PHARMACOLOGICAL EVALUATION ON THE EFFECT OF *SYZYGIUM POLYANTHUM* (WIGHT) WALP. LEAVES EXTRACT ON RAT'S BLOOD PRESSURE AND RELATED PARAMETERS

by

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induced vasorelaxations on isolated thoracic aorta rings of WKY (n=5) and their respective IC_{50} values

LIST OF SYMBOLS AND ABBREVIATIONS

±	plus minus
%	percentage
ºC	degree Celsius
Δ	capitalized delta sign, denoted changes
Δ Contraction	changes in the force of contraction
α	alpha
β	beta
δ	delta
α_{1A}	alpha-adrenergic receptor subtype 1A
α_{1B}	alpha-adrenergic receptor subtype 1B
α_{1C}	alpha-adrenergic receptor subtype 1C
α_{2A}	alpha-adrenergic receptor subtype 2A
$\alpha_{2A/D}$	alpha-adrenergic receptor subtype 2A/D
α_{2B}	alpha-adrenergic receptor subtype 2B
α_{2C}	alpha-adrenergic receptor subtype 2C
α_{2D}	alpha-adrenergic receptor subtype 2D
β_1	beta 1-adrenergic receptor
β_2	beta 2-adrenergic receptor
β_3	beta 3-adrenergic receptor
µg/ml	microgram per millilitre
µg/kg	microgram per kilogram
µl	microlitre
µM	microMolar
ACE	angiotensin-converting enzyme
AESP	aqueous extract of *S. polyanthum* leaves
ANOVA	Analysis of Variance
ANS	autonomic nervous system
AR	adrenergic receptor
AT_1	angiotensin II type 1
ATPase	adenosine 5'-triphosphatase

LIST OF SYMBOLS AND ABBREVIATIONS

Atr	atropine sulphate
AU	Absorption units
CAE	caffeic acid equivalent
$CaCl.2H_2O$	calcium chloride dihydrate
CARDIA	Coronary Artery Risk Development in Young Adults
CAS #	Identification number by Chemical Abstracts Services
CE	catechin equivalent
cGMP	cyclic guanylyl monophosphate
CMC	carboxymethylcellulose
CO_2	carbon dioxide
coA	coenzyme A
Crl: CD(BR)	a strain of rats which is used for multipurpose research
DBP	diastolic blood pressure
DDMP	4H-Pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl-
ddY	Deutschland, Denken, and Yoken (a strain of mice)
DMSO	dimethylsulfoxide
DOCA	deoxycorticosterone acetate
DPPH	diphenyl-1-picrylhydrazyl
E	chemical configuration when two groups of higher priority are on the opposite sides of double bond
EC_{50}	effective concentration of chemicals/drugs that causes 50 % of maximal response
EC_F	concentration of agonist that gives a response F percent of between bottom and top
ED_{50}	effective dose that causes 50 % of maximal response
eNOS	endothelial nitric oxide synthase
et al.	and others
eV	electron Volts
FE	flavonol equivalent
Force $_{after}$	force after addition of extracts

LIST OF SYMBOLS AND ABBREVIATIONS

Force $_{baseline}$	force before any addition of phenylephrine or serotonin concentration
Force $_{max}$	maximum force upon which phenylephrine or serotonin concentration is added
Force $_{max\ Phe/Serotonin}$	maximum force when phenylephrine or serotonin reaches plateau
FRIM	Forest Research Institute Malaysia
g	gram, a unit of tension
GAE	gallic acid equivalent
GC	gas chromatograph
g/kg	gram per kilogram
Hexa	hexamethonium bromide
HIV	human immunodeficiency virus
HP-5MS	5% diphenyl, 95% dimethylpolysiloxane
HPLC	high-performance liquid chromatography
HR	heart rate
IC_{50}	concentration that causes 50 % inhibition of maximal responses
i.d	internal diameter
INTERSALT	International Study of Salt and Blood Pressure
i.p	intraperitoneal
Isop	isoproterenol hydrochloride
IU/ ml	International standard unit per millilitre
K^+	potassium ion
KCl	potassium chloride
kg	kilogram
KH_2PO_4	potassium dihydrogen phosphate
KHS	Kreb's Henseleit solution
LC_{50}	lethal concentration that causes 50 % morbidity
L-NAME	Nω-Nitro-L-arginine methyl ester

