1.0 Introduction

Nowadays, herbal and medicinal plant products in Malaysia are getting popular and accepted by people. Although synthetic drugs are very effective and commonly used for industrial food processing they may give some side effects and toxic properties to human health. Therefore, in recent years, research has focused to extract the natural product from medicinal plant that can replace the synthetic additives (Atmani, *et al*., 2009). *Orthosiphon O. stamineus* from the family of Laminaceae is one of the famous medicinal plant in Southeast Asia. It is commonly known as 'Misai Kucing' in Malaysia, because of its pale purple flowers with long wispy stamens shaped like cat whiskers (Han, *et al.,* 2008).

O. stamineus is mainly used in traditional medicine to reduce bladder and kidney discomfort, arteriosclerosis, gout and rheumatism (Wiart, 2002). It can also be used in treatment of eruptive fever, influenza, hepatitis, jaundice, biliary lithiasis, tonsillitis, epilepsy, menstrual disorder, gonorrhea (Awale *et al*., 2004) and diabetic (Han, *et al*., 2008). However, there is no scientific research has been carried out to measure the potential of *O. stamineus* for antihypertension treatments and very few research has been done to measure their antioxidant activity.

O. stamineus is claimed to treat human diseases that related to oxidative stress including cardiovascular disease, cancer, diabetes mellitus and hypertension (Matkowski, 2008). Those diseases, especially hypertension are related to the reactive oxygen species (ROS) such as the superoxide radical ($O_2^{\bullet-}$) and hydroxyl radicals ($OH^{\bullet}$) that cause oxidative damage on lipids, proteins and nucleic acids (Wang, *et al*., 2008).

According to Tezuka, *et al*., 2000, *O. stamineus* contains several active chemical compounds such as terpenoids (diterpenes and triterpenes), polyphenol (flavonoid and phenolic acids) and sterols. Polyphenol is the most dominant compound in the leaf of *O. stamineus* and it has been reported to show high scavenging activity towards DPPH radical (Khamsah *et al*., 2006). The methanol extract from the leaves of *O. stamineus* contains high concentration of caffeic acid derivatives and it shows the ability to scavenge the DPPH free radicals (Zakaria *et al*., 2008). Moreover, research has also been done to measure the antioxidant activity and total phenolic contents of methanol extract of *O. stamineus* by measuring the bleaching rate by β-carotene or linoleic acid system.

Phenolics, triterpenes such as betulinic acid, oleanolic acid, and ursolic acid that present in the methanol extract from the leaves of *O. stamineus* have been found to play an important role in the antioxidant activity (Khamsah *et al*., 2006). There is no universal method which antioxidant activities can be measured accurately, because it may involve many reactions and mechanisms. Therefore, in this study, three in vitro assays were performed to determine the antioxidant activities of leaves crude extract of *O. stamineus* included DPPH radical scavenging assay, reducing power assay and metal chelating assay.

Hypertension is a major risk factor for cardiovascular and cerebrovascular disease (Branch, *et al*., 2000) and it is known as the third terminal disease in the world. In renin-angiotensin system, angiotensinogen converted to angiotensin I and the inactive decapeptide of angiotensin I will be converted to angiotensin II (active octapeptide vasoconstrictor) by angiotensin converting enzyme (ACE) and it caused contraction of blood vessels and increased the blood pressure (Lam, *et al*., 2007). To reduce the activity of ACE, natural ACE inhibitor from *O. stamineus* has been investigated to know its

potential. Therefore, in this study, the leaves extract of *O. stamineus* have been evaluated to determine their potentials as antioxidants and antihypertension agents (ACE inhibitor).

1.1 Antioxidants

Antioxidants are chemicals that reduce the rate of particular oxidation reactions that involve the transfer of electrons from a substance to an oxidising agent (Gulcin, *et al*., 2007). Antioxidants regulate various oxidative reactions that occur in tissues and are evaluated as a potential anti-aging agent. They can terminate or reduce the oxidation process by scavenging free radicals, chelating free radicals and also by acting as electron donors (Senevirathne, *et al*., 2006).

Antioxidants play an important role for maintaining healthy body. When human body is lack of antioxidants, the free radical will damage the cell (Valko, *et al*., 2006) because the reactive oxygen species (ROS) that is produced by all aerobic organisms can easily react with proteins, lipids and DNA. The generation of ROS proceeds to a variety of diseases such as arthritis, diabetes, inflammation, cancer as well as denegerative processes associated with aging (Turkoglu, *et al*., 2006).

Antioxidant activity can be described as combination of some chemical reactions including metal chelation, quenching free radicals by hydrogen donation from phenolic group, oxidation to a non-propagating radical, redox potential and enzyme inhibition. Antioxidants can prevent or reduce the oxidation that caused by free radicals and reactive oxygen species (ROS) via single or combination of the chemical events (Karagozler *et al*., 2008).

The most extensively used synthetic antioxidants are propylgallate (PG), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ). However, BHT and BHA have been suspected of being responsible for liver damage and carcinogenesis (Senevirathne, *et al*., 2006). Therefore medical experts believe that this disease can be prevented by eating natural antioxidants from our food supplement or plant. Natural antioxidants have multifunctions such as the reduction of chronic diseases like DNA damage, mutagenesis, and inhibitions of pathogenic bacteria growth (Gulcin, *et al*., 2007).

In this study, three types of antioxidant capacity estimation are used including DPPH radical scavenging assay, reducing power assay and metal chelating assay, to measure the antioxidant activity from the leaves crude extract of *Orthosiphon stamineus*. The DPPH assay is considered a valid and easy assay to evaluate scavenging activity of antioxidants, since the radical compound is stable and does not have to be generated as in other radical scavenging assays (Senevirathne, *et. al*., 2006).

The reducing power assay is used to evaluate the ability to reduce Fe^{3+} to Fe^{2+}. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action via the breaking of the free radical chain through donation of a hydrogen atom (Senevirathne, *et. al*., 2006). The metal chelating assay is used to assess the Fe^{2+} chelating capability. Measurement of the rate colour reduction allows estimation of the chelating activity of the chelator in the test samples (Gulchin, *et al*., 2007).

A) Free radicals and oxidation

Free radicals refers to the low molecular weight molecules (Poon, *et al.,* 2004) that contain one or more unpaired electrons (Valko, *et al.,* 2006), and an unpaired electron is one that presents in atomic hydrogen by itself (Halliwell, 2006). They indicate a considerable degree of reactivity of free radical. Those radicals are produced from oxygen, since reactive oxygen species (ROS) which is the most important class generated in our living system (Valko *et al.,* 2006), although most molecules in vivo are nonradical. It is continuosly produced in cells as accidental by-products of metabolism (Cheeseman and Slater, 1993).

There are many types of free radicals in living system (Halliwell, 2006). Reactive species can be divided into reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive chlorine species (RCS) (Cornelli, 2009). There has been a highly interest in the role of ROS and RNS in clinical and experimental medicine (Valko, *et al.,* 2006). Other reactive species such as C•, L•, or R• depend on the nature of the compounds including carbon, lipidic and generic radical. It is believed to be responsible to the development of some age-related disease and ageing by causing 'oxidative stress' and 'oxidative damage' (Halliwell and Whiteman, 2004).

