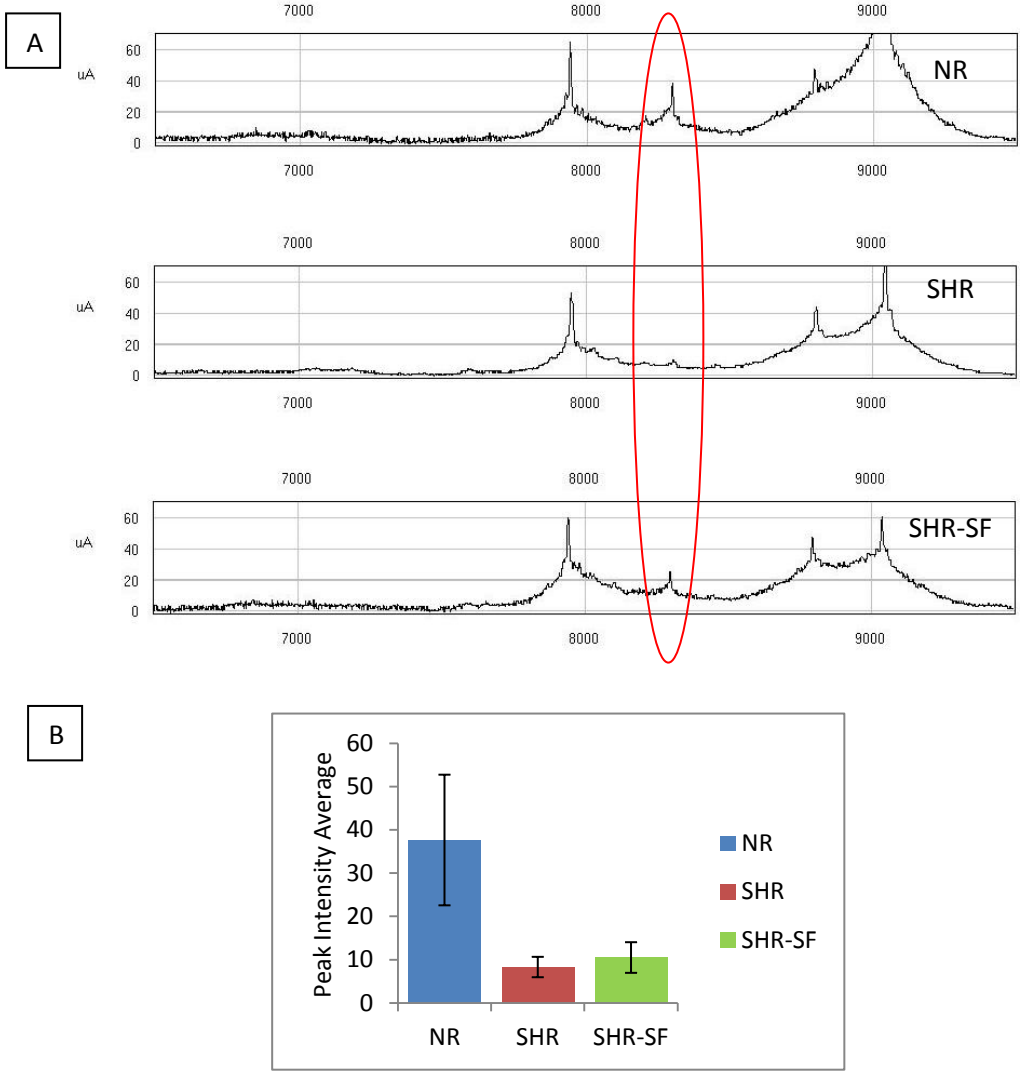


Figure 4.17: Changes of serum protein profiles (m/z 8303.3) upon administration of SF *F. deltoidea*. (A) Signal spectra of relative peak intensities at m/z 8303.3 in three different groups. Relative peak were exposed as mean, averaged m/z and intensity (n=4). (B) Histogram illustrating peak intensity average.

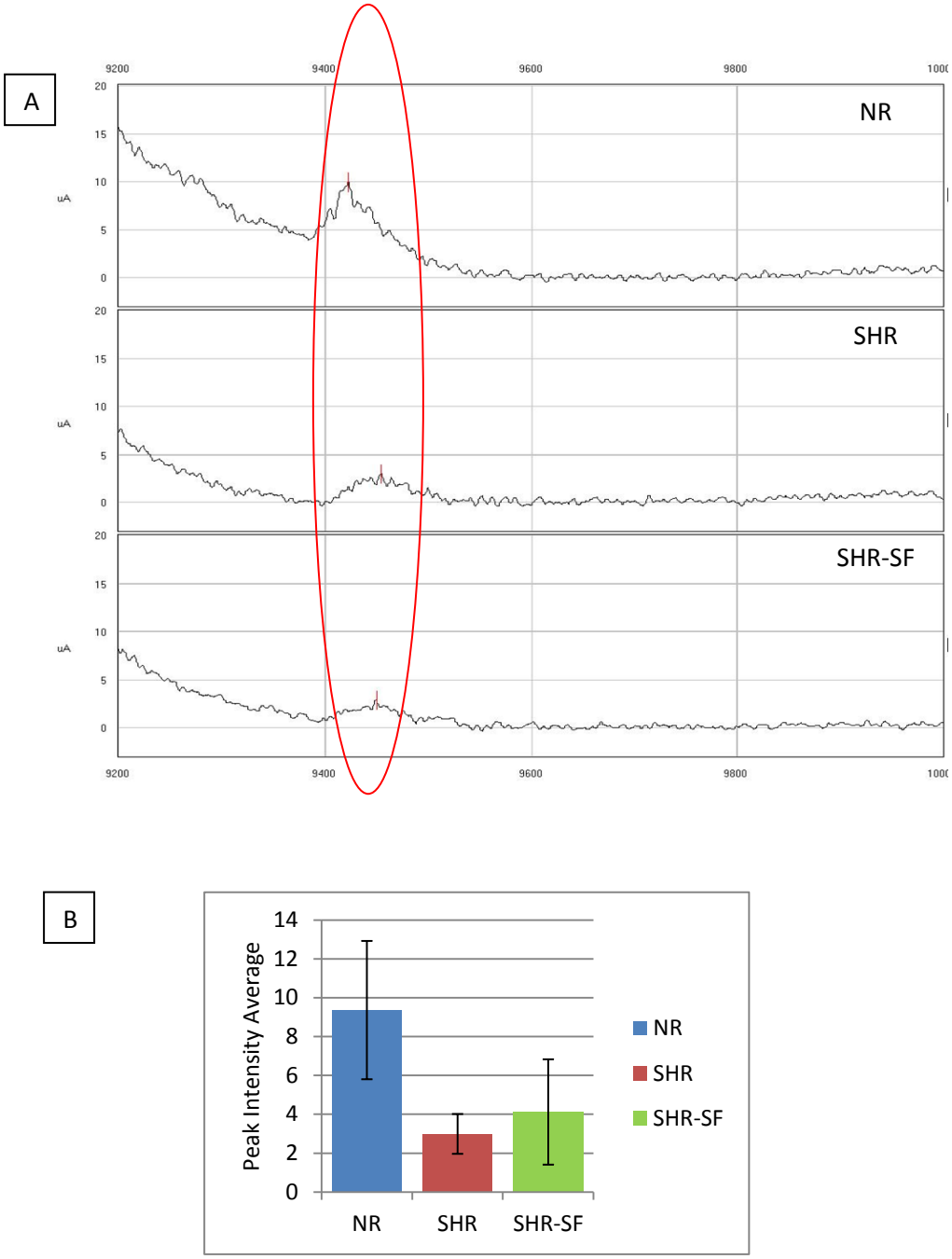


Figure 4.18: Changes of serum protein profiles (m/z 9445.0) upon administration of SF *F. deltoidea*. (A) Signal spectra of relative peak intensities at m/z 9445.0 in three different groups. Relative peak were exposed as mean, averaged m/z and intensity (n=4). (B) Histogram illustrating peak intensity average.

