HYPOTENSIVE EFFECT OF AQUEOUS AND METHANOLIC EXTRACTS FROM *SYZYGIUM POLYANTHUM* (WIGHT) WALP LEAVES ON THE EVALUATION OF NITRIC OXIDE PATHWAY - AN *in vivo* APPROACH

By

NURUL ATIQAH BINTI ZULAZMI

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Certificate

This is to certify that the dissertation entitled "HYPOTENSIVE EFFECT OF AQUEOUS AND METHANOLIC EXTRACTS FROM *SYZYGIUM POLYANTHUM* (WIGHT) WALP LEAVES ON THE EVALUATION OF NITRIC OXIDE PATHWAY - An *in vivo* APPROACH" is the bonafide record of research work done by Ms Nurul Atiqah bt Zulazmi during the period from October 2012 to May 2013 under my supervision.

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List of symbol and abbreviation

CVD	Cardiovascular disease
MBP	Mean blood pressure
SBP	Systolic blood pressure
DBP	Diastolic blood pressure
HR	Heart rate
CAM	Complementary and alternative medicine
NO	Nitric oxide
NOS	Nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
CaM	Intracellular calcium/ calmodulin
L-NAME	N^{w}-nitro-L-arginine methyl ester
DMSO	Dimethylsulfoxide
AESP	Aqueous extract
Meth-AESP	Methanolic extract
WKY	Wistar-Kyoto rats
NADPH	Nicotinamide adenine dinucleotide phosphate

Abstrak

Syzygium polyanthum (Serai kayu) ialah salah satu herba tradisional yang dimakan segar oleh rakyat Malaysia dan juga digunakan secara tradisional untuk mengubati tekanan darah tinggi, strok, kencing manis, kolesterol tinggi, katarak, ruam, gatal dan sakit perut. Data yang diperolehi daripada kajian lepas, medapati profil biologi dan analisis fitokimia bagi tumbuhan ini telah dilakukan dan terdapat beberapa bahan yang mempunyai potensi dalam menurunkan darah tinggi. Walaupun tumbuhan ini dibuktikan mempunyai potensi mengubati darah tinggi tetapi mekanisma farmakologinya masih tidak diketahui. Maka, dalam kajian ini, kami ingin menentukan kesan kardiovaskular *S. polyanthum* ekstrak pada tekanan darah tikus yang dibius dan penglibatan sistem nitric oksida dalam kesan penurunan darah tinggi. Dua penyediaan ekstrak telah dilakukan iaitu penyediaan ekstrak air (AESP) dan ekstrak metanol (Meth-AESP). AESP disediakan melalui proses beku-kering dan sisa dari penyediaan ini diteruskan menggunakan alat Soxhlet untuk pengekstrakan methanol (Meth-AESP). Kedua-dua ekstrak ini dikaji dengan melakukan suntikan ke urat leher kiri tikus dan tekanan darah direkodkan melalui arteri karotid kanan. Kedua-dua Meth-AESP dan AESP (100 mg/kg) mengurangkan tekanan darah tikus (MBP) ($p < 0.001$). Dengan tambahan L-NAME (20 mg/kg), hanya kesan penurunan tekanan darah oleh Meth-AESP telah dikurangkan dengan ketara ($p < 0.001$). Walau bagaimanapun, L-NAME tidak menjejaskan tekanan darah oleh AESP ($p > 0.05$).Kami membuat kesimpulan bahawa mekanisma penurunan tekanan darah oleh Meth-AESP mungkin melalui dan melibatkan sistem nitrik oksida.

Abstract

Syzygium polyanthum (Serai kayu) is one of the traditional herbs (ulam) that eaten freshly by Malaysian and traditionally used as a medicine for hypertension, stroke, diabetes, hypercholesterolemia, cataracts, rashes, itchy and sore stomach. Previous studies have established the biological profile and phytochemical analysis of *S. polyanthum* that contain anti-hypertensive associated compounds. Even though this plant has known to have potential in the treatment of hypertension but the pharmacological mechanism of it is still unclear. In this research, we want to determine the cardiovascular effects of *S. polyanthum* extracts on blood pressure of anaesthetised rat and the involvement of nitric oxide system in the hypotensive effect. *S. polyanthum* aqueous extract (AESP) was prepared by freezed-dried. The residue obtained was extract in Soxhlet extractor and successively fractionated using methanol to obtain the methanol extract (Meth-AESP). Both of the extracts were studied by administered through the left jugular vein of anaesthetised rat and the blood pressure is recorded through the cannulated right common carotid artery. Both Meth-AESP and AESP (100 mg/kg) significantly reduced the rat's mean blood pressure (MBP) ($p<0.001$). With the addition of L-NAME (20 mg/kg), the hypotensive response of Meth-AESP was significantly reduced ($p<0.001$). However, L-NAME did not affect the hypotensive response of AESP ($p>0.05$). We concluded that that hypotensive mechanism of Meth-AESP is likely mediated through the involvement of nitric oxide system.