Types of free radicals

i) Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) refers to reactive molecules that are derived from oxygen (O_2) (Poon, *et al.,* 2004). ROS is derived from both exogenous and endogenous

substances. Exposure to xenobiotics such as chlorinated compounds, metal ions, radiation and barbiturates may induce oxidative stress and damage. The potential endogenous sources include inflammatory cell activation, mitochondria, cytochrome P450 metabolism, and peroxisomes (Valko *et al.,* 2006). ROS include superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl) (Poon *et al.,* 2004). Fig.1.1 summarizes the formations and metabolisms of ROS at intracellular level.

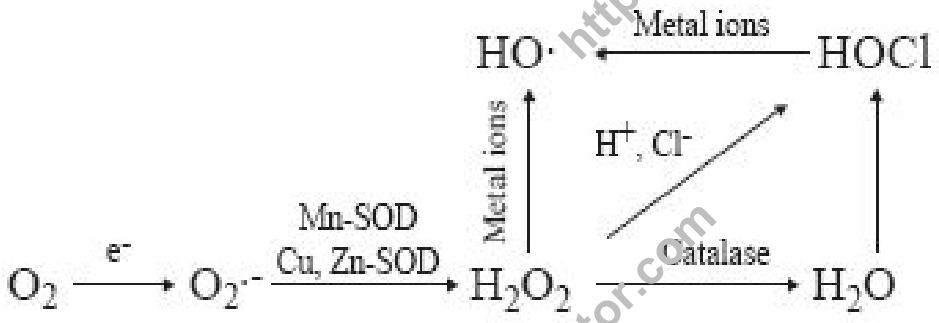


Fig.1.1. Metabolism and pathway of reactive oxygen species (ROS)

(Poon, *et al*., 2004)

ii) Superoxide radical ($O_2^{\bullet -}$)

Superoxide radical ($O_2^{\bullet -}$) is produced by the reaction of O_2 with an electron from respiratory chain in mitochondria (Poon *et al*., 2004). It is difficult to detect the presence of superoxide radical in intact mitochondria, because of the occurrence of high SOD activity. $O_2^{\bullet}$ can also be endogenously produced from xanthine oxidase, lipoxygenase, and cyclooxygenase (Poon *et al.,* 2004). Other endogenous sources of cellular ROS are neutrophils, eosinophils and macrophages (Valko *et al.,* 2006). $O_2^{\bullet -}$ is an anion and it is a modestly reactive compound that requires a specific transport system to penetrate lipid membranes to make more damage and it is considered to be less toxic when compared with

HO^- (Poon *et al*., 2004). Superoxide dismutase (SOD) is an enzymatic antioxidant that can be used to catalyze $O_2^{\bullet -}$ to O_2 and to the less reactive species H_2O_2 (Fig.1.2). It reduces $O_2^{\bullet -}$ by reduction of the metal ion transition at the active site (Valko, *et al.,* 2006).

$$2\ O_2 + 2H \xrightarrow{SOD} H_2O_2$$

Fig.1.2. Enzyme superoxide dismutase reaction

(Valko, *et al.,* 2006)

iii) Hydrogen peroxide (H_2O_2)

Mitochondria can generate significant quantities of hydrogen peroxide (H_2O_2) (Valko *et al.,* 2006). H_2O_2 is not a free radical, but it is still considered as ROS (Poon, *et al*., 2004). It is produced when cells are to detoxify $O_2^{\bullet -}$ by Cu, Zn-superoxide dismutases (SOD) in cytosol or manganese superoxide dismutase (Mn-SOD). It can also remodel the structure of cells and activate the transcription factor. When the production of H_2O_2 exceeds, it accumulates and becomes toxic to cells because of its oxidative nature. In some cases, it will form the hydroxyl radical and hypochlorous acid (HOCI) (Poon, *et al*., 2004). Catalase is an antioxidant that located in peroxisome and it promotes the conversion of hydrogen peroxide to water and molecular oxygen (Valko, *et al*., 2006).

B) Types of antioxidants

Antioxidants can be divided into 2 classes:

i. Preventive antioxidants which reduce the rate of chain reaction.

ii. Chain breaking antioxidants which interfere with chain propagation

(Murray, *et al*., 1998).

I) Preventive antioxidants (enzymatic antioxidant)

The antioxidant enzyme system is based on superoxide dismutase (SOD), catalase (Cat) and glutathione peroxidase (GPx). These primary enzymes have support enzymes such as glutathione reductase (GRD), glucose-6 phosphate dehydrogenase (G-6-PDH), and glutathione sulfur (S) transferase (GST) that supply the enzymes with reducing equivalents and substrate (Karlsson, 1997).

Type of preventive antioxidant

i) Superoxide dismutase (SOD)

Superoxide dismutase (SOD) is one of the most effective enzymatic antioxidants, and it catalyzes the dismutasion of $O_2^{\bullet -}$ to O_2 and to the less reactive species H_2O_2 (Valko, *et al.,* 2006). SOD destroys $O_2^{\bullet -}$ with high reaction rates, by successive oxidation and reduction of the transition metal ion at the active site. In humans there are three forms of SOD: cytosolic Cu, Zn-SOD, mitochondrial Mn-SOD and extracellular SOD (Valko, *et al.,* 2006).

ii) Catalase

Catalase is an enzyme that present in the cells of plants, animals and aerobic bacteria. It is found in blood, bone marrow, mucous membranes, kidney and liver. Catalase is located in peroxisome and it promotes the conversion of hydrogen peroxide to water and molecular oxygen (Valko, *et al*., 2006).

iii) Glutathione peroxidase

Glutathione peroxidase is an integral component that provides a second line of defense against hydroperoxidases before they can damage membranes and other cell components (Murray, *et al*., 2008). There are two forms of the glutathione peroxidase, selenium-independent and selenium-dependent (GPx, Se-dependent). It differs in the number of subunits, the bonding nature of the selenium at the active centre and their catalytic mechanisms (Valko, *et al*., 2006).

Humans have four types of Se-dependent glutathione peroxidases which are known to add two electrons to reduce peroxides by forming selenoles (Se-OH). The seleno-enzymes allow them to eliminate peroxides as potential substrates for the Fenton reaction (Fig.1.3). GPx acts with tripeptide glutathione (GSH) which is present in high concentration in cells. GPx competes with catalase for H_2O_2 as a substrate and it is important in protection against low levels of oxidative stress (Valko, *et al*., 2006).

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

$$2GSH + ROOH \xrightarrow{GPx} GSSG + ROH + H_2O$$

Fig.1.3.The glutathione peroxidase reaction

(Valko, *et al*., 2006).