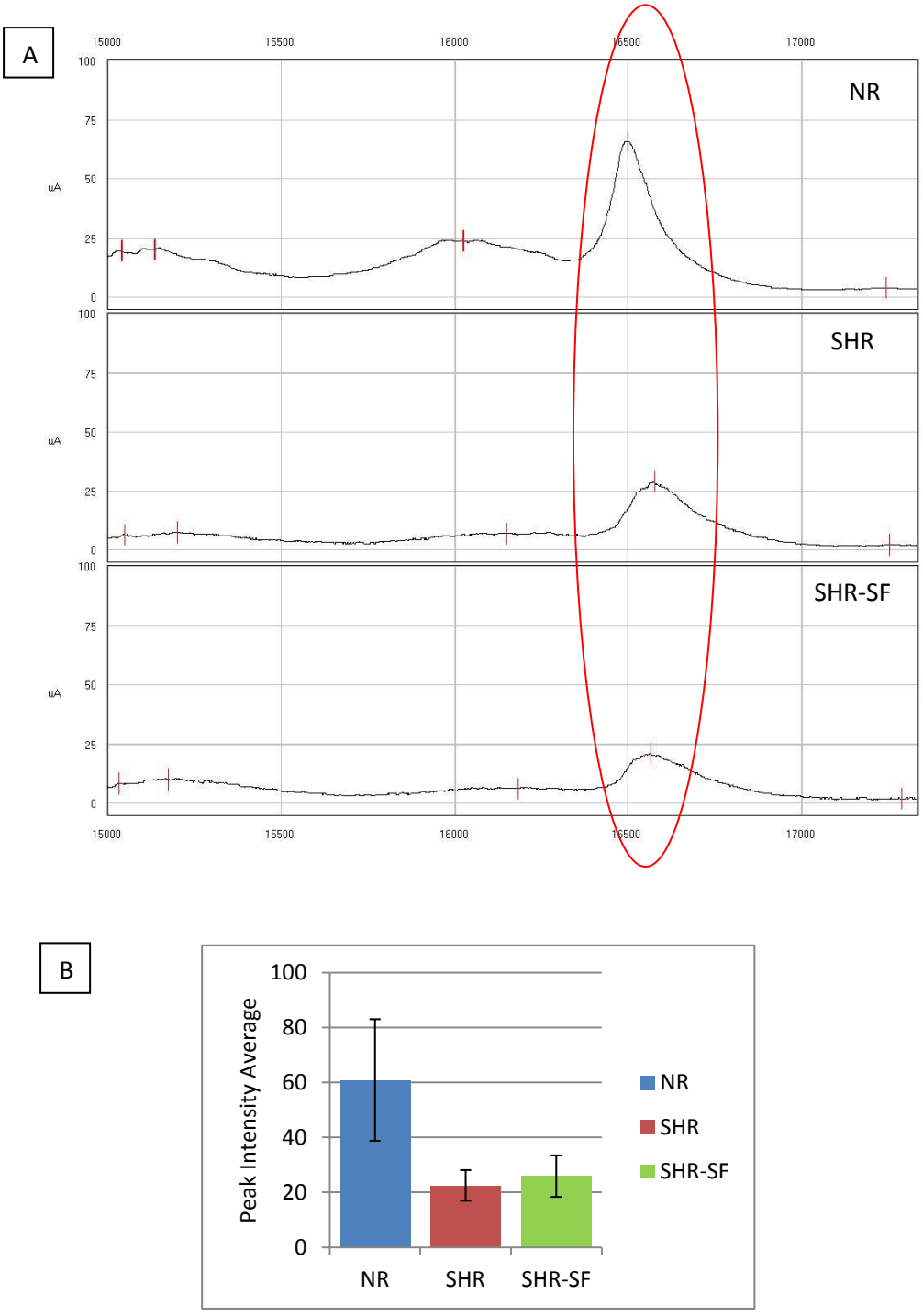


Figure 4.19: Changes of serum protein profiles (m/z 16,531.0) upon administration of SF *F. deltoidea*. (A) Signal spectra of relative peak intensities at m/z 16,531.0 in three different groups. Relative peak were exposed as mean, averaged m/z and intensity (n=4). (B) Histogram illustrating peak intensity average.

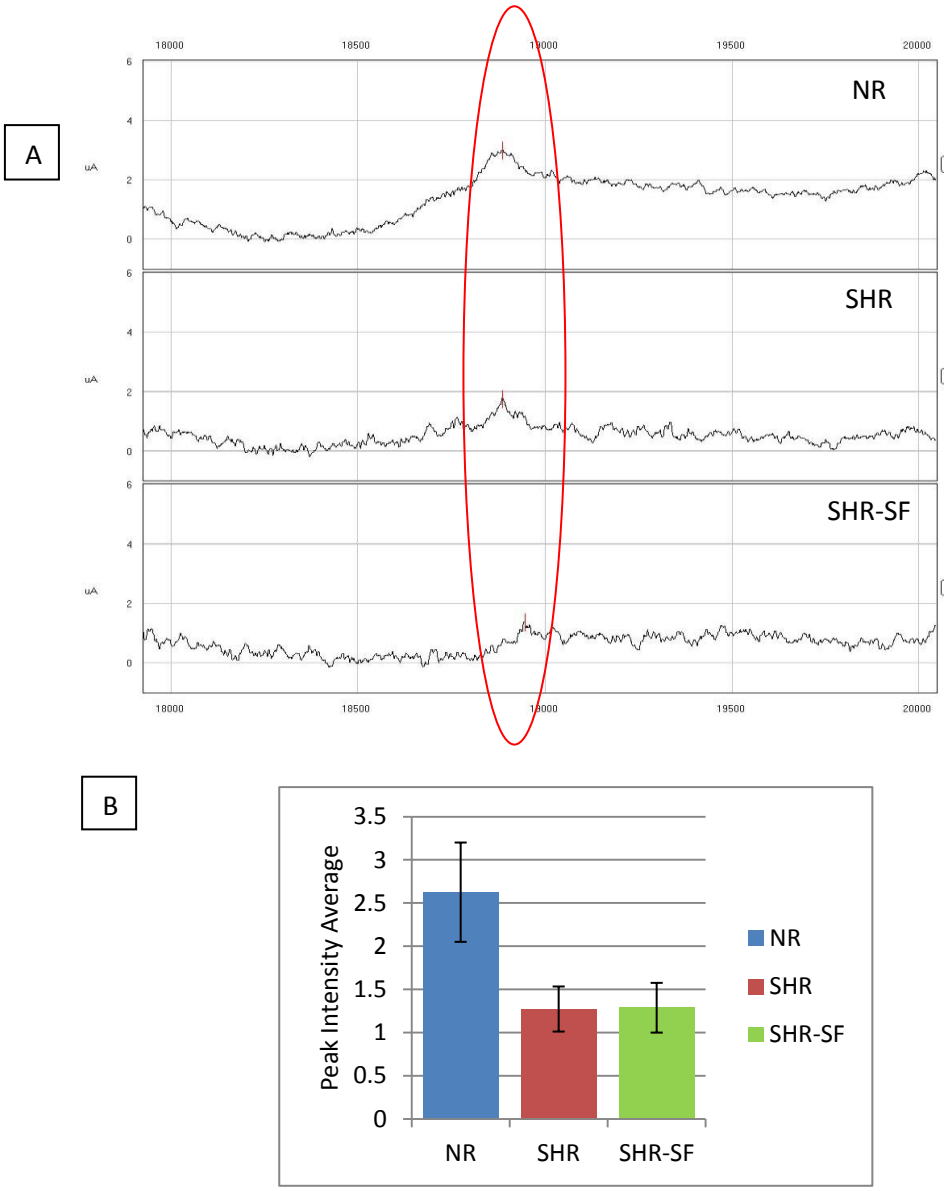


Figure 4.20: Changes of serum protein profiles (m/z 18,986) upon administration of SF *F. deltoidea*. (A) Signal spectra of relative peak intensities at m/z 18,986 in three different groups. Relative peak were exposed as mean, averaged m/z and intensity (n=4). (B) Histogram illustrating peak intensity average.

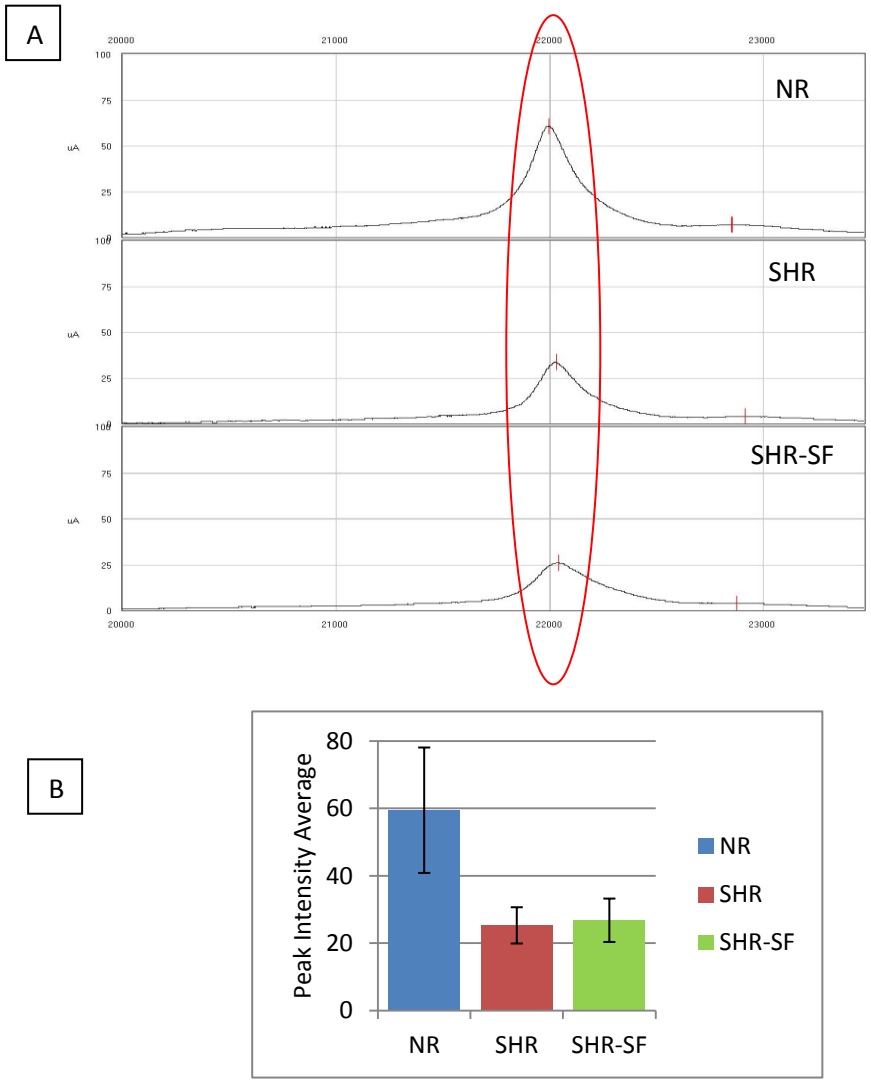


Figure 4.21: Changes of serum protein profiles (m/z 22,044) upon administration of SF *F. deltoidea*. (A) Signal spectra of relative peak intensities at m/z 22,044 in three different groups. Relative peak were exposed as mean, averaged m/z and intensity (n=4). (B) Histogram illustrating peak intensity average.