LIST OF SYMBOLS AND ABBREVIATIONS

m	metre
M	Molar
M_1	muscarinic receptor subtype 1
M_2	muscarinic receptor subtype 2
M_3	muscarinic receptor subtype 3
M_4	muscarinic receptor subtype 4
M_5	muscarinic receptor subtype 5
MAP	mean arterial blood pressure
met-AESP	residual methanolic extract of *S. polyanthum* leaves
Methox	methoxamine hydrochloride
mg	milligram
mg/kg	milligram per kilogram
mg/ml	milligram per millilitre
$MgSO_4.7H_2O$	magnesium sulphate heptahydrate
min	minute
ml	millilitre
ml/kg	millilitre per kilogram
ml/min	milliliter per minute
mm/ms	millimeter per milliseconds, a unit of pulse wave velocity
mmHg/sec	millimetre Mercury per second
mm	millimetre
mmHg	millimetre Mercury
mmol/L	milimol per litre
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MUPA	Makmal Unit Perkhidmatan Analisis
MW	molecular weight (in grams per mole)
m/z	mass-to-charge ratio
N_N	neuronal nicotinic
n	number of sample size
n.a	not available

LIST OF SYMBOLS AND ABBREVIATIONS

n.s	not significant
Na^+	sodium ion
NaCl	sodium chloride
$NaHCO_3$	sodium hydrogen carbonate
NAD(P)H	nicotinamide adenine dinucleotide phosphate
NHANES	National Health and Nutrition Examination Survey
NHLBI	National Heart, Lung, and Blood Institute
NHMS	National Health and Morbidity Survey
NIST	National Institute of Science and Technology of United States of America
nm	nanometre
nM	nanoMolar
O_2	oxygen
PGH_2	prostaglandin H_2
PGI_2	prostaglandin I_2
Phe	phenylephrine
Phento	phentolamine hydrochloride
Pk	Peak
ppm	parts per million
Prop	propranolol hydrochloride
QE	quercetin equivalent
RAAS	renin-angiotensin-aldosterone system
RE	rutin equivalent
ROS	reactive oxygen species
RT	retention time
SBP	systolic blood pressure
SD	standard deviation
S.E.M	standard error of mean
SHR	Spontaneously Hypertensive Rat
t=0	initial time
t=1	1 hour

LIST OF SYMBOLS AND ABBREVIATIONS

t=3	3 hours
t=5	5 hours
t=6	6 hours
t=24	24 hours
USD	US. Dollar
UV	ultraviolet
VIS	visible
vs.	versus
w=0	initial time
w=1	1 week
w=2	2 weeks
w=3	3 weeks
WHO	World Health Organization
WKY	Wistar-Kyoto rat
w/v	weight per volume
v/v	volume per volume
Z	chemical configuration when the two groups of higher priority are on the same side of the double bond

PENILAIAN FARMAKOLOGI KE ATAS KESAN EKSTRAK DAUN *SYZYGIUM POLYANTHUM* (WIGHT) WALP. TERHADAP TEKANAN DARAH TIKUS DAN PARAMETER BERKAITAN