II) Chain breaking antioxidants (non-enzymatic antioxidant)

The chain breaking antioxidants are often represented by phenols or aromatic amines. In vivo, chain breaking antioxidants such as superoxide dismutase will react in the aqueous phase to inhibit the superoxide free radicals ($O_2^{\bullet -}$) and vitamin E which acts in the lipid phase to inhibit $ROO^{\bullet}$ radicals (Murray, *et al*., 1998). Other non-enzymatic antioxidants included ascorbic acid (Vitamin C) and flavonoids (Valko, *et al.*, 2007).

Type of chain breaking antioxidants

i) Vitamin E

Vitamin E is found in the first line of defense against peroxidation of polyunsaturated fatty acids in cellular and sub-cellular membrane phospholipids (Murray, *et al*., 1998). Vitamin E is a fat soluble vitamin that exists in many different forms. In humans, α-Tocopherol is the most active form of vitamin E (Valko, *et al*., 2006) and it is an effective antioxidant by breaking free radical chain reactions as a result of its ability to transfer a phenolic hydrogen to a peroxyl free radical of peroxidized polyunsaturated fatty acid (Murray, *et al*., 1998). Vitamin E will help people who consume it to decrease hepatic

fibrosis, bile ductal proliferation and inflammatory infiltration (Olanlokun, 2008). There is no research done to see the effectiveness of natural vitamin E as antioxidant.

ii) Vitamin C

Vitamin C or ascorbic acid is a very important antioxidant that works in the aqueous environments of the body (Valko, *et al*., 2006). Vitamin C is a reducing agent that can reduce molecular oxygen, nitrate and cytochromes a and c and it may act as a general water soluble antioxidant (Murray, *et al*., 1998). In vitro study has shown Vitamin C able to promote metal ion-dependent hydroxyl radical formation in biological fluids. Majority of in vivo studies show that after consuming Vitamin E and Vitamin C, there is a reduction in markers of oxidative DNA, lipid and protein damage (Valko, *et al*., 2006). It shows that *Phyllanthus acidus* which exhibits high FRAP value contains the highest amount of Vitamin C (Gunawardena and Silva, 2009).

iii) Flavonoids

Flavonoids is a group of polyphenolic compounds (Pourmorad, *et al*., 2006). The structural components of flavonoids consist of two aromatic rings linked through three carbon atoms that formed an oxygenated heterocycle. The antioxidant capacity of phenolic compounds and flavonoids has been discovered and it gives beneficial implications in human health such as in the treatment and prevention of cancer and cardiovascular disease. Phenolic compounds act as terminators of free radical chains and as chelators of redox-active metal ions in lipid peroxidation (Valko, *et al*., 2006).

According to past years research, it was found that flavonoid in the leaf extracts of *Smilax excelsa* L. showed high percentage inhibition on DPPH radicals (DPPH radical scavenging activity), and it can inhibit the oxidation of linoleic acid (linoleic acid system method), bleaching of β-carotene (β-carotene bleaching method) and also scavenge on superoxide radicals (superoxide radical scavenging activity), hydroxyl radicals (hydroxyl radical scavenging activity) and hydrogen peroxide (hydrogen peroxide scavenging activity). It also has the ability in electron donating (reducing power assay) and the ability to chelate iron (II) ions (chelation activity on Fe^{2+}) (Ozsoy, *et al*., 2008). Moreover, polyphenol content in the leaf of *Orthosiphon stamineus* has been reported to play important role in reducing oxidative stress by inhibits the formation of lipid peroxidation products using DPPH assay (Akowuah *et al*., 2005).

C) Oxidative stress and antioxidants

The oxidative stress is caused by an excess of oxidation and lack of antioxidant defense mechanism. It can damage all the constituents of human body including proteins, lipids and DNA, so it has to be a temporary condition, under controlled by the antioxidant defense system (Cornelli, 2009).

I) Mechanism of oxidation in Lipid peroxidation

Peroxidation of lipids that exposed to oxygen is responsible for damage of the tissues in vivo, where it may be a cause of cancer, inflammatory diseases, atherosclerosis and ageing (Murray, *et al*., 1998). Lipids can be oxidized, nitrated and chlorinated by some types of reactive species (RS) (Halliwell and Whiteman, 2004). The mechanism of lipid

peroxidation can be divided into three stages; initiation, propagation and termination (Poon *et al.,* 2004). The initiation phase represented activation of oxygen and is rate limiting (Valko *et al*., 2006) since OH is a highly reactive ROS. It attacks hydrogen from nearly C-H bond to form H_2O. The OH with other radicals can generate racemic peroxyl radicals that attack the hydrogens from other polyunsaturated fatty acids and a chain reaction begin. The reactions are called propagation. Multiple aldehydes are formed with varying length of carbons and terminate the chain reaction, which is in termination stage (Poon, *et al*., 2004).

To control and reduce lipid peroxidation, humans need antioxidant in their activities and nature. Naturally occurring antioxidants include vitamin E (tocopherol), which is lipid soluble and vitamin C which is water soluble. Vitamin E (α-Tocopherol) and vitamin C function together in a cyclic-type process. α-Tocopherol is converted into an α-tocopherol radical by the donation of a labile hydrogen to a lipid or lipid peroxyl radical in antioxidant reaction. The α-tocopherol radical is reduced to the original α-tocopherol form by vitamin C (Valko, *et al*., 2006).

Vitamin C cooperates with vitamin E to regenerate α-tocopherol from α-tocopherol radicals in membranes and lipoproteins. Vitamin C has two ionisable hydroxyl groups known as di-acid ($AscH_2$) (Fig.1.4). 99.9% of Ascorbic Acid present as $AscH^-$, $AscH_2$ (0.05%) and Asc^{2-} (0.004%). $AscH^-$ is a donor antioxidant that reacts with radicals to produce the resonance stabilized tricarbonyl ascorbate free radical ($AscH^{\bullet}$) and it is not protonated. The product of ascorbate oxidation by many ROS is the semidehydroascorbate radical ($Asc^{\bullet-}$) a poorly reactive radical (Valko, *et al*., 2006).

Flavonoids is an ideal scavenger of peroxy radicals because of their reduction potentials relative to alkyl peroxyl radicals and are also effective inhibitors of lipid peroxidation (Ozsoy, *et al*., 2008). They have the ability to donate hydrogen and to scavenge a reactive radical with the presence of B-ring catechol group. In addition, the presence of functional groups included both hydroxyl groups of ring-B and the 5-hydroxygroup of ring-A are important to flavonoids in order to chelate redox-active metals and prevent catalytic breakdown of hydrogen peroxide (Valko, *et al*., 2006).