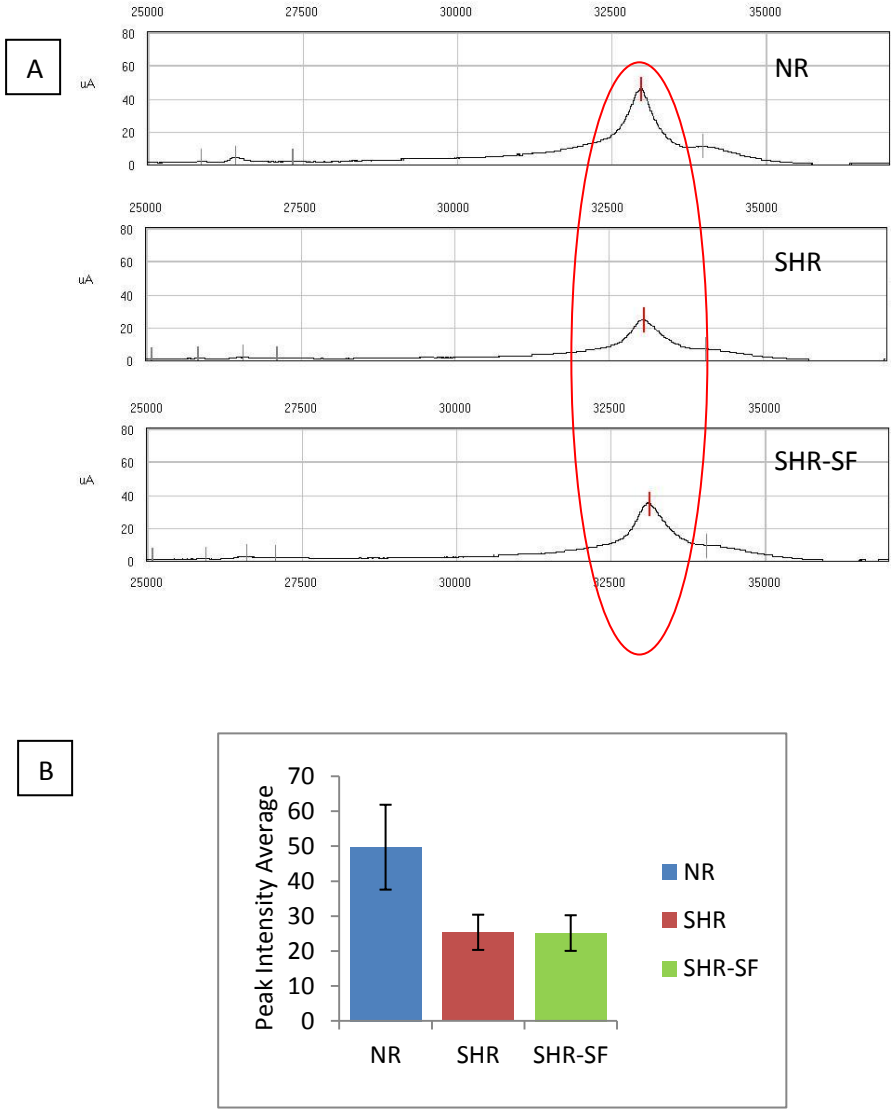


Figure 4.22: Changes of serum protein profiles (m/z 33,086) upon administration of SF *F. deltoidea*. (A) Signal spectra of relative peak intensities at m/z 33,086 in three different groups. Relative peak were exposed as mean, averaged m/z and intensity (n=4). (B) Histogram illustrating peak intensity average.

Modification of individual protein by administration of BL extract

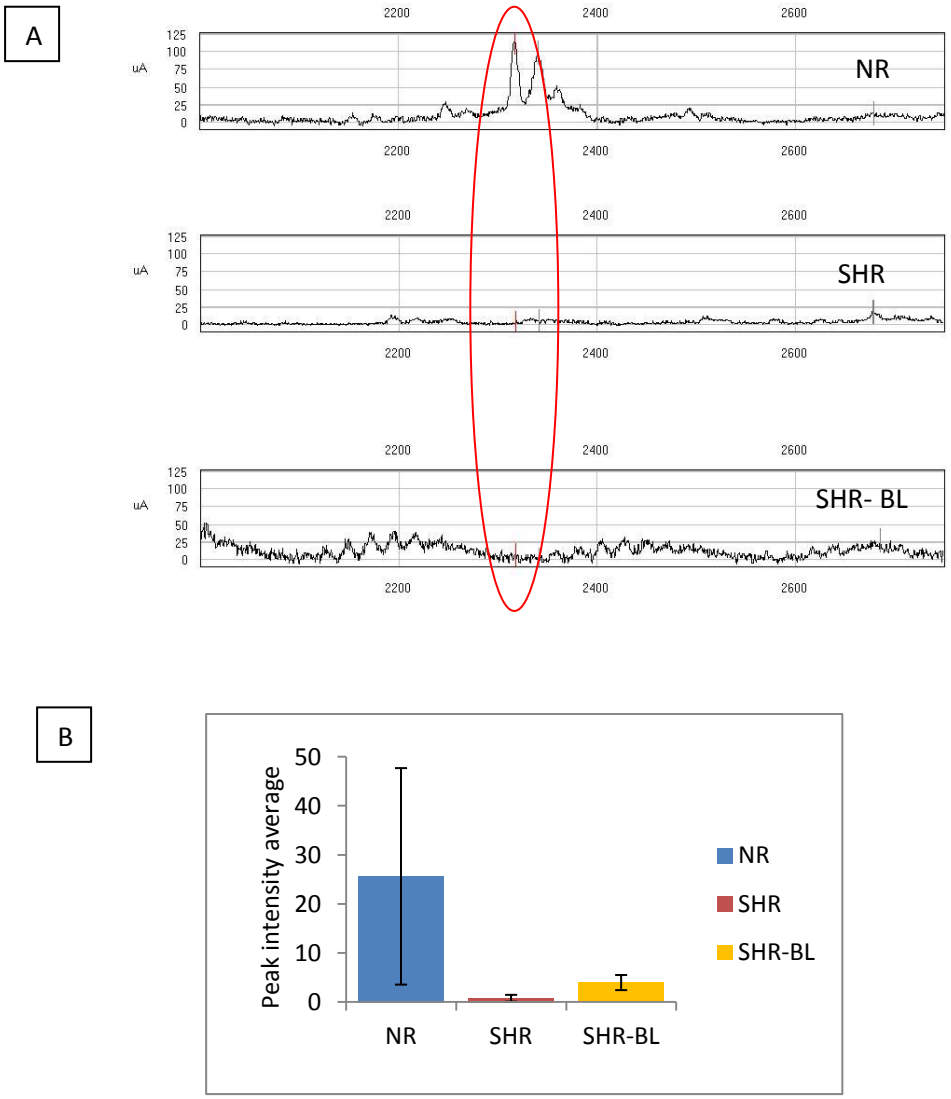


Figure 4.23: Changes of serum protein profiles (m/z 2317.6) upon administration of BL *F. deltoidea*. (A) Signal spectra of relative peak intensities at m/z 2317.6 in three different groups. Relative peak were exposed as mean, averaged m/z and intensity (n=4). (B) Histogram illustrating peak intensity average.