Chapter 1: Introduction

1.1- *Syzgium polyanthum* (Wight) Walp leaves

Syzgium polyanthum is a member of Myrtaceae family that is widely distributed in the subtropical, tropical and temperate regions of the world (Perumal *et al.*, 2012). According to website of World Agroforestery Centre, *S. polyanthum* can be found as understory tree in lowland primary and secondary forests, also in thickets, bamboo forest and teak plantations. In addition, this plant is widely distributed in Burma (Myanmar), Indo-China, Thailand, Malaysia, and Indonesia (Java, Sumatra, Kalimantan). This plant is also known as *Eugenia polyantha* and the synonym is *Eugenia lucidula.* It is well known by the Indonesian people because this plant is widely spread in many regions of Indonesia such as in mountains and lowland areas. In addition, the society usually calls the plant as laurel plant or pohon salam (Studiawan and Santosa, 2005a).

This tree is mostly grown in the forest and can be found in lowland until 1400 meters above the sea level. It can grow about 25 meters and have large straight root, round truck, and smooth surface (Figure 1.1). Its flowers are small, white, and fragrant while the leaves have 2.5-8 centimetres long leaf with flat margins. The tip of the leaf is blunt and the base is stretch along length and thigh (Sumono and Wulan, 2008) (Figure 1.2). This plant is usually planted for the leaves, bark and fruits (Dalimartha, 2007). Each of the part of the plant has its own benefits such as the leaves can be used not only

as spices for cooking but also can be used as medicine. The bark can be used to make bamboo while the fruits extract have the ability to neutralize hang over caused by too much alcohol consumption (Sumono and Wulan, 2008).

Figure 1.1: *Syzygium Polyanthum* trees.

Figure 1.2: *Syzygium polyanthum* leaves.

1.2- Herbal medicine and Hypertension

According to the guidelines issued by the World Health Organization (The International Society of Hypertension (WHO-ISH) and the sixth report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure), hypertension in adults is defined as a resting systolic blood pressure (SBP) of 140 mmHg or greater and/or a diastolic blood pressure (DBP) of 90 mmHg or greater in adults who are not taking antihypertensive medication (Chobanian *et al.*, 2003).

A survey based study was conducted to determine the status of hypertension in Malaysia and found that low awareness, low treatment and poor control of hypertension in this country could be one of the factors that CVD has been the leading cause of death for the last 40 years (Lim and Morad, 2004). In fact, the high prevalence and detrimental sequlae of hypertension, made it as an important public health problem worldwide. Besides, they also predicted that by the year 2025, 1.56 billion of people worldwide are expected to have hypertension (Chockalingam *et al.*, 2006).

The cost of treatment for this disease has become spot of worry for economic evaluations in Malaysia. Hence, hypertension is said to be one of the most expensive disease as far as treatment is concerned. This could be due to the complications that caused by the disease such as major organ damages that add huge costs to the overall costs of health care (Al-Efan, 2009). Some treatments that are too costly provoke some

patients to be dissatisfied with the conventional treatment and seek out an alternative medicine (Astin, 1998).

Complementary and alternative medicine (CAM) is increasingly popular and many CAM options exist that found to lower the blood pressure. Indeed, there are a large number of herbal remedies tested for anti-hypertensive effects with varying result. However, the pharmacological mechanism of most of these herbs is largely unknown (Ernst, 2005).

1.3- L-arginine-Nitric Oxide pathway

Between 1987 and 1988, researchers demonstrated that nitric oxide (NO) can be generated from mammalian cells. This is showed by the generation of NO from L-arginine by vascular endothelial cells that accounts for biological properties called endothelium-derived relaxing factor (EDRF). In addition, the chemical, pharmacological and biological properties of EDRF and NO are said to be identical (Moncada, 1992).

Through a lot of research that have been done to evaluate this NO pathway, the latest showed that the synthesis of NO from L-arginine can occur not only in vascular endothelial cells, but also in the macrophages, neutrophils, brain synaptosomes, adrenal glands and a number of other tissues. This pathways plays a role in neurotransmission,

mediates some of cytotoxic function, vascular tone and blood pressure (Radomski *et al.*, 1990).

This L-arginine-nitric oxide (L-arginine-NO) pathway is said to be one of the crucial regulatory system in the circulation. Interestingly, the more recent discovery towards nitric oxide production has shifted the focus of interest in the potential role of this pathway in the cardiovascular control (Rajapakse and Mattson, 2009). NO is produced by the endothelial form under basal conditions, shear stress and receptor-operated agonist such as acetylcholine (Kung *et al.*, 1995). In addition, most of the endogenous NO is derived largely from enzymatic pathways that are catalysed by nitric oxide synthase (NOS) through a series of redox reaction (Luiking *et al.*, 2010).

There are at least two distinct NO synthase in different tissues that are the endothelial or brain enzyme and also the phagocytic cell enzyme. The isoforms are referred by the most nomenclature of nNOS (neuronal), iNOS (inducible) and eNOS (endothelial) (Alderton *et al.*, 2001). The eNOS and nNOS are said to be Ca^{2+} dependent and iNOS is not Ca^{2+} dependent (Radomski *et al.*, 1990). The main concern for this project is eNOS because of its role in producing NO that is responsible for maintaining the low vascular tone and dilation of blood vessel. As said before, the activities of eNOS are controlled by intracellular calcium/ calmodulin (CaM) levels and it is activated by blood shear, or receptor stimulation (Guzik *et al.*, 2003). The increase in cytoplasmic calcium levels activates CaM that lead to efficient NO synthesis from eNOS (Sessa, 2004).