ABSTRAK

Daun *Syzygium polyanthum* (Wight) Walp. var. *Polyanthum* telah digunakan secara tradisional oleh masyarakat Melayu sebagai rawatan alternatif untuk penyakit darah tinggi. Bukti saintifik yang menyokong penggunaan tradisional ini masih terhad. Kajian ini menyiasat kesan ekstrak air (AESP) dan ekstrak "residual" metanol (met-AESP) bagi daun *S. polyanthum* ke atas tekanan darah tikus dan parameter berkaitan. Kedua ekstrak telah diberikan secara oral kepada tikus "Wistar-Kyoto" yang mempunyai tekanan darah biasa dan kepada tikus "Spontaneously Hypertensive" (SHR), kemudian tekanan darah sistolik diawasi dalam tempoh 24 jam. Seterusnya, kedua ekstrak telah diberikan setiap hari secara oral untuk 3 minggu kepada SHR, kemudian tekanan darah sistolik diawasi selama 3 minggu. Dalam kajian seterusnya, kedua ekstrak telah diberikan secara suntikan ke dalam salur darah vena WKY dan SHR yang disuntik pelali, kemudian, tekanan darah purata arteri, sistolik, dan diastolik dan juga kadar jantung direkodkan selama 20 minit. Dalam kajian berikutnya, kedua ekstrak telah ditambah pada torasik aorta yang diambil daripada WKY dan SHR yang telah dikontraksi oleh fenilefrin (1 μM) dan serotonin (10 μM). Kemungkinan penglibatan reseptor autonomik dan nitrik oksida dalam mediasi penurunan tekanan darah dan pengenduran salur darah oleh kedua ekstrak telah disiasat menggunakan penyekat yang berkaitan reseptor nikotinik- dan muskarinik-asetilkolin, α- dan β-adrenergik, dan nitrik oksida. Penyaringan bahan kimia tumbuhan, kromatografi gas spektrometri jisim, dan

kromatografi cecair berkualiti tinggi dijalankan untuk memperoleh profil bahan kimia ekstrak. Ekstrak yang diberikan secara oral, sama ada sebagai dos akut atau dos harian yang berulang telah menurunkan tekanan darah sistolik tikus SHR dengan signifikan. Kedua ekstrak telah menunjukkan kesan penurunan tekanan darah yang signifikan selepas tiga minggu rawatan, tetapi permulaan kesan penurunan darah oleh met-AESP adalah lebih cepat berbanding AESP. Kedua ekstrak yang diberikan secara suntikan melalui saluran darah vena telah menyebabkan kesan penurunan tekanan darah, tetapi hanya AESP pada dos yang paling tinggi telah menyebabkan sedikit penurunan kadar jantung pada WKY dan SHR yang disuntik pelali. Kesan penurunan tekanan darah oleh met-AESP lebih bertahan lama berbanding AESP dalam tikus SHR yang disuntik pelali. Kedua ekstrak juga menyebabkan pengenduran salur darah pada aorta daripada WKY dan SHR dengan kesan yang setara. Penurunan tekanan darah dan kadar jantung, serta kesan pengenduran salur darah oleh AESP dicadangkan sebahagiannya melibatkan reseptor α-adrenergik, sementara penurunan tekanan darah dan pengenduran salur darah oleh met-AESP sebahagiannya melibatkan reseptor muskarinik-asetilkolin dan β-adrenergik. Sebahagian dari kesan penurunan tekanan darah dan pengenduran salur darah oleh kedua ekstrak berkemungkinan melibatkan nitrik oksida. AESP mengandungi fenolik, tanin, flavonoid, saponin, dan kardiak glikosida; sementara met-AESP mengandungi fenolik, tanin, flavonoid, terpenoid dan resin. Gliserin, asid asetik, dan asid galik adalah bahan berpotensi di dalam AESP, sementara seselin, asid linolik, metil heksadekanoat, asid olik, dan asid galik adalah bahan berpotensi di dalam met-AESP yang berkemungkinan menyumbang kepada penurunan tekanan darah dan pengenduran salur darah oleh ekstrak ini. Kesimpulannya, kajian ini

menyokong penggunaan tradisional daun *S. polyanthum* sebagai rawatan alternatif untuk penyakit darah tinggi.