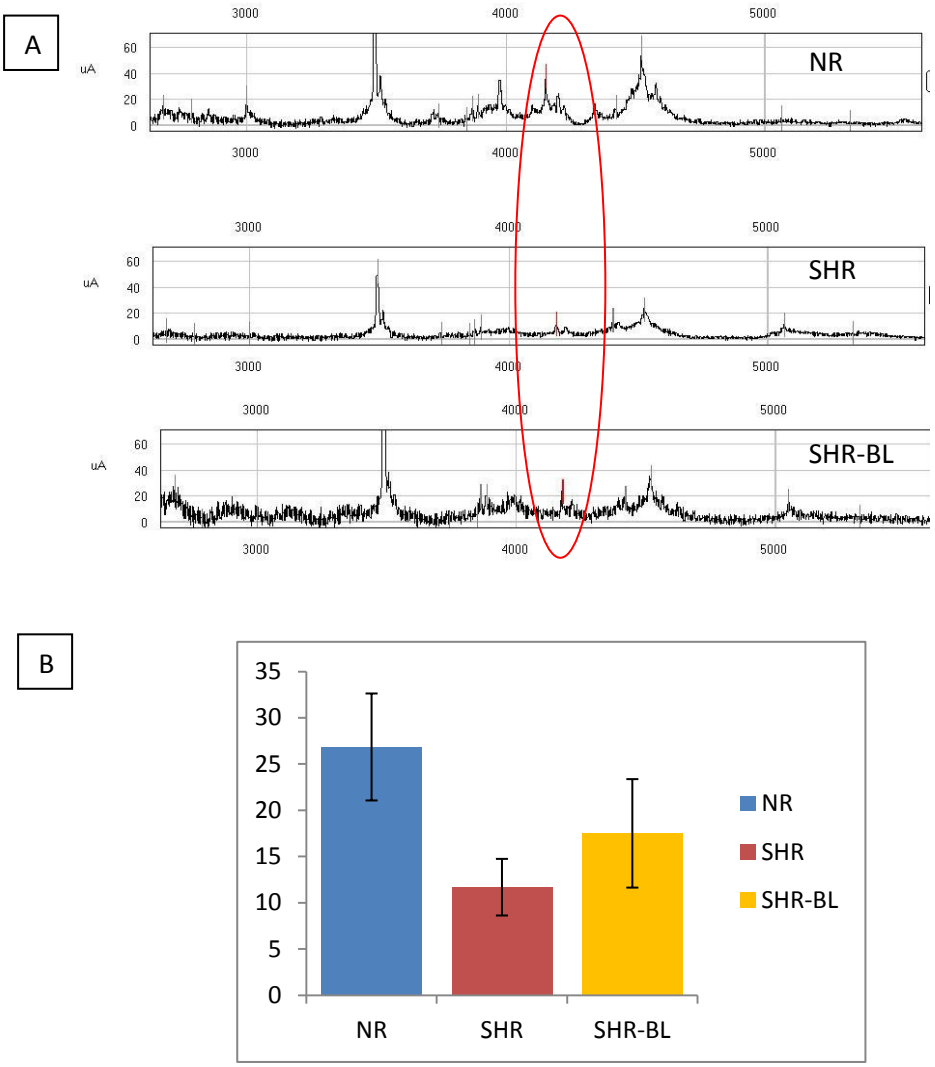


Figure 4.24: Changes of serum protein profiles (m/z 4180.6) upon administration of BL *F. deltoidea*. (A) Signal spectra of relative peak intensities at m/z 4180.6 in three different groups. Relative peak were exposed as mean, averaged m/z and intensity (n=4). (B) Histogram illustrating peak intensity average.

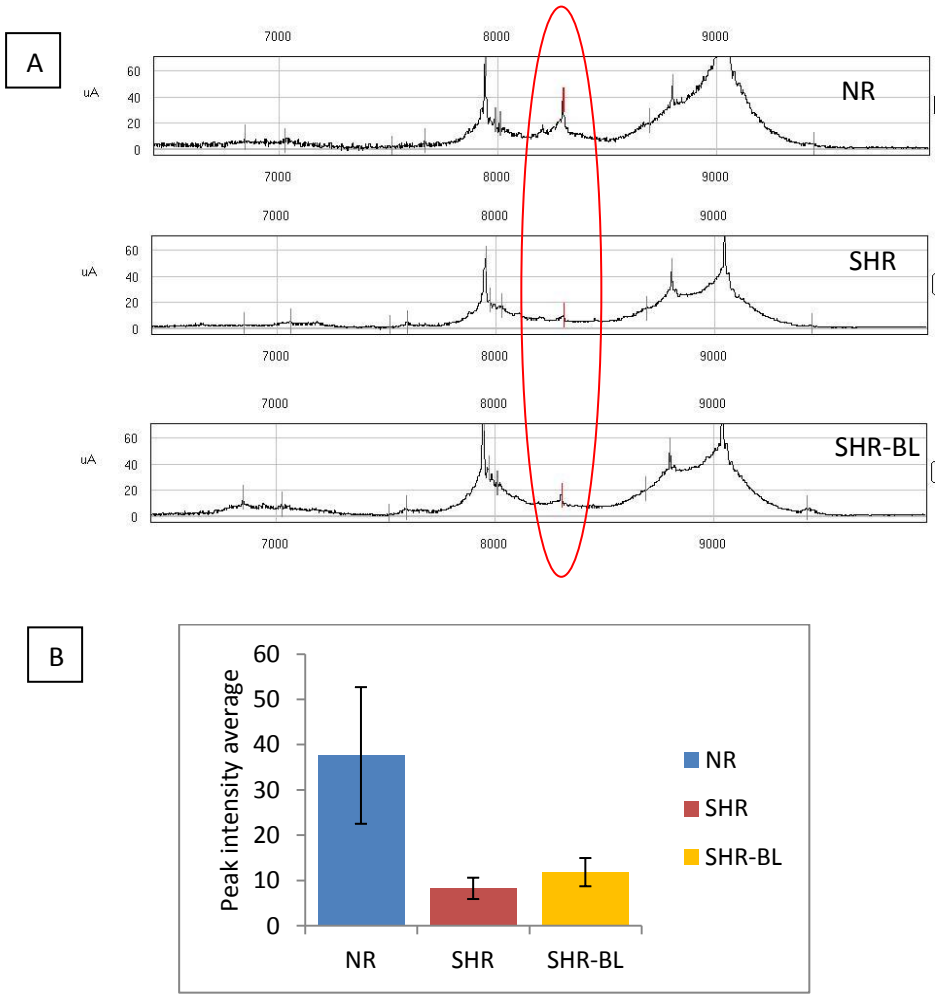


Figure 4.25: Changes of serum protein profiles (m/z 8303.3) upon administration of BL *F. deltoidea*. (A) Signal spectra of relative peak intensities at m/z 8303.3 in three different groups. Relative peak were exposed as mean, averaged m/z and intensity (n=4). (B) Histogram illustrating peak intensity average.

4.6.2 Multivariate analysis

4.6.2.1 Comparison of healthy rat (normotensive rat) versus diseased rats (nontreated SHR)

Comparison of treated and nontreated SHR in multivariate analysis using Biomarker Protein Software (Ciphergen) to generate classification and regression tree (CART) model were shown in Figures 4.26-4.28. CART model 1 was generated from 67 spectra. Interestingly, the model generated from BPS produced only one decision node based on a protein with m/z 4180.6 (2.30-fold down-regulated in the univariate analysis [Table 4.17]). In cross-validation, a sensitivity and specificity of 16.6% and 37.5% in identifying normotensive rats (NR/SD) against SHR were observed in this CART model (Figure 4.26).

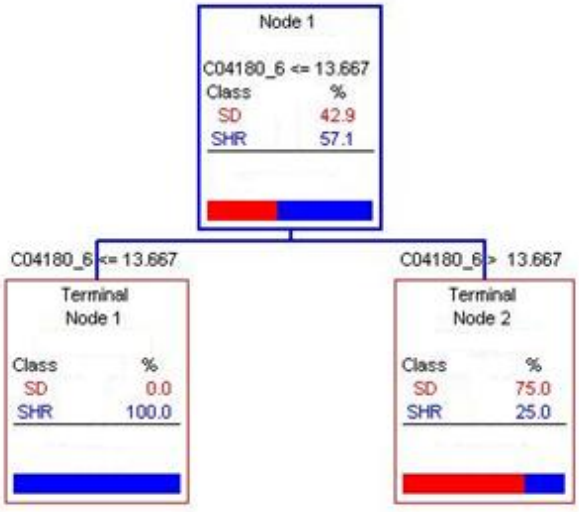


Figure 4.26: CART model 1: Normotensive rats (NR) versus nontreated SHRs.

SD – normotensive rats, SHR – nontreated SHR. Figure was directly extracted from the software.

4.6.2.1 Comparison of healthy rat (normotensive rat) versus diseased rats (nontreated SHR) and treated rats (SHR-SF)

Comparison of NR, SHR and SHR-BL generated CART model using 67 spectra derived on CM-10. This model has two nodes with m/z of 33086 and 25076 (Figure 4.27). However, from these results, no decision node could be produced. Therefore, no specify and sensitivity values were obtained.

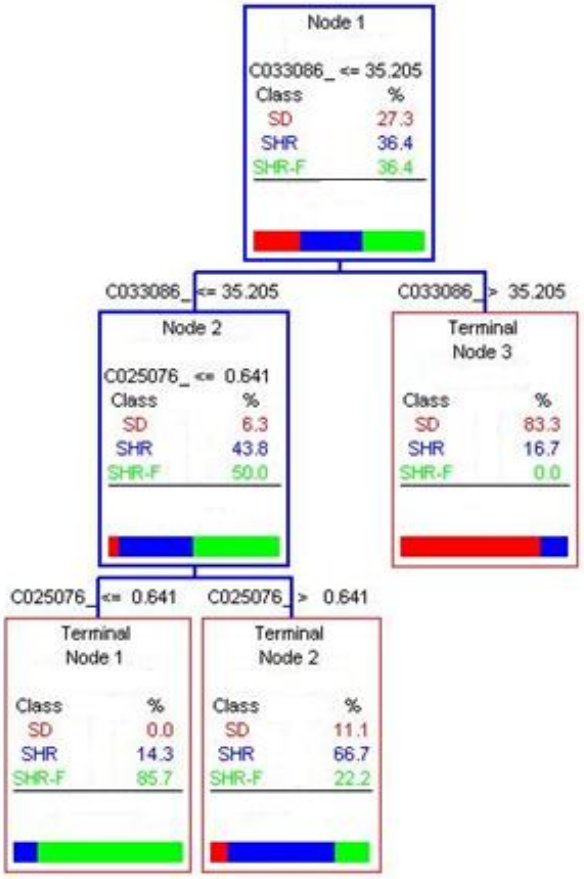


Figure 4.27: CART model: comparison of normotensive rats, nontreated SHRs and SHRs treated with SF.