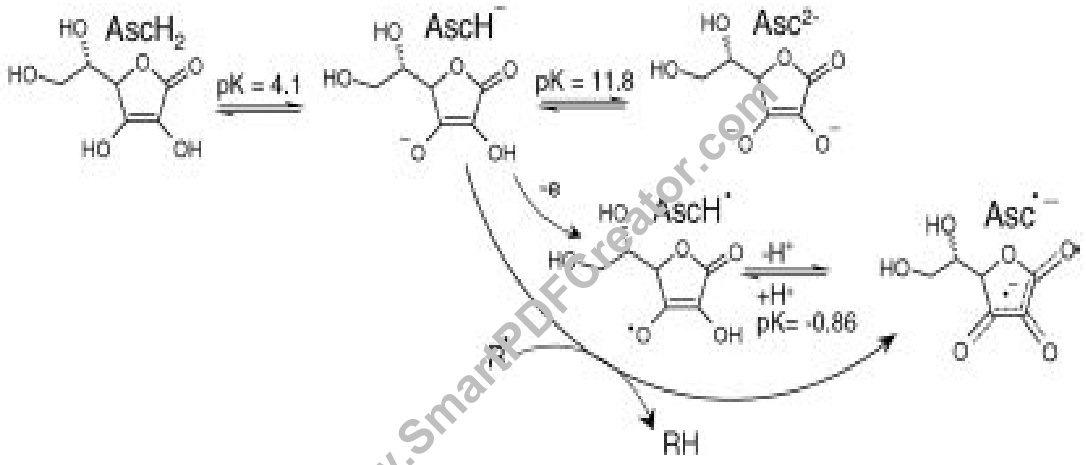


Fig.1.4.Various forms of Ascorbic Acid (Vitamin C) and its reaction with radicals (R•) (Valko et al., 2006)

II) Human diseases related to oxidative stress

There are various diseases that related to ROS like ageing, cancers, coronary heart disease, Alzheimer's disease, neurodegenerative disease disorders, atherosclerosis, cataracts, inflammation (Chang, *et* al., 2007), diabetes mellitus, myocardial infarction (Adedapo, *et al.,* 2008) and AIDS (Pourmorad *et al*., 2006). Diseases related to reactive oxygen species (ROS):

i) Cancer

Cancer is the second most common cause of death after cardiovascular disease. Cancer cells can be classified by three properties:

a. inhibited or uncontrolled of growth.

b. invasion of local tissues.

c. spread to other parts of the body (Murray, *et al*., 1998)

High endogenous level of oxidative substance, deficiency of antioxidants and mitogenesis are important risk factor of cancer (Scandalios, *et al*., 1992). Ageing is caused by cancer can be characterized into radiant energy, chemical compounds, and viruses. Ultraviolet rays and x-rays that are mutagenic and carcinogenic can form free radicals in tissues. Free radicals can interact with DNA and other macromolecules, leads to molecular damage and may contribute to carcinogenic effects of radiant energy (Murray, *et al.,* 1998). A cellular redox imbalance is induced by oxidative stress in various cancer cells when compared to normal cells. DNA damage by ROS involves single or double stranded DNA breaks, pyrimidines, purine or deoxyribose modifications and DNA cross-link (Valko, *et al*., 2007).

Anticancer drugs can be taken to inhibit the process of carcinogenesis, but if the redox state in the body is imbalance it can cause the secondary cancer. Therefore, consumption of antioxidant such as vitamin E, vitamin C and β-carotene is useful in preventing the carcinogenesis in the cancer treatment (Noda and Wakasugi, 2001).

ii) Cardiovascular disease

Cardiovascular disease is a term describing all diseases that involve the heart and circulatory system including coronary heart diseases, congestive heart failure and peripheral vascular diseases. It is the major cause of death in developed countries (Goh, *et al*., 1995). The oxidative stress in cardiac and vascular myocytes induced by ROS has been linked with cardiovascular tissue injury. The ROS-induced play a role in many types of cardiovascular diseases like atherosclerosis, hypertension, and congestive heart failure. The major sources of oxidative stress that associated with cardiovascular system involve xanthine oxidoreductase (XOR), NAD(P)H oxidase, NOS, mitochondrial cytochromes and hemoglobin (Valko, *et al*., 2007). In the previous study it was found that antioxidants played an important role in cardiovascular diseases. Glutathione, a radical scavenger was found to inhibit endothelin-induced ROS generation in cardiovascular diseases (Valko, *et al*., 2007).

iii) Hypertension

Hypertension develops as the result of disturbance of the body's blood pressure regulating system (Duncan, *et al*., 1999). It is a major risk factor for the development of cardiovascular disease (Chiong, *et al*., 2008). Hypertension can be classified into two types, essential or primary hypertension and secondary hypertension (Goh, *et al*., 1995). Primary hypertension is the most frequently type of hypertension. The symptom of this disease can not be identified but it has been linked to family history of hypertension and obesity. Secondary hypertension affects a small but significant number of the hypertensive

population and unlike primary hypertension, is a potentially curable condition (Chiong, *et al*., 2008).

Renin-angiotensin system has an important role in the development of hypertension and cardiovascular diseases. Excessive oxidation of this system is the main cause of hypertension and this system is regulated by angiotensin converting enzyme (Loizzo, *et al.*, 2008). In clinical medications, a lot of antihypertensive drug were used to prevent and cure hypertension, but they can cause side effects such as hypokalemia and hyperglycemia. Several classes of drugs such as angiotensin converting enzyme inhibitors, angiotensin receptor blockers, beta-blockers, calcium-channel blockers and thiazide-type diuretics are used in medical treatment of hypertension (Goh, *et al*., 1995).

In addition, antioxidants are also important in hypertension treatment because they can improve the vascular and renal function to reduce blood pressure. The reactive oxygen species (ROS) is important in severe hypertension. When the level of ROS scavengers such as vitamin E and glutathione is reduced, the activity of antioxidant is decreased and it contributes to oxidative stress. Therapeutic blood pressure lowering action such as AT_1 receptor blockers and angiotensin converting enzyme inhibitor (ACEI) have been attributed to NA(D)PH oxidase inhibition and decreased ROS production (Touyz, 2004).

1.2 Angiotensin Converting Enzyme (ACE)

A) Introduction

Angiotensin converting enzyme (ACE) is a 150 to 180 kDa ectoenzyme expressed in many organism tissues, including vascular endothelium, renal proximal tubular endothelium, heart, lung, activated macrophages and brains (Lapointe and Rouleau, 2002) . The active site of ACE consists of 3 parts:

a) A carboxylate binding functionality.

b) A pocket that accommodates a hydrophobic side chain of C-terminal amino acid residues.

c) A zinc ion that coordinates to the carbonyl of the penultimate peptide bond of the substrate. The carbonyl groups become polarized and they are subjected to a nucleophilic attack (Loizzo, *et al*., 2008).

ACE is a major link between the renin-angiotensin system (RAS) and the kinin systems (Watanabe, *et al*., 2005). As a bioactive component of renin-angiotensin system (RAS), ACE plays a significant role in blood pressure regulation, fluid and electrolyte balancing, cardiovascular system development and vascular remodeling by hydrolyzing angiotensin I into a potent vasopressor peptide angiotensin II. Moreover, it also deactivates the vasodepressor peptide bradykinin (Zhao and Xu, 2008).