The eNOS is known as a major weapon of endothelial cell to fight vascular disease and generate the vasoprotective molecule nitric oxide (Förstermann and Münzel, 2006). Thus, the blockade of NO synthesis by the NOS inhibitors such as L-monomethyl arginine (L-NMMA) or N^{w}-nitro-L-arginine methyl ester (L-NAME) can cause endothelium dependent contractions and lead to hypertension. Other activities that can elevate the blood pressure include the reduce activity of nitric oxide synthase (NOS) activity and also the decrease of NO concentration (Sakai *et al.*, 2003).

1.4- Scope of the research

Syzygium polyanthum (serai kayu) is known as one of the traditional herbs (ulam) that eaten freshly by the Malaysian and traditionally used as a medicine of hypertension (A Ismail *et al.*, 2012). Besides that, it is also used as a medicine for diabetic, diarrhoea, gastritis, drunks and skin disease (Sumono and Wulan, 2008). Previous studies have established the biological profile and phytochemical analysis of *S. polyanthum* that might be the factor that contribute to the traditional used of this herb (Kusuma *et al.*, 2011). Even though, it might be used as one of the alternative medicine for hypertension but the pharmacological mechanism of it is still unclear. L-arginine-NO pathway is an important signalling pathway in depression. With this information, the present study aimed to study the involvement of this pathway in the hypotensive effect of *S. polyanthum* (Kulkarni and Dhir, 2007). In this research, *in vivo* approach has been employed to see the overall effect of this plant extracts on the normotensive Wistar-Kyoto rats.

1.5- Aim of the study

Syzygium polyanthum is broadly use especially in Singapore, Indonesia and Malaysia in local cooking as a spice and as a flavour enhancer. *S. polyanthum* is also said to be one of the potential alternative medicine due to its biological properties (Kusuma *et al.*, 2011).

Despite of the availability of information regarding the biological capabilities of this *S. polyanthum*, none had discussed the effect on the cardiovascular system. Since, *S. polyanthum* has been used as the traditional medicine for hypertension, so it is possible to determine scientifically the benefits of this plant on the cardiovascular system. In fact, the screening of the potential benefits of locally consumed folkloric medicine might be valuable because a few successes of phytochemical screening of medicinal plants had revealed the popular hypertensive medicines such as digitoxin (*Digitalis purpurea*),digoxin (*Digitalis lanata*) and atropine (*Atropa belladonna*) (Pan *et al.*, 2009).

There are several mechanisms could explain the hypotensive effect of this plant such as the central and peripheral sympatholytic effect; blockade of the Renin-Angiotensin-System (RAS), direct or indirect enhancement of NO production, changes in calcium homeostasis, among other mechanisms (Lima-Landman *et al.*, 2007). In this study the enhancement of NO production is chosen as a model to explain the *in vivo* cardiovascular effects of *S. polyanthum*.

This study also serves as a basis to study the active compounds in *S. polyanthum* that affecting the coronary blood flow. In addition, the experimental model of hypertension that permits the evaluation of the NO pathway to study the mechanisms of cardiovascular effects of other plants will be established.

Therefore the objectives of this study are:

i. To determine the cardiovascular effects of aqueous and methanolic extracts from *Syzygium polyanthum sp.* leaves on the anaesthetized male adult normotensive Wistar-Kyoto rats.

ii. To determine the involvement of Nitric Oxide (NO) system in the hypotensive effect of *Syzygium polyanthum sp.* extracts

1.6- Hypothesis

The nitric oxide system mediated partly the hypotensive effect of *Syzygium polyanthum* sp. extracts.

Chapter 2: Literature Review

2.1- *Syzygium polyanthum Wight (walp)* leaves.

The leaves of *Syzygium polyanthum* are recognized as one of the popular medicinal plants in Indonesia and it has been used as a culinary additive among the society. In addition, the leaves can also be used for diabetes and skin infection treatment (Kusuma *et al.*, 2011). Besides, they also have been used for anti-ulcer, anti-diabetes, anti-inflammatory, anti-diarrhoea treatments (Lelono *et al.*, 2009). In Malaysia the leaves is common as one of the traditional herbs (ulam) that is eaten freshly by the Kelantanese.

The plant has been known since long time ago as a species that can be used as therapy and the leaf has been developed medically as an alternative medical plant. The chemical properties contain in this leaf has been associated with many medicinal properties. One of it is the anti-hypertensive properties and vasorelaxant properties as previously shown by the ability to mediate the hypotensive effect *in vivo* (Lahlou *et al.*, 2004). This leaf also has many pharmacological activities that are useful in dentistry such as it can reduce pain after tooth extraction (Sumono and Wulan, 2008). The extract of the leaves also can decrease the blood glucose level (Studiawan and Santosa, 2005a). In addition, they also had a significant *in vitro* anti-oxidative activity (Lelono *et al.*, 2009).

2.2- Chemical Properties of *Syzygium polyanthum Wight (walp.)* leaves.

Syzygium polyanthum leaves have been examined for the biological activities and some of the active substances and chemical composition have been isolated and identified. The plant extracts phytochemical screening showed that the crude extracts contained alkaloid, carbohydrate, tannin, alkaloid, steroid, triterpenoid, flavonoid and saponin (Kusuma *et al.*, 2011).

In addition, based on the antioxidant research that conducted on the methanolic crude extracts of *S. polyanthum* leaves has revealed that this leaves showed mild antioxidant property due to both of detected phenolic acids, gallic and caffeic acid (Lee Wei HAR and ISMAIL, 2012).