SD – normotensive rats; SHR – nontreated SHRs; SHR-F – SHRs treated with SF. Figure was directly extracted from the software.

4.6.3.3 Potential biomarker following administration of BL extracts

Similarly, 67 spectra from CM-10 arrays of NR, SHR and SHR-BL were used to generate another CART model. Two nodes were produced; m/z 1617.5 and 5059.7. However, from these results, no decision node could be produced. Therefore, no specifity and sensitivity values were obtained. This model achieves the correct classification of NR, SHR and SHR-BL with 16.7%, 25% and 25% respectively.

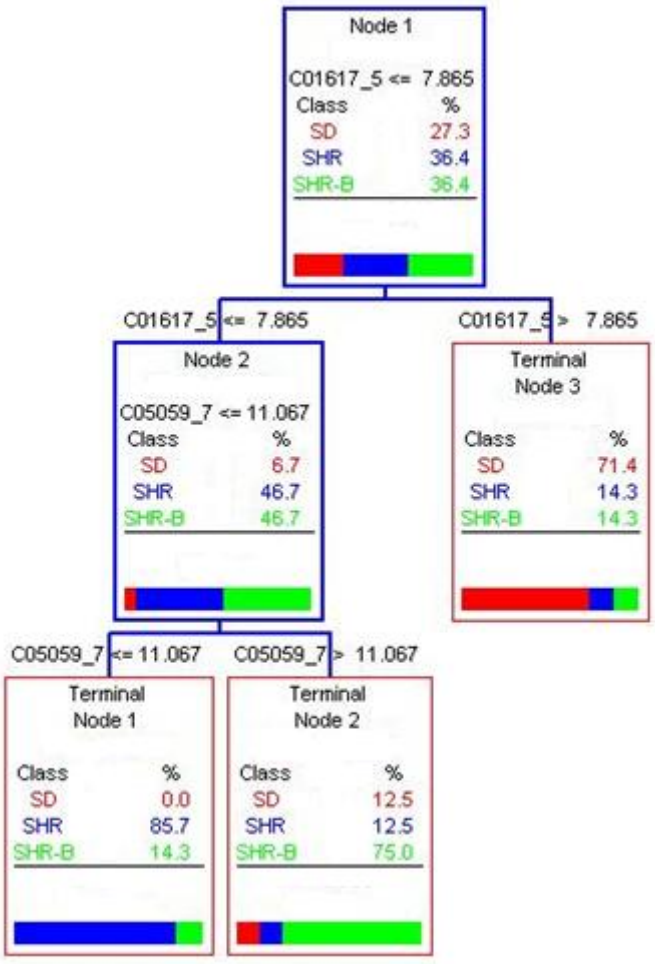


Figure 4.28: CART model: comparison of normotensive rat, nontreated SHR and SHR treated with BL.

SD – normotensive rats; SHR – nontreated SHRs; SHR-B – SHRs treated with BL. Figure was directly extracted from the software.

DISCUSSION

5.0 Discussion

5.1 ACE activity inhibition

ACE inhibitors are the first-line drug recommended in antihypertensive therapy (Joint National Committee, 1988). Thus, the ACE activity inhibitory assay was used as the preliminary assay in monitoring and evaluating the effect of SF and BL as antihypertensive agents. The potential of SF and BL aqueous extracts from *F. deltoidea* ACE activity inhibitory were assayed *in vitro.* This assay represents the *in vivo* activity of ACE, whereby HHL was used as a synthetic substrate in place of Angiotensin I (Ang I). If not inhibited, ACE will convert Ang I to Angiotensin II (Ang II) which is translated by this assay as the hydrolysis of HHL to form hippurate. Hippurate is a detectable compound and thus, could be measured spectrophotometrically (Cushman & Cheung, 1971). Ang II is a vasoconstrictor that promotes the constriction of the blood vessel and thus, leads to the increase in blood pressure. Therefore, inhibition of ACE activity could reduce the formation of Ang II and hence lowers the blood pressure.

The usage of HHL in ACE activity inhibitory evaluation is common in the determination of hypertensive properties of natural product extracts, for examples, the use of ripe strawberries in Brazil (Pinto *et al.*, 2008), *Rabdosia coetsa* – a plant used in Chinese folk medicine (Li *et al.*, 2008) and cheeses (Sieber *et al.*, 2010). In this study, addition of *F. deltoidea* extract(s) in the incubation exerted a significant reduction of hippurate. This could be explained as the reduction of HHL binding to ACE and thus, lowering the amount of hippurate produced. This indicated the inhibition of ACE activity; the higher the inhibition rate, the lower the amount of hippurate produced.

Results demonstrated that both extracts (SF and BL) produced the highest inhibition of ACE activity (75.3% and 78.4%) at a concentration of 0.4 mg/ml (Figures 4.2 - 4.3). ACE inhibition by both extracts increased as the concentrations were

increased. The effectiveness of the extracts in inhibiting ACE activity was measured by the half maximal inhibitory concentration (IC_{50}). In this study, SF and BL resulted in the following IC_{50} values; 0.13 mg/ml and 0.18 mg/ml respectively (Table 4.1). These results suggested that these extracts may contain active compound(s) that have potential in the inhibition of ACE activity, thus may help to reduce or control the development of hypertension.

5.2 *In vivo* anti-hypertensive study

Hypertension is a complex disease as it involves various mechanisms as its factor apart from ACE. *In vivo* study was performed with the aim to gather more knowledge and precisely understand the actual mechanism(s) of how SF and BL affect hypertension. Spontaneously Hypertensive Rat (SHR) is the animal model that is usually used to study essential or primary hypertension (Pinto *et al.,* 1998). The hypertensive state for SHR begins at the age around 5-6 weeks and the systolic pressure will keep on increasing until it reaches 180 and 200 mmHg or higher in the adult phase (Conrad *et al.,* 1995). In this study, SHR aged between eight to ten weeks were used for *in vivo* study as it was reported to be the most suitable experimental age (Canby *et al.*, 1989; Sanchez & Pettinger, 1981). The hypertensive condition of this model only allowed us to administer the extracts (SF and BL) in the form of drinking water at a dosage of 75 mg/kg supplied in ten days to minimize environmental stress during the treatment. The same protocol has been used in the study of the effect of potassium supplement in SHR (Jin *et al.,* 1999) and the delivery method for melatonin to SHR in another drug discovery investigation (Nava *et al.,* 2003). In order to validate the effect of SF and BL towards SHR, normal untreated Sprague Dawley (SD) rats were included in this study. The sera collected from all rats were then used in proteomics study to monitor the alteration of the protein profiles. Three different techniques were performed - SDS-PAGE, 2-DE and SELDI-TOF.

5.3 SDS-PAGE

Protein expression in SHR have been primarily studied using SDS-PAGE (Gros *et al.*, 2000; Jin, *et al.*, 1999). Electrophoresis, such as SDS-PAGE, is usually performed to monitor or observe the expression of specific protein(s) in a biological sample before proceeding to another technique (Iwahashi *et al.*, 2002). In this study, SDS-PAGE analysis of rats' serum revealed that the proteins were differentially expressed following the treatments as compared to non-treated SHR. The deregulated expression could be clearly seen in three different molecular weight (MW) regions; 6.9, 29 and 35.8 kDa (Figure 4.4). Protein expression in SF treated SHR (SHR-SF) and BL treated SHR (SHR-BL) serum (Figure 4.4) showed similar pattern with protein expression displayed by normotensive rats and also in Captopril treated SHR (SHR-Cp). All groups significantly showed distinguished pattern compared to nontreated SHR (diseased group). Comparing the serum protein profiles with the profile of normotensive rats; SHR-SF serum protein profile showed similar pattern in all three regions (Figures 4.5a-c), which suggested a reversed/ normalised pattern of the SHR profile. However, SHR-BL only showed the changes in MW regions 35.8 kDa and 29 kDa (Figures 4.5a-b). Thus, from this study it was hypothesized that both the extracts (SF and BL) may contain anti-hypertensive compound(s) or agent(s) that are affecting a few proteins in the serum protein in SHR.

However, it is also important to note that SDS-PAGE may separate similar density proteins in one band. This usually indicates that the proteins are having the same MW or it could be isoforms of the same proteins. This limitation may reduce the sensitivity of protein identification due to two or more proteins being identified in one particular band as SDS-PAGE only separates the proteins based on its MW compared to 2-DE;which separates proteins based on the MW and charges of the proteins. Therefore, a higher resolution technique, 2-DE was performed to overcome this limitation.