B) Mechanism of Angiotensin Converting Enzyme (ACE) in regulating hypertension

i) Renin-angiotensin-aldosterone system (RAAS)

Renin angiotensin-aldosterone system (RAAS) is a circulating and hormonal system that regulates blood pressure, electrolyte and fluid homeostasis in the body of organism and it begins with the biosynthesis of renin by the juxtaglomerular (JG) cells. Renin functions as an unusual endocrine axis when the active hormone, angiotensin II is formed (Fig.1.5). This reaction is initiated by the regulated secretion of renin, the rate limiting processing enzyme (Atlas, 2007).

Renin is a monospecific enzyme that specific for its substrate, angiotensinogen. Angiotensinogen is a 60kD glycoprotein of the α-globulin fraction in plasma protein. It is synthesized and released mainly from liver (Atlas, 2007). It consists of 2 homologous lobes with the active site residing in the deep cleft located between them. The active site can accommodate 7 amino acid units of the substrate, angiotensinogen and cleave the Leu10-vall peptide bond within angiotensinogen to generate angiotensin I (Murray, *et al*., 1998).

The renin release from secretory granules is regulated by four factors:

a) Renal baroreceptor mechanism in the afferent arteriole that senses changes in renal pressure.

b) Changes in delivery of NaCl.

c) Sympathetic nerve stimulation through beta-1 adrenergic receptors

d) Negative feedback by direct action of angiotensin II on the juxtaglomerular (JG) cells.

Once it is secreted, renin cleaves the N-terminal portion of angiotensinogen to form the biologically non-active decapeptide angiotensin I (Atlas, 2007). In the RAAS, the inactive decapeptide, angiotensin I is hydrolyzed by angiotensin converting enzyme (ACE), which removes the C-terminal dipeptide to form the octapeptide angiotensin II, that is biologically active and potent vasoconstrictor (Atlas, 2007). Angiotensin II is more than a hormone that expresses hemodynamic and renal actions but that it is also a local, biologically active mediator that has direct effects on endothelial and smooth muscle cells. It plays a key role in the initiation of pathobiological events that lead to vascular disease.

Recent clinical trials of ACE inhibitors have consistently reported the salutary effects of this class of agents in treating and preventing cardiovascular disease and its modest effect on blood pressure lowering (Dzau, 2001). Angiotensin II is the primary effector of a variety of RAAS-induced physiological and pathophysiological actions. There are four types of angiotensin II receptor subtypes and the type 1 (AT1) receptor mediates most of the action in angiotensin II. This includes actions on cardiovascular system, in increasing the blood pressure (Atlas, 2007). Angiotensin II stimulates the synthesis and the releases aldosterone from the adrenal cortex through AT_1 receptor, which increases the blood pressure through sodium retention (Atmani, *et al.,* 2009).

Aldosterone is a major regulator of sodium and potassium balance; therefore it plays a major role in regulating extracellular volume. It enhances the reabsorption of sodium and water in the distal tubules and collecting ducts to promote potassium excretion (Palmer and Williams, 2005). Angiotensin II actively raises blood pressure through two main effects:

a) Angiotensin II acts directly on the walls of blood vessels making them contract and causing them to narrow. By doing this, blood cannot flow so freely and therefore blood pressure increases. Because of this function, angiotensin II is also called a vasoconstrictor.

b) Angiotensin II stimulates adrenal glands to release another hormone called aldosterone. Aldosterone causes sodium to be reclaimed by kidney, which in turn attracts water through osmosis leading to an increase in blood pressure. (Palmer and Williams, 2005).

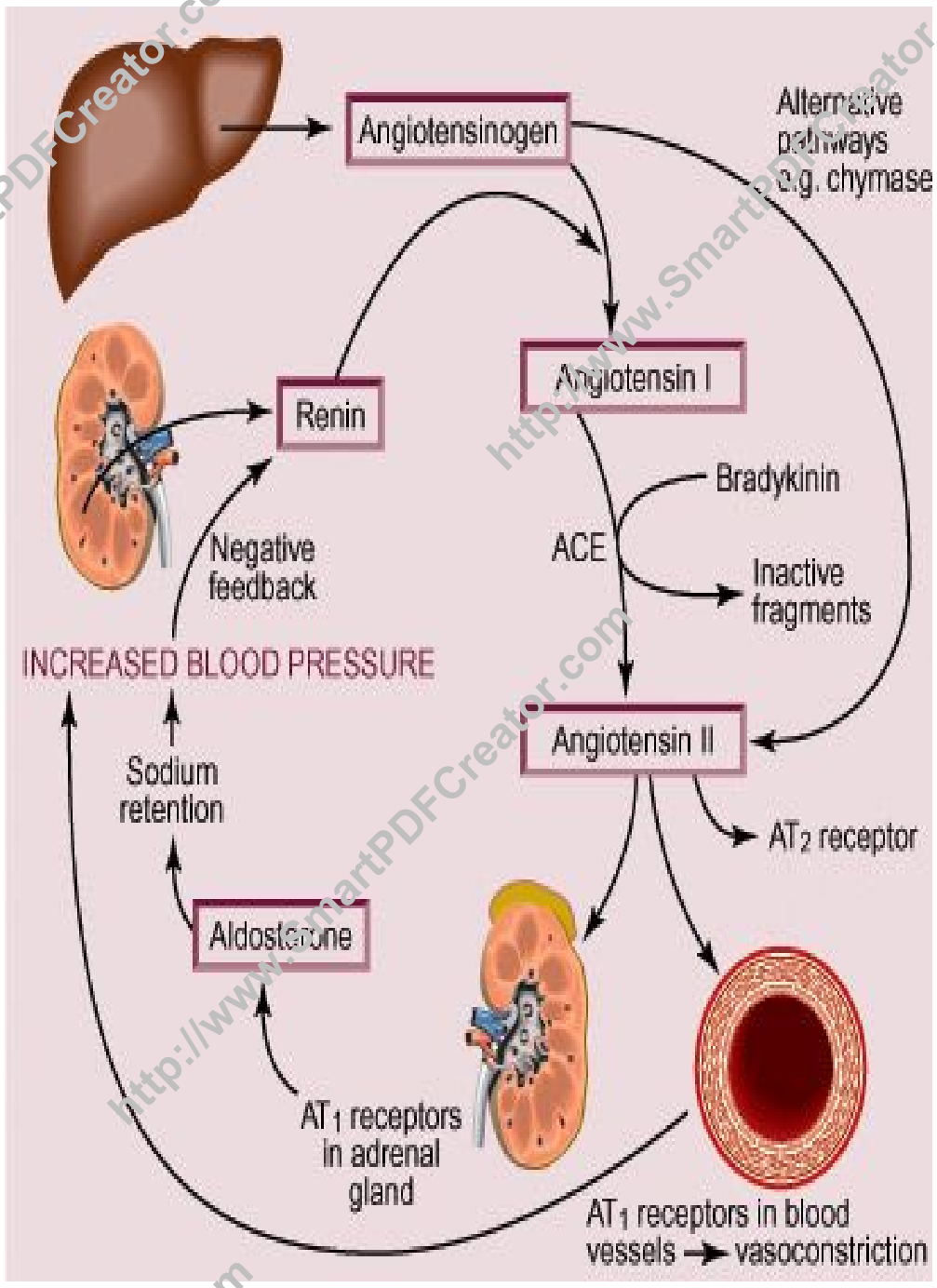


Fig.1.5. Renin-angiotensin-aldosterone system (RAAS)

(Atlas, 2007).