PHARMACOLOGICAL EVALUATION ON THE EFFECT OF *SYZYGIUM POLYANTHUM* (WIGHT) WALP. LEAVES EXTRACT ON RAT'S BLOOD PRESSURE AND RELATED PARAMETERS

ABSTRACT

Syzygium polyanthum (Wight) Walp. var. *Polyanthum* leaves are traditionally consumed by the Malays as an alternative treatment for hypertension. However, the scientific evidence to support this claim is scarcely reported. The present study investigated the effects of aqueous (AESP) and residual methanolic extracts (met-AESP) of *S. polyanthum* leaves on rat's blood pressure and related parameters. Both extracts were orally administered on Wistar-Kyoto (WKY) and Spontaneously Hypertensive rats (SHR), then systolic blood pressure (SBP) were monitored within 24 hours. Later, both extracts were daily administered *via* oral administration for 3 weeks in SHR, then the SBP were monitored within 3 weeks. In the subsequent study, both extracts were intravenously administered in anaesthetized WKY and SHR, then, mean arterial, systolic, and diastolic blood pressures, as well as the heart rate were recorded within 20 minutes. In the following study, both extracts were added on the phenylephrine (1 µM) - and serotonin (10 µM)-contracted isolated thoracic aorta rings from WKY and SHR. Possible involvement of autonomic receptors and nitric oxide in mediating the blood pressure reduction and vasorelaxation by both extracts was investigated by using respective blockers for nicotinic-and muscarinic-acetylcholine, α- and β-adrenergic receptors, and nitric oxide. Phytochemical screening, gas chromatography mass spectrometry, and high

performance liquid chromatography were carried out to obtain phytochemical profiles of the extracts. The orally-administered extracts, either as an acute dose or repeated-dose significantly reduced blood pressure of conscious SHR. Both extracts showed significant hypotensive effects after 3-week treatment, but the onset of hypotensive effect by met-AESP was faster than AESP. Both of the intravenously-administered extracts caused significant reduction in blood pressure, but only AESP at the highest dose caused mild reduction in heart rate of anaesthetized WKY and SHR. The blood pressure reduction by met-AESP was more sustained than AESP in anaesthetized SHR. Both extracts also caused vasorelaxation on aorta rings from WKY and SHR with comparable effects. The reductions in blood pressure and heart rate, and also vasorelaxation by AESP, were suggested to partly involve α-adrenergic receptors, while the reduction in blood pressure and vasorelaxation by met-AESP partly involved muscarinic-acetylcholine and β-adrenergic receptors. A part of the reduction in blood pressure and the vasorelaxation effects by both extracts possibly involve nitric oxide. AESP contained phenolics, tannins, flavonoids, saponins, and cardiac glycosides; while met-AESP contained phenolics, tannins, flavonoids, terpenoids, and resins. Glycerine, acetic acid, and gallic acid were the potential compounds in AESP, while seselin, linoleic acid, methyl hexadecanoate, oleic acid, and gallic acid were the potential compounds in met-AESP that possibly contributed to the reduction in blood pressure and vasorelaxation by these extracts. In conclusion, this study supported the traditional use of *S. polyanthum* leaves extracts as an alternative treatment for hypertension.

CHAPTER 1

INTRODUCTION

1.1 Hypertension: A worldwide problem

Hypertension is a condition that describes a chronic elevation of blood pressure (Guyenet, 2006; Dugdale, 2011). It is a global dilemma with a worldwide prevalence of 40.0 % among adults aged 25 and above in 2008, as reported by the World Health Organization (WHO) (2014). Kearney and co-authors (2005) projected that, by the year 2025, the number of adults with hypertension will increase to a total of 1·56 billion. This projection has envisioned the future burden to be faced by the global population. Hypertension is a major risk factor for coronary heart disease and stroke. In fact, the WHO (2007) disclosed that 30.0 % from 58 millions of deaths worldwide in 2005 was due to various types of cardiovascular diseases with hypertension as a major underlying risk factor.

Despite the high prevalence of hypertension, proper blood pressure control by hypertensive patients is still not achieved. In 'National Health and Nutrition Examination Survey' which was conducted in the United States, 47.5 % of those with high blood pressure did not have it controlled within year 2007 to 2010 (Go *et al.*, 2013). Meanwhile, a recent report by the WHO (2014) stated that the number of people worldwide with uncontrolled hypertension rose from 600 million in 1980 to nearly 1 billion in 2008. With the alarming increase in the incidence of hypertension, the global control over the raised blood pressure is still unsatisfactory.