5.4 2-DE and Protein Identification using MALDI-TOF/TOF MS

The application of 2-DE as a tool to separate serum proteins resulted in highly reproducible gels inter sample and inter treatment groups (Figure 4.6). In the present study, analysis of 2-DE gel protein spots revealed that the expression levels of twelve protein spots that were significantly altered when the 2-DE serum protein profiles of normotensive rats were compared to the profiles of nontreated SHR rats. The spots were subsequently identified as plasma retinol binding protein, serum albumin fragment, clusterin, complement c3, haptoglobin, alpha 1 macroglobulin (2 spots), actin, alpha 1 antiproteinase, alpha 2HS glycoprotein and Apolipoprotein H. With the exception of clusterin, α2HS glycoprotein and actin, all other proteins have been previously identified to be associated with hypertension. Their levels were also reported to be altered in SHR as well as in patients with hypertension. In this study, the expression of the seven proteins appeared to be altered when similar experiments were performed on the sera of SHR treated with SF and BL. These proteins were alpha 1 antiproteinase, haptoglobin, alpha 1 macroglobulin, apolipoprotein H, complement C3, serum albumin and plasma retinol binding protein. However, protein with the spot number 96 was not identified by MALDI-TOF/TOF due to low spot volume.

5.5 Serum proteins related to hypertension

5.5.1 Alpha 1 antiproteinase (A1AT)

Alpha 1 antiproteinase is also known as alpha 1 antitrypsin (A1AT). This protein belongs to a family of serine protease inhibitors (serpins) (Gettins, 2002). A1AT is an acute phase protein produced in response to inflammation and is produced by the liver to protect the immune system. A1AT down-regulates inflammation by inhibiting elastase, an enzyme produced by inflammatory cells which will freely break down the elastin in the pulmonary and lung systems (Kushner & Mackiewicz, 1993).

Based on the results, this protein was up-regulated by three folds in SHR rat serum as compared to normotensive rat serum (Table 4.4 and Figure 4.13). However, the expression of A1AT in SHR was down-regulated following the treatments with SF and BL of *F. deltoidea*; a closer pattern to the expression of A1AT in normotensive rat serum (Figure 4.13).

A1AT may be associated to hypertension as the elevation of inflammation-sensitive plasma protein (ISPs) i.e. orosomucoid, fibrinogen, haptoglobin, ceruplasmin including A1AT has been observed in virtually all hypertensive incidences (Engström *et al.,* 2002). The level of A1AT was observed to elevate as the human blood pressure increased (Engström *et al.*, 2006). These studies help to strengthen the observed pattern revealed in this study. A1AT was also found to be elevated in increased incidence of cardiovascular diseases (Engström *et al.*, 2002). Another study by Friedman (1990), reported that the widely accepted marker of inflammation, leukocytes, is associated in the development of hypertension. Based on these studies, it could be suggested that hypertension promotes inflammation (Chae *et al.*, 2001). However, the significance of this association is still unclear due to the complex relationship between systemic inflammation, vascular cells activation and structural changes in the arteries. Therefore,

the link between these conditions needs to be explored (Pauletto & Rattazzi, 2006). Result from this study suggested that, SF and BL extracts may help in regulating inflammation or the causes of inflammation since the expression of this protein in SHR was down-regulated after the administration of the extracts. [#FDR]

5.5.2 Haptoglobin (HPT)

Besides A1AT, haptoglobin (HPT) is another inflammation-sensitive protein found to be elevated in expression during the incidence of cardiovascular disease and cardiovascular risk. These incidences may be associated with cholesterol level which contributes to arteriosclerosis leading to damage of the arteries. HPT is highly synthesized by hepatocytes and is also found in adipose tissues (Chiellini *et al.*, 2004; Hanley *et al.*, 1983; Haugen *et al.*, 1981). However, there are also evidences showing that it could be found in the skin, lungs, kidneys and heart (D'Arniento *et al.*, 1997; Kalmovarin *et al.*, 1991). HPT found in the arterial tissues is normally bound to free haemoglobin released by erythrocytes in blood system. Serum HPT may be involved in hypertension based on its nature as an anti-inflammatory protein released during acute-phase condition. Any inflammation may increase the level of plasma HPT. However, the amount and role of liver-derived plasma HPT protein compared to artery-derived plasma HPT protein in arterial wall remains unclear (deKleijin *et al.*, 2002). de Kleijin and his group reported the artery-derived plasma HPT involved in arterial restructuring as the serum HPT mRNA and protein expression were elevated after a sustained increase and decrease in blood flow. They have also suggested that the function of HPT

Note: [#FDR] indicates proteins which were significant based on ANOVA but does not comply with FDR significance level.

in arterial restructuring is facilitated by cell migration confirmed that HPT was involved in artery restructuring as the expression of HPT was found to be elevated following balloon dilation during angioplasty (Smeets *et al.*, 2003).

From this study, HPT (spot 99) was down-regulated in normotensive rat serum compared to SHR rat (Table 4.4 and Figure 4.10). This suggested that the normal blood flow prevents damage of the arterial structure thus, require very minimal repair by HPT. As a result, less HPT will be present. SHR rats experience increase of blood flow, which then causes damage to the artery and thus increasing the demand for restructuring and anti-inflammatory activity. Expression of HPT increased following administration of the extracts (SF and BL). One possible explanation would be an arterial repairing action/mechanism triggered by the extracts. The presence of these extracts probably stimulates the expression of HPT, and thus promotes repair or restructuring of the artery, which then triggers dilation of the artery. Eventually this condition may promote lowering of blood pressure in the artery of SHR rats.

5.5.3 Alpha 1 macroglobulin (A1M)

Alpha 1 or α_1-macroglobulin (A1M) belongs to a family of large glycoproteins that inhibits all four types of proteinases by trapping mechanism (Sottrup-Jensen, 1989). Three commonly expressed α-macroglobulins in rats are α_1-macroglobulin, α_2-macroglobulin and α_1-inhibitor 3 monomer (Eggertsen *et al.*, 1991; Warmegard & Johansson, 1992). In several rat tissues, α_1-macroglobulin inhibit serine proteinases group namely tonin (Boucher & Genest, 1974). The amount of A1M correlates with the amount of tonin.

Tonin activity is characterised by release of the vasoconstrictor peptide, Ang II directly from Angiotensinogen (AG) (Grise *et al.,* 1981) or from other peptides presenting the sequence of the N-terminal of AG (Pesquero *et al.,* 1982). Findings by Ikeda and Arakawa (Ikeda & Arakawa, 1984) demonstrated the capability of tonin to also release bradykinin – a potent vasodilator. Thus, tonin was proposed to be involved in the kinin-tensin system; a system that is able to generate both pressor (Ang II) and depressor (bradykinin) directly. The formation of tonin-A1M complex however, still allows the catalytic site to accept low molecular weight substrates but the bigger ones will be inhibited (Pesquero, *et al.,* 1982). Taking into consideration that most substrates are small in size, the tonin-A1M complex is still able to generate Ang II from Ang I, even when Ang I converting enzyme (ACE) activity is being inhibited by the enzyme inhibitors. The complex (tonin-A1M) probably still retains esteroprotease activity of tonin, therefore direct production of kinin continues as opposed to the Ang II release. The complex therefore, may play an important role in the control of blood pressure or perfusion in the circulating blood, vascular or tissue surfaces (Ikeda *et al.,* 1988). Result of this study demonstrated the importance of A1M as inhibitor for tonin reaction, which in turn help to regulate the release of Ang II and aldosterone secretion and kinin production, thus its expression was displayed in normotensive rat (Figure 4.11).

Hypertension in SHR rat probably occur as a result of Ang II accumulation via two directions; firstly the lack of A1M expression hence tonin activity is not inhibited relating to the increase of Ang II release and secondly the Ang II release by ACE activity. The uncontrolled release of Ang II promotes constriction of the blood vessels and finally increases the blood pressure. Treatment with the extracts (SF and BL) displayed their effect towards the up-regulation of A1M expression levels (spots $108^{\#FDR}$ and 117) (Figure 4.11), and subsequently may help to lower the blood pressure in SHR rats receiving SF and BL treatments.

5.5.4 Apoliporotein H (APOH)

Apolipoprotein H is formerly known as β_2-Glycoprotein I (β_2GI). In previous studies, β_2GI has been identified as a component of circulating plasma protein and characterized as a lipoprotein, thus it was named as Apolipoprotein H (APOH). This lipoprotein is a unique lipoprotein as the APOH characteristic differ from other flexible lipoproteins (Lee *et al.*, 1983). APOH is synthesized in the liver and distributed freely in serum (Polz & Kostner, 1979).

The precise function(s) of APOH are yet to be determined. APOH binds to negatively charged substances such as heparin, phospholipids and dextran sulfate. APOH is involved in anti-coagulation activity in serum by preventing the activation of the intrinstic blood coagulation cascade whereby it will bind to phospholipids on the damaged cells surface. APOH appears to inhibit the release of serotonin by the platelets

Note: $^{\#FDR}$ indicates proteins which were significant based on ANOVA but does not comply with FDR significance level.

thus preventing the activation of the ADP-induced aggregation. It's binding to the agglutinating and negatively charged compounds causes the inhibition of agglutination by contact activation of the intrinsic blood coagulation pathway (Schousboe, 1985).