C) Angiotensin Converting Enzyme Inhibitors (ACEI)

Angiotensin converting enzyme inhibitors (ACEI) are used as antihypertensive drugs because they can inhibit the activity of angiotensin converting enzyme (ACE) which is regulates the conversion of angiotensin I to angiotensin II (Lapointe and Rouleau, 2002). In the treatment of hypertension disease, ACEI is first utilized from the venom of the Brazilian viper (Nyman, *et al*., 1998). The natural peptide-inhibitors of ACE from the snake venom with C-terminal of proline, succinylproline are synthesized as the first target compound. However, after modifying the structure of the compounds, an oral nonpeptide compound, captopril, enalapril, benazepril and fosinopril are found as a new ideal hypotensive. But some side effects have been reported such as cough and sexual hypoacusis when they are used for a long time (Zhao and Xu, 2008).

ACEI have shown to be beneficial in a wide range of cardiovascular diseases, whether chronic heart failure (CHF), hypertension, atherosclerosis or diabetes. Evidence from experimental and clinical studies would suggest that the beneficial cardiovascular effects of ACE inhibitors are the result of their effects on the conversion of Angiotensin-I to Angiotensin-II (Lapointe and Rouleau, 2002). ACEI are considered effective and safe for the treatment of hypertension. Their anti-hypertensive effect is enhanced by a low salt diet (Duncan, *et al.,* 1999). The ACEI inhibit the production of Angiotensin II that is a potent vasoconstrictor, by blocking the conversion of Angiotensin I to Angiotensin II. Inhibition of ACE results in a decrease in blood pressure (Pihlanto, *et al*., 2008).

ACEI inhibit the activity of ACE in myocardium, kidney, vessel wall through decreasing blood pressure and inhibiting myocardial and vascular hypertrophy (Fig. 1.6). They can also improve the autonomic nervous activity of patients with chronic heart failure. ACEI are not only used as antihypertensive drug, but also for the treatment of cardiovascular system, endocrine and urinary system because it has been tested that myocardium hypertrophy and myocardial fibrosis are reduced and ventrical remodeling is improved in the treatment by using ACEI (Zhao and Xu, 2008).

However, the side effects such as cough and angioneurotic edema associated with clinically used synthetic ACEI have been addressed. Synthetic ACEI are also known to be deleterious in pregnancy (Oh, *et al*., 2002). Therefore the screening and the development of new ACE inhibitors would be beneficial in the treatment of hypertension. ACEI have been identified and isolated from plant and animal sources such as mushrooms, skeletal muscle, fish scales, fermented foods, sunflower seeds, chickpeas and peas (Quist, *et al*., 2009). A number of compounds from plants have been identified to possess in vitro ACE inhibitory activity including flavonoids, xanthones, fatty acids, terpenoids, and alkaloids (Braga *et al*., 2007).

No chemical compound from *O. stamineus* have been evaluated as active principles for the antihypertensive action. The main objective of this study is to isolate compounds from the leaves of *O. stamineus*. The result is an evident of the important of *O. stamineus* in the treatment of hypertension.

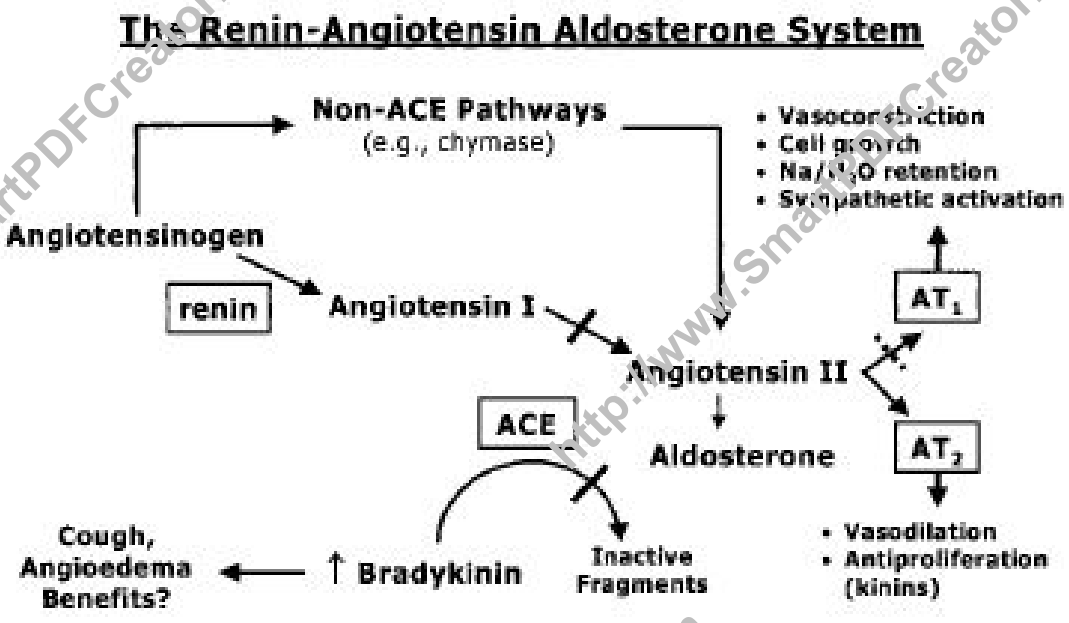


Fig.1.6. Schematic of RAAS system and site action of ACE inhibitors

(McMurray, *et al*., 2004)