Likewise, this scenario is almost similar to the condition in Malaysia. Two national scale-studies conducted in Malaysia in year 1996 and in year 2006 have shown that the awareness, the treatment, and the control rates for hypertension were poor, in spite of the high incidence of hypertension (Lim *et al.*, 2004; Rampal *et al.*, 2008). Lim and colleagues (2004) reported that only 26.0 % of those with high blood pressure and on anti-hypertensive medication in 1996 had a controlled range of blood pressure, while in 2006, Rampal and co-authors (2008) stated that the number only increased to 26.8 %. These findings indicated that hypertension still remains at stake both globally and locally.

Even though the poor control of blood pressure maybe affected by some non-compliance issues among hypertensive patients (Hassan *et al.*, 2006), there also arises concerns regarding the efficacy of the available anti-hypertensive drugs, as well as, if there are needs to seek for more effective anti-hypertensive agents. In addition to the issue of efficacy, WHO (2013) has identified that the side effect of conventional anti-hypertensive drugs is one of the main factors that influence people into seeking for alternative traditional medicines. While some of the side effects are treatable by adding another medication, but some are incurable and may lead to a discontinuation of the prescribed medicines. The next major concern is the cost of anti-hypertensive drugs especially in countries where medicines are not subsidized. According to the American Heart Association, the estimated direct and indirect costs of hypertensive medication in the United States in year 2009 reached up to USD 51.0 billion (Go *et al.*, 2013).

1.2 Plant: A source of alternative anti-hypertensive medicines

Since hypertension has remained as an unresolved problem, as a result, tremendous amount of researches have been conducted in looking for alternative anti-hypertensive medicines, especially from natural sources, such as plants. Most of the studies explored into traditional ethno-medicinal plants, which were evidenced over time. In fact, there are numerous conventional medicines at present such as papaverine (vasodilator), theobromine (diuretic), theophylline (mild diuretic), protoveratrins A and B (anti-hypertensive), rescinamine (angiotensin converting enzyme inhibitor), and reserpine (adrenolytic, anti-hypertensive) that were originally discovered through observations of traditional remedial methods of indigenous people (Aftab, 1995; Gilani and Atta-ur-Rahman, 2005; Lahlou, 2013).

Since the search into traditional pharmacopoeia has led to successful discovery of modern anti-hypertensive medicines today, a lot of potential ethno-medicinal plants have undergone various scientific investigations in proving the therapeutic claim to alleviate hypertension. As a preliminary step, various herbal preparations or extracts have been tested for their abilities to reduce blood pressure of rats (Ajay *et al.*, 2007; Lima-Landman *et al.*, 2007; Ameer *et al.*, 2010a; Soncini *et al.*, 2011). Furthermore, some of the investigations on the crude herbal preparations have led to bio-assay guided fractionation, leading to identification of the fractions that attribute to the utmost significant reduction in blood pressure of rats (Lima-Landman *et al.*, 2007; Ameer *et al.*, 2010b). Meanwhile, some other researches have successfully isolated the bioactive compounds that are responsible for the reduction in blood pressure of rats, such as eugenol (Lahlou *et al.*, 2004b;

Interaminense *et al.*, 2007), methyleugenol (Lahlou *et al.*, 2004a), dodoneine (Ouedraogo *et al.*, 2011), eucalyptol or 1, 8-cineolle (Lahlou *et al.*, 2002b), α-pinene (El Tahir Kamal and Al-Ajmi, 2003), and artemetin (de Souza *et al.*, 2011).

1.3 *Syzygium polyanthum* (Wight) Walp. var. *Polyanthum*

Although there are numerous claims made with regards to blood pressure lowering abilities by some Malay ethnomedicinal plants, it is for sure that, these claims entail for further scientific clarification. One of the ethno-medicinal plants that have been traditionally claimed by the Malays as an alternative treatment for hypertension is *Syzygium polyanthum* (Wight) Walp. var. *Polyanthum* (Noraida, 2005; Yusuf, 2013). The shoots are commonly consumed as a fresh salad or 'ulam', while the decoction of *S. polyanthum* leaves is traditionally consumed as a hypertension remedy. This plant is popular among the Indonesians and the popularity is evidenced by the emergence of various *S. polyanthum*-based supplement products, such as drinks and herbal capsules in the market.