In normal blood clotting process, blood coagulation occurs in damaged blood vessels to prevent loss of blood. However, the formation of blood clots due to the formation of thrombin may block the flow of blood and oxygen through the blood vessel. Small blood clots that break off may get stuck in the tiny capillaries and totally block the blood flow. Blood pressure will increase due to these phenomena. APOH causes a reduction of prothrombinase binding sites on platelets and reduces the activation caused by collagen when thrombin is present at physiological serum concentrations of APOH suggesting a regulatory role for APOH in coagulation (Nimpf *et al*., 1986).

APOH is also considered as a clinical marker for cardiovascular risk as the concentration of APOH is associated to metabolic syndrome alterations and vascular diseases in type 2 diabetes. Besides, APOH also involved in activation of lipoprotein lipase in lipid metabolism (Nakaya *et al.*, 1980).

In this study, the level of APOH expression in SHR was down-regulated compared to the normotensive rat (Table 4.4 and Figure 4.13). This indicated that reduction of APOH in SHR may cause the agglutination and coagulation of blood therefore, increasing the pressure of blood flow. The expression dynamic result (spot 259) suggested the normalising effect of both extracts towards APOH as the expression was up-regulated compared to the SHR and eventually follow the normotensive rat's expression pattern (Figure 4.13). Therefore, it could be suggested that the administration of SF and BL extracts may contribute to the lowering of blood pressure by controlling the coagulation activity in blood.

5.5.5 Complement C3 precursor (CO3)

Complement C3 (CO3) is a protein that will be cleaved to produce two chains (alpha chain [CO3a] anaphylatoxin and beta chain [CO3b]) following removal of four Arginine residues by CO3 convertase (Nakagawa & Komorita, 1993).

CO3 mainly involved in the activation of complement system in both classical and alternative complement pathways. Activated CO3b will bind to cell surface carbohydrates and also involve in immune aggregation. CO3a anaphylatoxin is a mediator of local inflammatory process by enhancing contraction of the smooth muscles, increases vascular permeability and causes histamine release from mast cells and basophilic leukocytes (Nakagawa & Komorita, 1993). These mechanisms cause phagocytosis of opsonised particles, activate later stages of complement (from formation of CO5 convertase) and induce inflammation.

Elevation of CO3 was found in diabetes cases related to insulin resistance (Muscari *et al*., 2007; Ylitalo *et al.*, 2001). Most of them hypothesised that the elevation of CO3 may be induced by inflammation caused by deposition of lipid in the plasma. However, none of the researchers confirmed this mechanism.

Nevertheless, the role of CO3 or complement in hypertensive have also been studied in kidney of SHRs given deoxycorticosterone acetate (DOCA) and salt (Takagi *et al*., 1990). Since CO3 plays a role in IgA nephropathy, CO3 was found to be necessary for renal injury in DOCA-salt mice (Takagi, *et al.*, 1990) as the kidney injury of CO5-deficient mice administrated with DOCA and was not as severe as wild-type mice treated with DOCA and salt (Raij *et al.*, 1989). In renovascular hypertensive rats, CO3 shows its association in the left ventricular perivascular inflammation (Nicoletti *et al.*, 1996) and also stimulated the pulmonary vascular constriction in rats (Morganroth *et al.*, 1990). However, among these studies, only Zhi-Hong Lin and partners (2004)

reported the involvement of CO3 in arterial smooth muscle cell growth in hypertensive disease.

Folkow (1987) and Sen *et. al.* (1974) reported that SHR showed exaggerated growth of cardiovascular organ in comparison with normotensive rats. Enhanced DNA synthesis and organ hypertrophy was observed in SHR rats before the elevation of blood pressure (Walter & Hamet, 1989). In cell culture studies, SHR-derived vascular smooth muscle cells (VSMCs) indicated exaggerated growth compared to cells from normotensive rats (Fukuda, Hu, *et al.*, 1999; W. Y. Hu *et al.*, 2002) and production of Ang II in homogenous cell was shown in the SHR derived VSMCs (Fukuda, *et al.*, 1999; Fukuda, Satoh, *et al.*, 1999). Hu and team (2000) suggested the generation of the Ang II in VSMCs from SHR may due to the contractile changes to the synthetic phenotype in VSMCs from normotensive rats. Lin and partners hypothesized the exaggerated growth and synthetic phenotype of VSMCs is caused by genetic abnormalities. From their study, they have found that mRNA encoding CO3 is expressed only in VSMCs from SHR. This may explain the significant elevation of CO3 (spot 85) in SHRs compared to normotensive rats observed in this study. CO3 was highly expressed in SHR compared to the normotensive rat with fold changes 3.52 (Table 4.4). Similar pattern was observed in SHR-SF (Figure 4.9). However, the expression of CO3 in SHR treated with BL exhibited different pattern as compared to nontreated SHR (Figure 4.9). Thus, it could be suggested that CO3 may act as a potential target in SF treatment due to the decrease of CO3 expression after the treatment. However, treatment with BL did not affect the expression of CO3. The association of complement system with hypertension still remains a question even after Lin and partners found the association CO3 in arterial smooth muscle cells in SHR.

5.5.6 Serum albumin

Serum albumin levels have been previously associated with hypertension. In an epidemiological investigation, increasing albumin concentration in the serum within the physiological range was found to correlate with increase in systolic and diastolic blood pressure in men and women in all age groups (Høstmark *et al.*, 2005). In this study results showed that the albumin spot was reduced significantly in normotensive rats and SHR-SF. This data is analogous to the epidemiology study by Høstmark and colleagues in which serum albumin in SHR is higher compared to the serum albumin in normotensive rats. However, the serum albumin appeared to be a 19.9 kDa fragment of the serum protein (68.7 kDa) (Figure 4.12). This fragment may be the results of the proteolysis of serum albumin although currently no information is available on the association of serum albumin with hypertension in rats.

5.5.7 Plasma Retinol Binding protein (RETBP)

Plasma retinol binding protein (RETBP) was known as a protein for delivery of retinol to tissues (Quadro *et al.*, 1999). Recently, endrocinology and metabolism study showed that RETBP could be associated with variables related to insulin resistance and diabetic complications (Takebayashi *et al.*, 2007). However, none of the studies reported on the direct association of serum RETBP with hypertension. Buzio *et al.* (1994) reported that there was no difference in the level of RETBP in serum of patient given protein diet. Another study reported that RETBP level was elevated in pregnancy-induced hypertension (Inoue *et al.*, 2009). However, in the evaluation of the relationship between RETBP and essential hypertension in women, Solini *et al.* (2009) reported that RETBP level increased in naïve hypertension women and this correlates with the degree of intima-media thickness. Other study reported that the increased level of RETBP is

associated with subclinical inflammation in childhood obesity, inflammatory markers and other variables related to atherosclerosis. These findings imply that RETBP could be involved in the development of atherosclerosis which could also relate to hypertension. Our study showed a contrasting effect towards RETBP whereby the expressions in SHR were down-regulated as compared to normotensive rats, SHR-SF and SHR-BL (Figure 4.15). This difference could be explained by the fact that RETBP found in this study could have undergone some modifications. The protein was found to be present in the region of pI=6.51 instead of the actual pI= 4.69.

From this study, it can be hypothesized that the extracts may have the capability to act as anti-hypertensive agent based on the similar trend of RETBP expression with normotensive rats.

5.6 SELDI-TOF analysis and data interpretation

In the present study, *F. deltoidea* extracts (SF and BL) demonstrated a revealing antihypertensive effect based on *in vitro* ACE activity inhibitory study and 2-DE – MALDI-TOF/TOF analysis from *in vivo* samples. Realising the positive potential of these extracts, the serum protein profiles of all rats in all tested groups were further explored using SELDI-TOF. This technique was applied as it is a proteomic tool that enables the measurement of various sizes and characteristic of proteins in a blood sample especially for the low MW protein which could not be detected in 2-DE.

Preliminary SELDI-TOF analysis was performed in order to observe the profiles of low MW serum proteins present in SHRs and normotensive rats. Differences between these profiles enable the determination of deregulated serum proteins. Characterisation was done by looking at the signal peaks which will give their exact mass per charge ratio (m/z). Only mass values within the range of 2,500 and 40,000 Da were considered in the analysis. Values less than 2,500 Da were excluded as it represents the signal noise from the matrix, buffers and reagents. All data were generated using Ciphergen Express Data Manager and univariate analysis of the protein expression was performed using Expression Dynamic Mapping (EDM) application. A total of 16 peaks were found to be significantly altered in hypertensive state. All the proteins showed down regulation in the sera from SHR rats as compared to the sera from normotensive rats (Table 4.7).

The second part of analysis was aimed at identifying protein peaks which were affected by the administration of SF and BL extracts. The analysis was done by directly comparing the profiles of SHR, normotensive and treated SHR. Following treatment with BL, three protein peaks with the m/z 2317.6, 4180.6 and 8303.3 demonstrated 5.02, 1.50 and 1.43 folds changes in their expression (Figures 4.23 - 4.25). These proteins showed a normalisation effect as the expression of the affected protein

increased after the administration BL. The administration of SF affected more protein peaks (seven peaks), however, the level of alteration was somewhat minimal and less significant. The difference in fold change was only 1.38, 1.27 and 1.15 as shown by peaks with m/z 9445.0, 8303.3 and 16,531 (Figures 4.16 – 4.22).