Agus and Agustin (2008), have shown the leaves has many chemical properties. Some of the properties that have been stated are the tannins, flavonoid, and essential oils, including citric acid and eugenol. Indeed, these properties are basic matters for therapy and treatment that are used in almost every section in dentistry.

In addition, previous research examined that this leaves have potential in decreasing the glucose level activity on mice due to the chemical constituents that present is the essential oils such as sitral, eugenol, tannins and flavonoids (Studiawan and Santosa, 2005b). Twenty seven compounds were detected in this leaves extracts using two different methods of distillation by steam distillation with and without n-

hexane. The main compounds found are the cis-4- decene, oktanal, α-pinene, farnesol, β-osimen, and nonanal (Wartini and Made, 2009).

2.3- Nitric oxide pathway in hypertension

Generally, it's now has been accepted the fact that endothelium-derived relaxing factor (EDRF) is identical to nitric oxide (NO). While an amino acid called L-arginine serves as the precursor for NO. Vascular NO has many functions in vessels such as it causes the relaxation of blood vessels (Förstermann, 2010). While an amino acid called L-arginine serves as the precursor for nitric oxide. Recent discovery showed that nitric oxide derived from L-arginine is a major endothelium-derived relaxing factor in the vessel and has a greater potential in cardiovascular control (Rajapakse and Mattson, 2009).

Endogenous NO is derived largely from enzymatic pathway and is catalysed by NO synthase (NOS). The enzymatic pathway described is a series of redox reaction that include the degradation of L-arginine to L-citrulline and NO. In addition, this NO production is dependent on the availability of the arginine precursor and the activity of various NOS enzymes (Figure 2.1). The decrease of constitutive NO production in the vasculature can cause development of vascular disease (Luiking *et al.*, 2010). Hence, increasing NO is a potential therapeutic target in hypertension (Rajapakse and Mattson, 2009).

Based on the brief mechanism, NO that has been formed will rapidly diffuses into the blood where it binds to heme moiety of haemoglobin. It also diffuses into the vascular smooth muscle cells besides to the endothelium where it binds to and activates enzyme guanylyl cyclase to catalyzes the dephosphorylation of GTP to cGMP. This process serves as a second messenger for many important cellular functions, particularly for signalling smooth muscle relaxation. There are some mechanism found that explain the smooth muscle relaxation such as the increases of cGMP associates with decreases intracellular Ca^{2+} concentration. Next, K^{+} channel activation leads to hyperpolarization and the mechanism where the stimulation of a cGMP-dependent protein kinase activates enzyme that dephosphorylates myosin light chains (Lincoln and Cornwell, 1991).

N-ω-nitro-l arginine methyl ester (L-NAME) is one of the non-selective inhibitor for nitric acid synthase (NOS). In fact, the administration of L-NAME will cause chronic inhibition of NOS on the normotensive rat and induce a sustained hypertension. Thus, loss of NO-mediated vasodilation may be involved in diseases states such as essential hypertension. In agreement, Dowell *et al.*, (1996) have shown that acute nitric oxide synthase inhibition will enhance the actions of range vasoconstrictor agents both *in vitro* and *in vivo*. The relaxation induced by L-arginine on the vessel such as on the aorta was also antagonized by the inhibitor of NO biosynthesis (Moritoki *et al.*, 2012).