1.3 Medicinal plant - *O. stamineus* (Misai Kucing)

A) Description

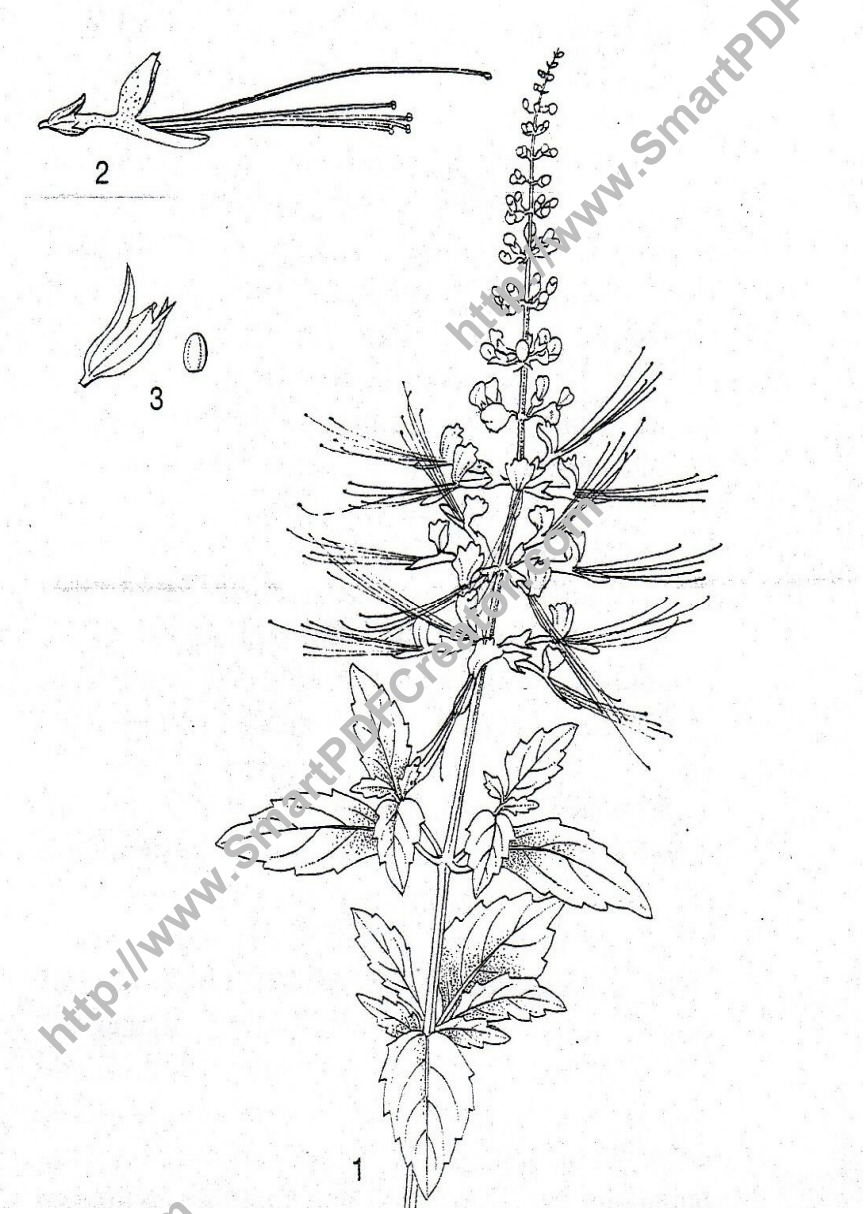


Fig.1.7. Parts of *O. stamineus*

1. flowering stem; 2. flower; 3. fruiting calyx and nutlet

(de Padua *et al*., 1999)

O. stamineus from the family of Lamiaceae (Zakaria, *et al.,* 2008) and subfamily of *Nepetoideae* (de Padua, *et al*., 1999) is a medicinal plant that commonly grown in Southeast Asia and mostly cultivated in Indonesia (Fig.1.7). The synonyms name of *O. stamineus* is *O. aristatus* and the other common name is Java Tea (Matkowski, 2008). The *Lamiaceae* mint family is a large taxon of several thousand species which includes numerous popular and less known herbs with pronounced therapeutic properties (Matkowski, 2008).

O. stamineus is a perennial herb, 25 to 200 cm of tall (de Padua, *et al.,* 1999). The flowers of *O. stamineus* borne on verticals about 16 cm length, white to bluish in colour with long far-exerted filaments make them known as cat's whiskers or 'Misai Kucing' in Malaysia (Han, *et al*., 2009). The leaves of *O. stamineus* are arranged in opposite pairs and its petiole is short, about 0.3 cm in length and reddish purple in colour (Han, *et al*., 2009).

The leaves are ovate or rhombic, cuneate at base, acute or acuminate at apex, serrate, glabrous or minutely pubescent and glandular-punctate. The fruits of *O. stamineus* splitting into 4 oblong-ovoid nutlets in 1.5 to 2 mm long and brownish in colour (de Padua, *et al*., 1999). The dried leaves and stem tips of *O. stamineus* contains 12% of minerals with high contents of potassium, lipophilic flavones included sinensitin, flavonol glycosides, caffeic acid derivatives, inositol and saponins (de Padua, *et al*., 1999).

B) Medical uses

In Malaysia as well as in some other countries traditional medicine is accepted as one of the various treatment systems and it is being practiced widely by every level of the society. *O. stamineus* is one of the most valuable local medicinal plants which provide bioactive medicinal compounds. The entire part of the *O. stamineus* plant is used for medicinal formulation and it is basically used to treat diabetes, hypertension and rheumatism (Tezuka, *et al*., 2000). Leaves of *O. stamineus* are consumed because of its mild diuretic, anti-fungal and bacteriostatic activity (Hossain, *et al*., 2008). In Vietnam, the aerial part of *O. stamineus* is used in treating many diseases including edema, hepatitis, urinary lithiasis, influenza and jaundice (Tezuka, *et al*., 2009).

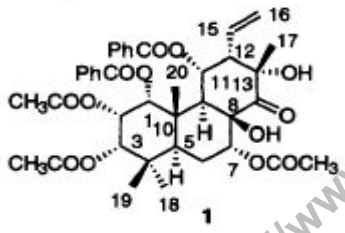
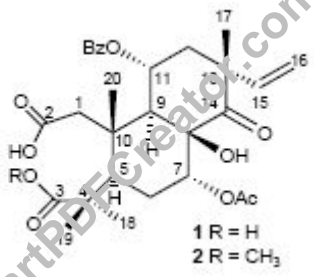
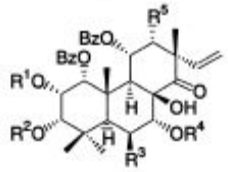
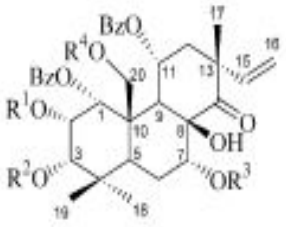
The leaves of *O. stamineus* have been widely used as a diuretic in tea and treatment against various kidney diseases and gallstones. Various tests have been performed to demonstrate the diuretic activity of *O. stamineus* in animals and man. In Europe, *O. stamineus* has been taken to reduce inflammation and in treatment of bacterial infections of urinary tract. The leaves of *O. stamineus* are boiled together with *Andrographis paniculata* 'Hempedu bumi' and consumed as tea to treat diabetes (de Padua, et al., 1999).

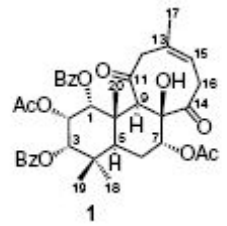
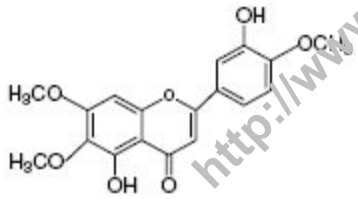
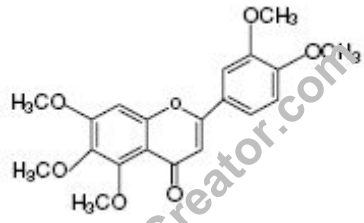
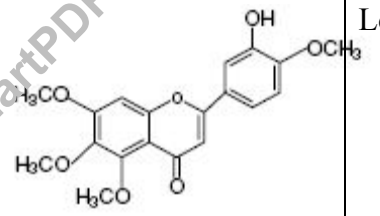
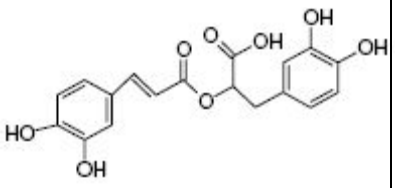
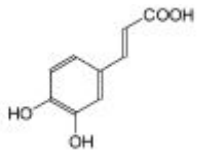
In scientific research, *O. stamineus* has been reported showing anti-fungal properties. The oils and methanol extract of *O. stamineus* show great potentials of anti-fungal activity against phytopathogenic fungi (Hossain *et al*., 2008). Aqueous extract of *O. stamineus* has antimicrobial properties, and it can inhibit the growth of gram positive and gram negative bacteria. The lipophilic flavonoids that present in *O. stamineus* have shown inhibitory effect against tumour cells (de Padua, *et al*., 1999). However, nothing is known yet about the antihypertensive components of *O. stamineus*.