Even though SELDI-TOF analysis allows researcher to confidently distinguish protein profiles of several subject groups, the identities of these proteins are not determined. Further characterisation and identification will require another technique. Nonetheless, the information in the form of protein mass (m/z) could be further used to investigate potential disease biomarker. In SELDI-TOF multivariate analysis, potential biomarker candidates were investigated using Biomarker Pattern Software™ (BPS). This software potentially allows researchers to establish a decision tree that could correctly classify the samples group/origin. Statistically significant peaks obtained from EDM univariate analysis will be used to generate the Class and Regression Tree (CART) model. This software will analyse all data step wise by looking at the intensity ratio and the frequency of a specific peak that is present in different groups.

Comparison between SHR and normotensive (NR) data analysed by BPS exhibited only one potential biomarker candidate in these groups which is the peak with m/z 4,180.6 (Figure 4.26). Based on collated data, the software automatically set the cut off intensity level of m/z 4,180.6 at 13.667 whereby serum samples with the intensity > 13.667 indicated NR subjects and those with the intensity ≤ 13.667 will be grouped as SHR subjects. Referring to Figure 4.26, CART model generated a small decision tree, with only a single node (Node 1) and two terminal nodes. Terminal Node 1 grouped all the serum with the intensity of m/z 4,180.6 ≤ 13.667. This terminal node classified 100% of the serum sample as SHR and 0% as NR. Terminal Node 2 grouped all the serum with the intensity > 13.667, and hence 75% were classified as NR and 25% as SHR. In biomarker determination, a group with 100% subject differentiation is

considered absolute while the group that did not achieve 100% will be subjected to the next evaluation using other potential peak(s). The decision tree generated from this study suggested that 25% of SHR subject have been misdiagnosed as NR.

Administration of SF to SHR generated a decision tree with three terminal nodes (Figure 4.27). Two peaks were chosen as potential biomarker candidates; m/z 33,086 and 25,706. Node 2 classified the groups as NR or SHR/treated SHR based on m/z 33 086 at the intensity cut off of 32.205. A total of 83.3% was correctly classified as NR in Terminal Node 3 (33,086 > 32.205). Node 2 were further analysed using m/z 25 706 at the intensity cut off of 0.641 to further distinguished SHR and treated SHR. In Terminal Node 1 (25,706 ≤ 0.641), none of the NR was misdiagnosed as SHR and 85.7% was classified as treated SHR. Terminal Node 2 (25,706 > 0.641) classified 75% as SHR. Based on this CART model, it could be suggested that these two m/z peaks were potential biomarker candidates. However, the use of these candidates could not produce an absolute classification and resulted with several misdiagnosed cases.

On the other hand, administration of BL generated a decision tree with three terminal nodes but two different peaks were chosen as potential biomarkers; m/z 1617.5 and 5059.7 (Figure 4.28). Node 2 classified the groups as NR or SHR/treated SHR based on m/z 1617.5 at the intensity cut off of 7.865. Only 71.4% was correctly classified as NR in Terminal Node 3 (1617.5 > 7.865). Node 2 was then further analysed using m/z 5059.7 at the intensity cut off of 11.067 to distinguished SHR and treated SHR. SHR comprised 85.7% of the group classified as SHR in Terminal Node 1 (5059.7 ≤ 11.067) and 75% classified as treated SHR in Terminal Node 2 (5059.7 > 11.067). Similarly as in the SF treatment, the two protein peaks were suggested as potential biomarker candidates could not produce an absolute classification and resulted with several misdiagnosed cases.

In this study, generation of this model serve as a trial method to identify potential biomarker candidates in research involving more than two groups of subjects (i.e. normal, diseased and diseased-treated). The decision trees produced were not absolute and could not uniquely distinguish the subjects. This could be due to the small size number used in the experiment. Increasing the sample size could lower the deviation between peak m/z values as well as intensities and in turn will improve the decision tree model.

As a whole, treatment of SHR with SF and BL extracts demonstrated alteration of some low MW proteins expressions. These alterations leads to the identification of several potential biomarkers, alas these proteins could not highly distinguish each group as modelled by CART decision tree.

5.7 General Discussion

Four different techniques were used to study the potential of *F. deltoidea* extracts with anti-hypertensive properties; ACE assay, SDS-PAGE, 2-DE and SELDI-TOF-MS. The preliminary study using ACE inhibitory assay was conducted to monitor the ability of these extracts in inhibits ACE as a part of hypertension study. Initial observation of changes in protein profiles in the serum of treated SHRs (SHR-SF and BL) was performed using SDS-PAGE. To give better resolution, 2-DE was carried out. Differentially expressed proteins were identified by MALDI-TOF/TOF. Knowing the identities of these differentially expressed proteins will lead to an understanding on how they are involved in the anti-hypertensive effects of *F. deltoidea* extracts towards SHR. The limitation of 2-DE in observing low molecular weight proteins was overcome by the usage of SELDI-TOF.

Results from the three approaches showed a degree of association with each other. In SDS-PAGE, treatment with BL resulted in minimum change in protein sera expression at MW region of 6.9 kDa (Figure 4.5c). This was also observed in 2-DE as treatment with BL mostly altered proteins expression in the middle MW region; range 21-101 kDa (Figure 4.7). Specifically, the expression of ALBU and RETBP in SHR-BL exhibited similar expression as in non-treated SHR (Figure 4.12 and 4.15). A lesser number of significant peaks were altered in SHR-BL compared to SHR-SF in SELDI-TOF – EDM application.

It was apparent that some SELDI-TOF results may be similar to MALDI-TOF/TOF results. The m/z of native protein peaks found in SELDI-TOF could be associated with the theoretical MW of proteins obtained from the SWISS-PROT database for proteins identified in MALDI-TOF/TOF analysis. Peaks at m/z 22,044 and

33,086 found in SHR-SF protein profile could be RETBP (MW: 23,220 Da) and APOH (MW: 33,197 Da) respectively.

This study had revealed the anti-hypertensive potential of *F. deltoidea* extracts (SF and BL). Even though the direct mechanism on how these extracts affects hypertension remains unclear, this study has shown the effect of SF and BL of F. deltoidea extracts on several mechanisms - (1) ACE inhibition, (2) inflammation, (3) vasodilation and (4) anti-coagulation.

Based on the ACE activity inhibitory assay, it could be postulated that both extracts affect the hypertension primarily via ACE pathway. *F. deltoidea* may contain a certain compound(s) that mimic the characteristics of ACE substrate, thus, their binding will block enzyme activity. Another possibility would be allosteric binding of the compound to the enzyme, causing changes the in enzyme conformation and hence reduce the affinity of the substrate for the enzyme active site.

Administration of *F. deltoidea* extracts to SHR rats caused alterations in sera protein profile. Based on the profile, it could be hypothesized that *F. deltoidea* may be acting as an anti-hypertensive through regulation of the kinin-tensin system via alteration of A1M expression. This will reduce the production of vasoconstrictors and promote the release of vasodilators thus lowering or regulating blood pressure. This observation correlates well with the observed results in ACE *in vitro* assay. Besides the kinin-tensin system, *F. deltoidea* extracts also showed noticeable effects towards inflammatory proteins (A1AT and HAPT) thus potentially may prevent arterial damage. *F. deltoidea* extracts may also posses anti-coagulation activity (through up-regulation of APOH) that reduces pressure in blood flow by minimizing the blockage of the vascular system. It has to be noted that these postulations are made based on the known function of the differentially expressed protein. Besides these three mechanisms, *F. deltoidea*

also affects the complement system (CO3) involved in inflammation pathway. However, the exact relation with hypertension could not be clearly postulated at this point.

Even though this primary investigation on the effects of *F. deltoidea* towards hypertension gave informative clues, the actual mechanism of action could not yet be conclusively determined. While the mechanism of action remains unresolved, identification of potential biomarkers could complement this investigation. This study managed to identify several low MW proteins that could be used as potential biomarkers for hypertension. Further characterisation of these proteins will provide insights regarding on the actual mechanism(s) involved.

CONCLUSION

6.0 Conclusion

In summary, *F. deltoidea* aqueous extracts (SF and BL) exhibited potential as anti-hypertensive property. It can be concluded that;

- *F. deltoidea* extracts showed an inhibitory activity towards ACE *in vitro*.
- *F. deltoidea* extracts may act as anti-hypertensive agents via alterations of sera protein profiles of SHR rats as observed in 2-DE after the administration of aqueous extracts.
- *F. deltoidea* extracts had also altered low MW proteins (LMWP) in SHR. SF altered proteins expressions at m/z 3493.2, 8303.3, 9445, 16 531, 18 986, 22 044 and 33 086 Da and BL altered smaller proteins in expressions at m/z 2317, 4180.6 and 8303.3 Da. These LMWPs might be the potential biomarker candidates.

As a while, SF may be more effective as anti-hypertensive agent when compared to BL. In SDS-PAGE, SF exhibited preliminary sera protein profiles changes in all MW regions. However, BL affected only two MW regions. The protein expression of SHR treated with SF showed normalizing effects as observed in 2-DE. BL did not resulted in normalizing effects towards CO3. Lastly, SF affects more LMWP compared to BL.

In conclusion, administration of aqueous extracts of *F. deltoidea* showed anti-hypertensive effects. However, the exact mechanisms involved remain unclear. The results however, do support traditional claims that *F. deltoidea* helps in improving blood circulation.