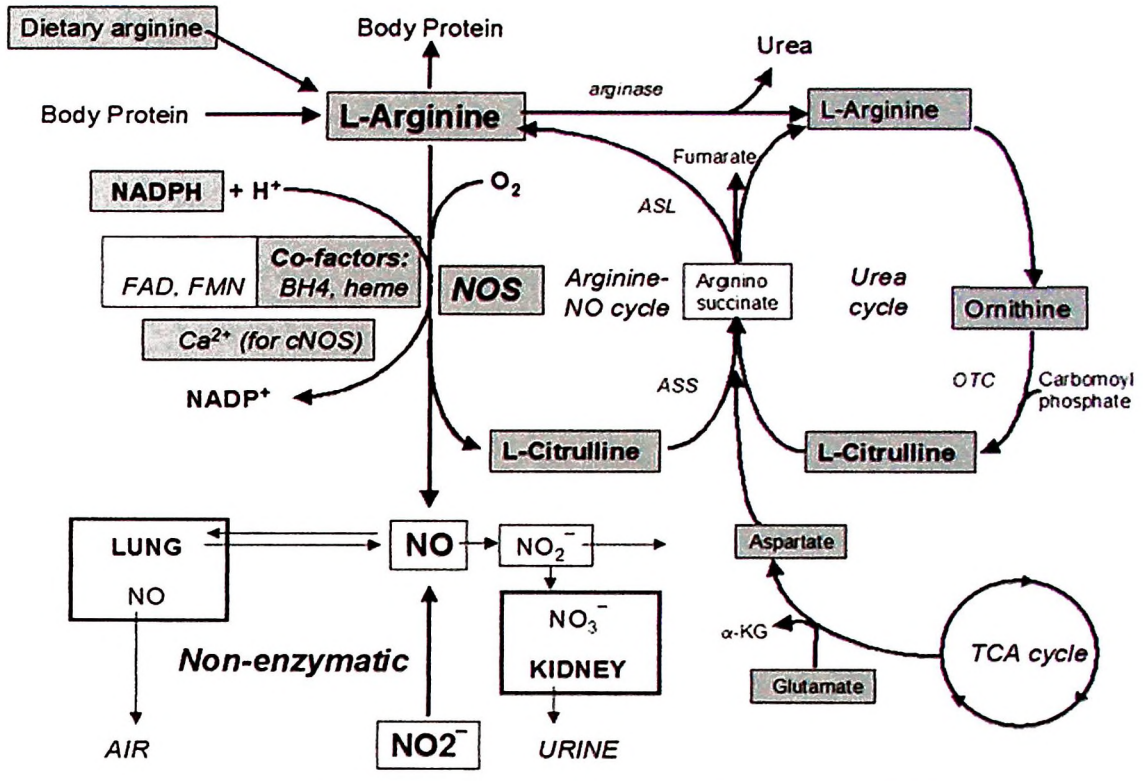


Figure 2.1: NO synthesis pathway; the schematic overview of the NO synthesis pathway that involve both systematic (NOS: the major pathway) and non-enzymatic pathways. L-arginine is converted to NO and citrulline with the presence of NADPH and Oxygen. Essential cofactors (BH4, FAD, FMN and heme) is important for the activity of NOS Ca^{2+} dependant pathways (endothelial (eNOS) and neuronal (nNOS)) (Luiking *et al.*, 2010).

Chapter 3: Materials and Methods

3.1- Plant Materials

3.1.1- Plant Collection

The plant materials were collected from the District of Bachok Kelantan, Malaysia in October 2012. The plant was authenticated by Dr. Richard Chung from Forest Research Institute Malaysia (FRIM). The herbal specimen (dried leaves) was deposited into FRIM herbarium (Sample number: PID-171011-10).

3.1.2- Preparation of standardized extracts

One-fifth kilograms (0.2 Kg) of *S. polyanthum* leaves were weighed using digital weighing balance (A&D® HV60KGL, Columbia). Next the plant material was washed and cleaned under tap water before rinsed with distilled water. The excess water was dried in an incubator (Memmert Gmbh + Co.KG,German) at a pre-set temperature of 50°C for three (3) consecutive days. The dried leaves were ground into powder in a laboratory blender (WARING Commercial® USA) and the filtrate was sieved-off by mechanical siever. The powdered sample is obtained from the #10 stack of sieve (Figure 3.1).

3.1.3- Plant extraction

The powdered sample is immersed in distilled water (with ratio of solute to solvent 1:10) for the preparation of aqueous crude extracts. Then, it was heated on a hot plate (Erla® EMS-HP-700, Illinois) at 90 °C with continuing stirring by magnetic stirrer for 30 minutes. The extract was then filtered through tea filter before filtered through Whatman No.41 filter paper (Whatman® Schleicher and Schuell, Malaysia). The liquid filtrate obtained was packed in universal bottles and lyophilized in a freeze dryer (ilShin®, Korea). The lyophilized sample produced was designated as the aqueous extract of *S. polyanthum* leaves (AESP).

The residue was dried at 50 °C in incubator to get the powdered leaves sample. Then it is further subjected to methanolic extraction (using 95% methanol (v/v), Merck, Germany) via continuous hot extraction technique. The residue powdered leaves was packed into a Soxhlet thimble and successively extracted with methanol using Soxhlet apparatus (Favorit®, Thailand) (Figure 3.2). The extract was then concentrated and dried in the incubator (Memmert GmbH + Co.KG, Germany) at a pre-set temperature of 50°C. The extract obtained at this stage was designated as the methanolic extract of *S. polyanthum* leaves (Meth-AESP). AESP and Meth-AESP were finally kept in an air-tight bottle and stored in a refrigerator (National NR-B53FE, Malaysia) at 4 °C until use.

Figure 3.1: Mechanical siever that was used to filter the powdered sample

Figure 3.2: Soxhlet apparatus was used for methanol extraction

3.2- Reagents

3.2.1- Drugs and chemicals

For extraction process, methanol was purchased from Merck, Malaysia. For *in vitro* experiments, dimethylsulfoxide (DMSO) and 95 % methanol (v/v) were purchased from Merck, Malaysia. N-ω-nitro-l arginine methyl ester (L-NAME) was purchased from Sigma®, USA. Heparin (Heparinol®-5000, Malaysia) was bought from Ain Medicare Sdn. Bhd, Malaysia and sodium pentobarbital (DORMINAL 20%) was bought from Alfasan Woerden-Holland. Normal saline and distilled water were prepared by the Unit Pengurusan Makmal Sains (UPMS) or Science Lab Management PPSK USM.

3.2.2- Preparation of drug

The aqueous extract (AESP) and methanolic extract (Meth-AESP) of *S. polyanthum* leaves were freshly prepared immediately before use by dissolving the extracts into 0.9 % NaCl or normal saline. AESP (100 mg/kg) was diluted with 1000 µL of normal saline while Meth-AESP (100 mg/kg) was diluted with 950 µL of normal saline and 50 µL of DMSO. Negative control for both of the reagents were prepared that involves the negative control for Meth-AESP (950 uL NaCl + 50 µL DMSO) and negative control for AESP (1000 µL NaCl). Six (6 %) dilution of sodium pentobarbital (200 mg/ml) was prepared. Fifty milligram per kilogram (50 mg/kg) of sodium pentobarbital was used to anaesthetize each rat intraperitoneally. L-NAME (20 mg/kg)

was dissolved in normal saline. The AESP, Meth-AESP and L-NAME that has been prepared were fixed to 0.2 ml per injection.