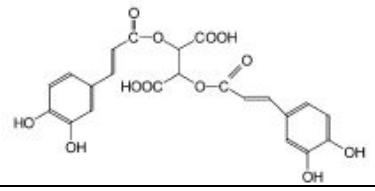
C) Chemical constituents

O. stamineus contains a number of potentially bioactive compounds, especially from polyphenolic group (Matkowski, 2008). The main components of leaves of *O. stamineus* are the pharmacologically active polyphenols: the polymethoxylated flavonoids and the caffeic acid derivatives (Olah, *et al.,* 2003). Rosmarinic acid, lipophilic flavones with highly methoxylated substation patterns such as eupatorin, sinensitin, teramethylscutellarein, and 3'-hydroxy-5,6,7,4'-tetramethoxyflavone are the major compounds in *O. stamineus*. Other compounds such as diterpenoids including isopimarane and staminane skeleton-based have also been found in *O. stamineus* (Matkowski, 2008). Table 1.1 shows compounds isolated from *O. stamineus*. Among these compounds, the flavonoids and caffeic acid derivatives are found to possess potential therapeutic properties, as they are shown to exert diuretic and uricosuric actions in rats (Loon, *et al*., 2005).

Table 1.1.Chemical compounds isolated from *O. stamineus*

Compound	Chemical Structure	References
Diterpene: (Staminol A)		Stampoulis, *et al.,* 1999
Diterpene: (Secoorthosiphols A-C)		Awale, *et al*., 2002
Orthosiphols R-T		Awale *et al*., 2002
Diterpene: (Siphonols A-E)		Awale *et al.,* 2003
Diterpene: (Neoorthosiphonone A)		Awale, *et al.,* 2004

	 1	
Polyphenol: Polymethoxylated flavonoid (Eupatorin)		Olah, *et al*., 2003 Akowuah, *et al.,* 2004 Loon, *et al.,* 2005
Polyphenol: Polymethoxylated flavonoid (Sinensetin)		Olah, *et al*., 2003 Akowuah, *et al.,* 2004 Loon, *et al*., 2005
3'-hydroxy-5,6,7,4'-tetramethoxyflavone (TMF)		Akowuah, *et al.,* 2004 Loon, *et al.,* 2005
Caffeic acid derivative: (Rosmarinic acid)		Olah, *et al*., 2003 Akowuah, *et al.,* 2004
(Caffeic acid)		Olah, *et al*., 2003

Caffeic acid derivative : (Cichoric acid)		Olah, *et al*., 2003

Methylripariochromene A, a flavonoid characterized by *O. stamineus* exhibits hypotensive and vasodilating properties and decreases the cardiac output in animals. This flavonoid has been isolated as the major constituent of an aqueous extract of leaves of *O. stamineus*. It inhibits the contractile response of the smooth muscle of thoracic aorta of rats stimulated with potassium chloride. It also reduces the systolic blood pressure and the heart rate in spontaneously hypertensive rats (Wiart, 2002). This compound has exhibited anti-hypertensive activity (Wai-Leng, *et al*., 2004). Flavonoids is a class of low molecular weight secondary plant metabolites found in most land plants. Most of the protective effects of flavonoids in biological systems are their antioxidant abilities, capacity to transfer electrons, free radicals and chelating abilities, activate antioxidant enzymes, reduce alpha-tocopherol radicals and inhibit oxidases (Akowuah, *et al.,* 2004).

Phenolic phytochemicals are secondary metabolites of plant origin which constitute one of the most abundant groups of natural metabolites and are synthesized by plants in order to protect themselves from biological and environmental stresses. Recent studies have shown that phenolic phytochemicals have high antioxidant activity and certain therapeutic properties including antihypertension activity (Apostolidis, *et al.,* 2006). Twenty phenolic compounds are isolated from *O. stamineus* including nine lipophilic flavones, two flavonol glycoside, nine caffeic acid derivatives and 2,3-dicaffeoyltartaric acid are identified and quantified by high performance liquid chromatography (HPLC) (Akowuah, *et al*., 2004).

Three main flavonoids found in *O. stamineus* such as sinensitin, eupatorin, and 3' hydroxyl-5, 6,7,4'-tetramethoxyflavone are also shown to posses cytotoxic, antifungal and antioxidant activities. Moreover, sinesitin has recently been reported to reverse the P-glycoprotein –mediated multidrug resistance in the absorption of drugs. Several HPLC methods for the analysis of these flavonoids have been reported in the literature (Loon, *et al*., 2005).

Awale *et al*., 2004 reported that separation of the methanol extract led to the isolation of four novels highly oxygenated isopimarane-type diterpenes named siphonols A-D and a novel norisopimarane-type diterpene, siphonol. They also investigated the constituents of *O. stamineus* cultivated in Okinawa and isolated three new highly oxygenated 2,3-secoisopimarane-type diterpenes named secoorthosiphols A-C as extremely minor constituents, together with three staminane-type and five isopimarane-type diterpenes (Awale, *et al*., 2002). *O. stamineus* plays an important role in free radical scavenging and antioxidant activities (Zakaria, *et al*., 2008). Total phenolics content and antioxidant activity of methanol extract of *O. stamineus* are screened and its antioxidant activity is higher than synthetic antioxidant butylated hydroxylanisole (BHA) (Khamsah, *et al*., 2006).

1.4. Research Objectives

1. To extract chemical compounds from leaves of *O. stamineus* with methanol, chloroform, hexane and water.
2. To separate chemical compounds with Thin Layer Chromatography (TLC), Column Chromatography (CC) and High Performance Liquid Chromatography (HPLC) and identify the presence of chemical compounds with chemical reagents.
3. To determine the antioxidant activities in the crude extract of *O. stamineus* by using 3 different methods:

   a) DPPH free radical scavenging assay

   b) Reducing power assay

   c) Metal chelating assay
4. To determine percentage of ACE inhibition and ACE activity from crude extract and compound isolated from *O. stamineus*.
5. To determine total phenol content.
6. To determine LC_{50} value of the crude extract of *O. stamineus* from Brine Shrimp Lethality Assay (BSLA).