3.3- Animals

3.3.1- Animals preparation

The procedure and protocol described below were approved by Animal Ethics Committee USM (USM/Animal Ethics Approval/ 2010/ (59) (244)). Ten (10) male normotensive Wistar-Kyoto rats weighing 300-400g were used in this investigation. The animals were bred and obtained from the Animal Research and Service Centre (ARASC), USM Kelantan. The animals were kept at 20-24°C under 12 h light-dark cycles and were allowed free access to tap water and commercial pellet. Animal handling and all procedure on animals were carried out accordance with the guidelines of the Animal Ethics Committee USM.

3.3.2- Animals surgical procedure and experimental protocol

The rats were separated into two (2) groups (AESP and Meth-AESP) consist of 5 rats for each group. The rats were then anesthetized intra-peritoneal with pentobarbital sodium (50 mg/kg, i.p.) for the surgical preparation. This surgery routinely included the intubation of trachea with polyethylene tube to facilitate the ventilation. Next, the cannulation of right common carotid artery is done to measure the mean arterial blood pressure (MBP) and the cannulation of left internal jugular vein is to administer the

extracts and control in solution (Figure 3.3 and Figure 3.5). The arterial catheter was connected to a pressure transducer (BIOPAC Inc., USA) coupled to BIOPAC Student Lab Pro® Software (Figure 3.4). The left internal jugular vein was cannulated with a tubing to enable administration of AESP/Meth-AESP (100 mg/kg), L-NAME (20 mg/kg) and vehicle. Plus, this arterial and vein catheter was connected with heparinized saline to clear the tubing after the chemical injection.

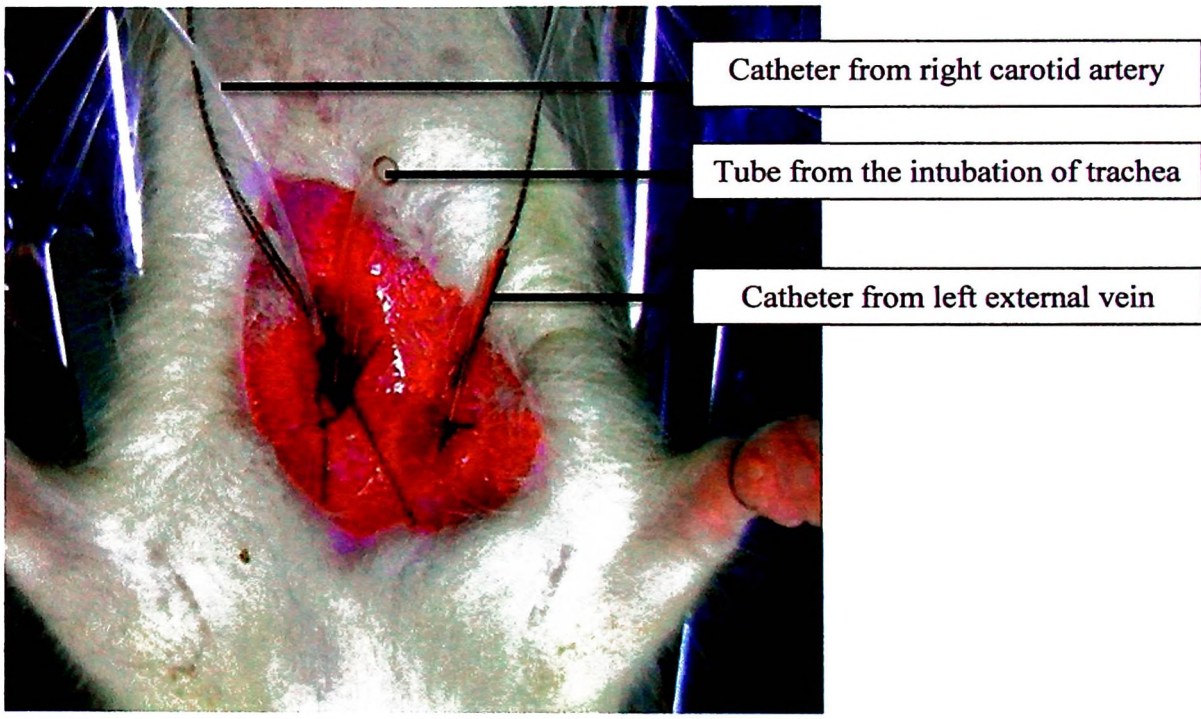


Figure 3.3: Mini surgical operation showed cannulation of right carotid artery, left external vein and intubation of the trachea (refer to label)

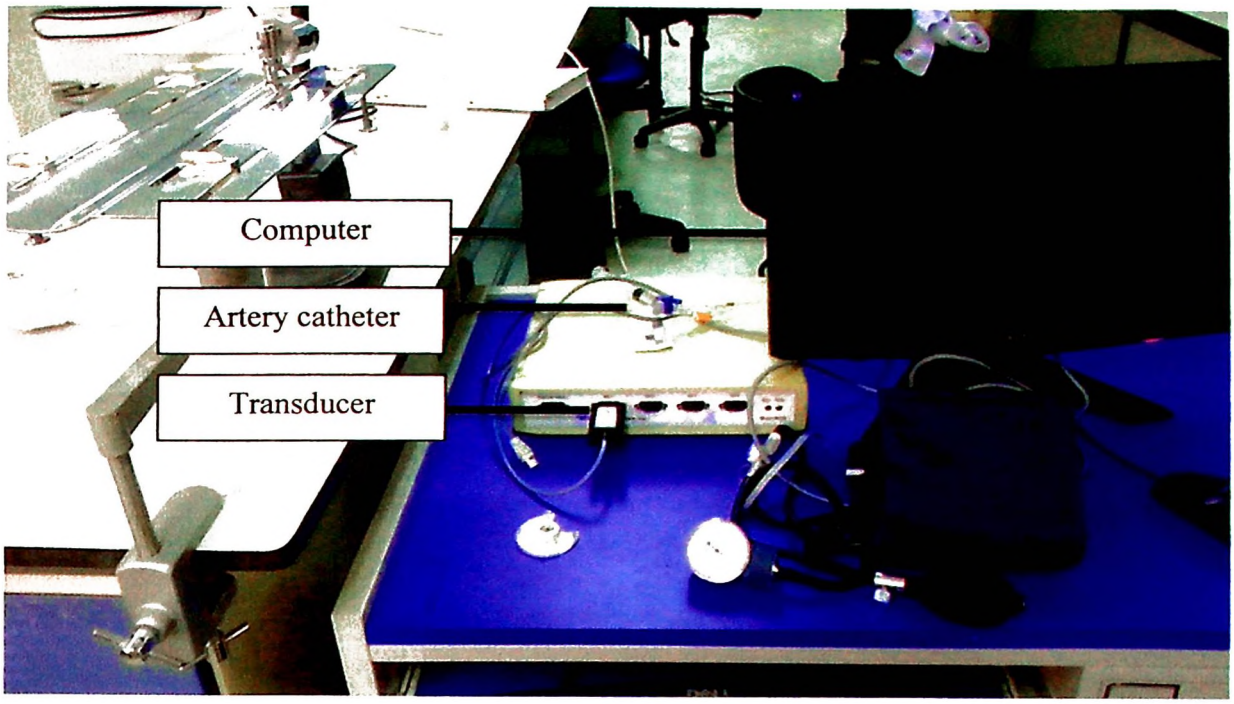


Figure 3.4: The setting for the experiment showed transducer connected with the computer and the artery catheter.

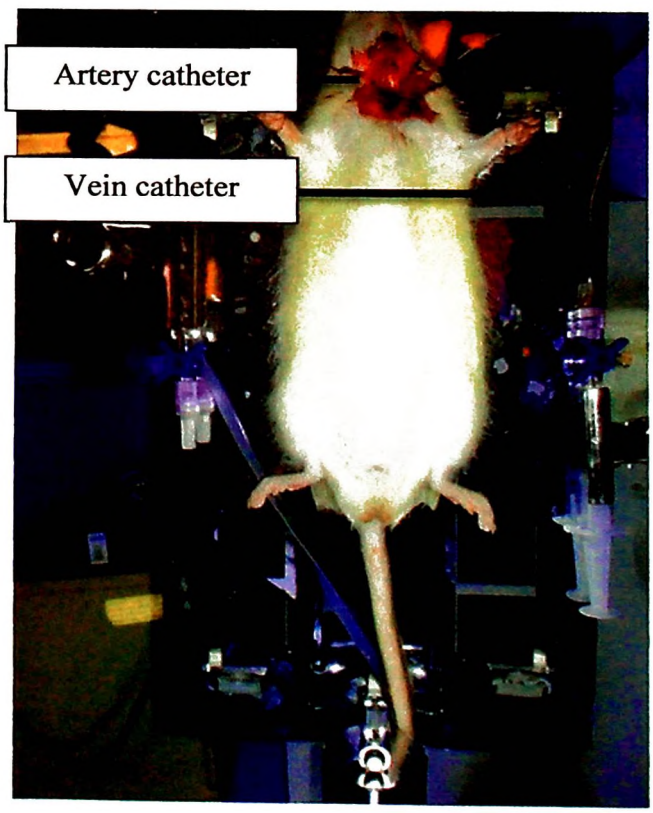


Figure 3.5: The anesthetized rat connected with the vein and artery catheter.

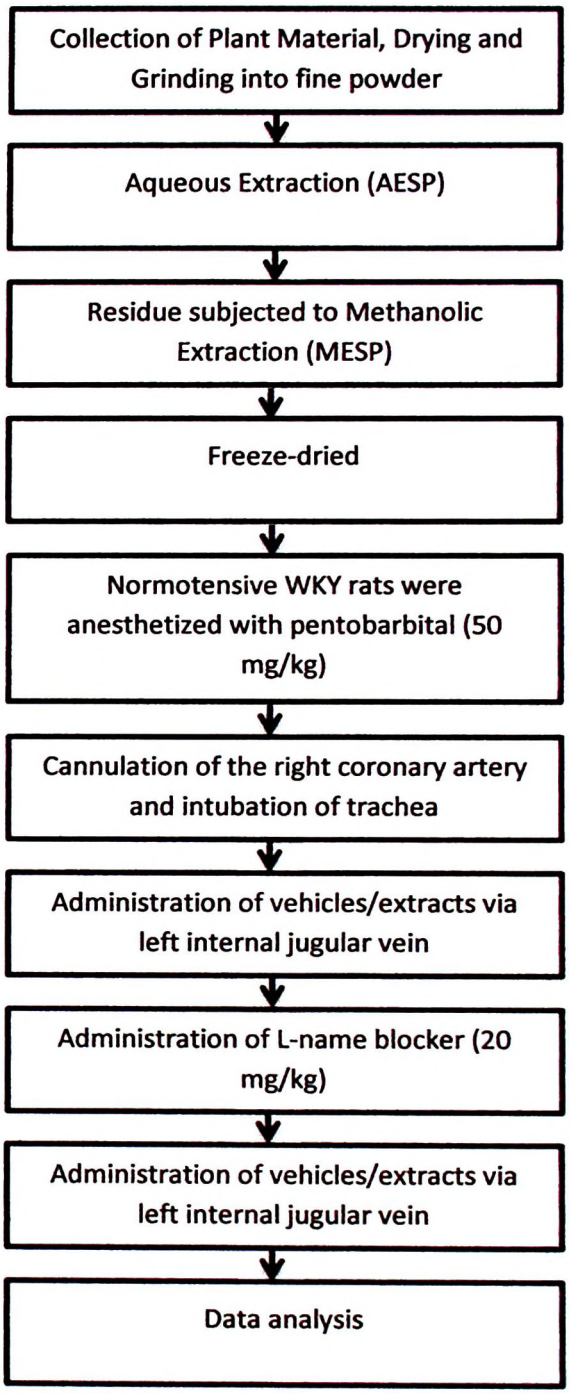
3.4- Blood Pressure recording

Before each experiment, blood pressure and heart rate (HR) were allowed to stabilize and were recorded. The negative control reagents (DMSO+NaCl / NaCl) were administered prior to (Meth-AESP / AESP) extracts. The control value was calculated by comparing the basal value of Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP), Mean Blood Pressure (MBP) and Heart Rate (HR) to the value after the negative control reagents administration. Next, the value of SBP, DBP, MBP and HR were allowed to achieve the normal baseline. This is followed by the administration of extracts (Meth-AESP/AESP) and the value of changes is calculated. The basal values were achieved once again to prepare for the pre-treatment with L-NAME (20 mg/kg). The changes of the value for SBP, DBP, MBP and HR were evaluated for 30 minutes. Each injection was given at a fixed volume of 0.2 ml to reduce the possible volume effects.

3.5- Statistical analysis

All values are expressed as mean ± S.E.M in each experiment. In both of the experimental groups, baseline changes in the hemodynamic parameters before chemical treatment were assessed using one way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test using a GraphPad-Prism v5.0 software (GraphPad, San Diego, CA). All tests were two-tailed and the significance level was set at $p < 0.05$.

3.6- Flow chart of research experimental design



Chapter 4: Results

4.1 The effects of AESP and Methanol-AESP (100 mg/kg) extract on Mean Blood Pressure (MBP), Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP) and Heart Rate (HR)

The hypotensive effect of *S. polyanthum* extracts was evaluated by the reduction of blood pressure from the baseline. The anaesthetised rats were given intravenous injection of AESP or Meth-AESP and the blood pressure changes are recorded and compared with the control. The percentage of reduction is calculated and the results are shown in Table 4.1 and Table 4.2:

Table 4.1: Group of rat that given AESP extraction (Figure 4.1 and Figure 4.2)

Mean Blood Pressure

(% of reduction)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5
Normal saline	0.00	0.00	0.82	0.00	0.00
AESP (100mg/kg)	41.08	33.07	32.99	50.10	29.39

Systolic Blood Pressure

(% of reduction)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5
Normal saline	0.00	0.00	0.50	0.00	0.00
AESP (100mg/kg)	39.46	32.11	36.94	45.42	32.81

Diastolic Blood Pressure

(% of reduction)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5
Normal saline	0.00	1.23	1.13	0.00	0.00
AESP (100mg/kg)	44.00	36.62	30.68	58.03	30.35

Heart Rate

(% of reduction)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5
Normal saline	1.46	0.68	1.29	0.00	1.79
AESP (100mg/kg)	26.76	11.60	23.38	28.42	8.76

Table 4.2: Group of rats that given Meth-AESP extraction (Figure 4.3 and Figure 4.4)

Mean Blood Pressure

(% of reduction)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5
Normal saline+ DMSO	4.32	0.41	0.09	0.00	0.99
Meth-AESP (100mg/kg)	25.53	54.12	56.08	63.33	48.51

Systolic Blood Pressure

(% of reduction)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5
Normal saline+ DMSO	2.39	0.04	0.00	0.00	1.33
Meth-AESP (100mg/kg)	11.90	30.69	43.78	53.72	46.39

Diastolic Blood Pressure

(% of reduction)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5
Normal saline + DMSO	5.90	0.45	0.40	0.00	1.01
Meth-AESP (100mg/kg)	32.91	66.26	64.11	70.81	52.62

Heart Rate

(% of reduction)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5
Normal saline + DMSO	1.85	0.63	0.60	1.29	1.54
Meth-AESP (100mg/kg)	4.94	7.18	3.43	0.00	0.37