Despite so, there is a dearth of scientific information that is available and accessible in the literature on the anti-hypertensive potential of *S. polyanthum* leaves. At the beginning of the present study, the only scientific rationale purporting on the potential of *S. polyanthum* leaves was indicated in a compilation book, entitled "Penelitian Tanaman Obat di Beberapa Perguruan Tinggi di Indonesia". In this book, Hajar (1996), as cited in Sundari and co-authors (2000) reported that the infusion of *S. polyanthum* leaves caused a significant decrement in the blood pressure of Wistar rats, which were induced hypertensive by feeding with high-sodium diet.

In addition, previous researches have shown that there were a few potential compounds in the essential oil of *S. polyanthum* leaves, such as eugenol, eucalyptol, and α-pinene (Agusta, 2000; Arintawati, 2000; Kato *et al.*, 2013) that might reduce the blood pressure. Eugenol was demonstrated to cause immediate and dose-dependent reduction in blood pressure and heart rate, in either anaesthetized or conscious rats (Lahlou *et al.*, 2004b), and to exhibit vasorelaxation effects in rat's (Damiani *et al.*, 2003; Interaminense *et al.*, 2007) and rabbit's thoracic aorta (Nishijima *et al.*, 1999), as well as on rat's mesenteric vascular bed (Criddle *et al.*, 2003). Meanwhile, α-pinene was reported to cause reduction in blood pressure and increment of heart rate in non-anaesthetized rats (Menezes *et al.*, 2010), and in urethane-anaesthetized rats (El Tahir Kamal and Al-Ajmi, 2003). Furthermore, eucalyptol was shown to cause an endothelium-dependent vasorelaxation on rat's thoracic aorta (Lahlou *et al.*, 2002b; Pinto *et al.*, 2009); a blood pressure reduction in anaesthetized and in conscious normotensive rats (Lahlou *et al.*, 2002b); and a reduction in myocardial contractility (Soares *et al.*, 2005).

Although these compounds might correlate to the proclaimed traditional use of *S. polyanthum* leaves as an alternative anti-hypertensive medicines, these studied compounds are compositions of the essential oil, which are sparingly soluble in water, and may not be present in the traditionally consumed *S. polyanthum* leaves decoction. Altogether, the traditional therapeutic claim on the use of *S. polyanthum* leaves decoction as an alternative anti-hypertensive medication still requires scientific validation by embracing its pharmacological effect on the cardiovascular system, especially on the blood pressure, and also by analyzing its phytochemical constituents.

1.4 Scope of study

This study was divided into two important scopes, the phytochemical analysis and the pharmacological evaluation. The phytochemical analysis was instigated by a preliminary phytochemical screening for a rapid and convenient determination of the phytochemical groups present in *S. polyanthum* leaves extracts. A more comprehensive quantitative identification was then preceded by means of gas chromatography mass spectrometry (GCMS), and high performance liquid chromatography (HPLC). GCMS is commonly used for determination of volatile and semi-volatile constituents in plants (Al-Hashmi *et al.*, 2013; Geetha *et al.*, 2013; Sarikurkcu *et al.*, 2013), while HPLC is used for separation of highly polar and large molecular weight materials that have very low volatility, and thus cannot be separated by gas chromatography (Scott, 2003). Thus, numerous researches have widely applied HPLC in the process of identification of chemical constituents in plants (Ameer *et al.*, 2010b; Leeya *et al.*, 2010; Alam *et al.*, 2011; Praman *et al.*, 2011; Soncini *et al.*, 2011; Shie and Lay, 2013). Gallic acid was introduced as a reference compound in this study as it was initially identified as the highest peak in the HPLC chromatograms of *S. polyanthum* leaves extracts, and therefore was quantified. In addition, Har and Ismail (2012) have previously detected the presence of gallic acid in the methanolic extract of *S. polyanthum* leaves.

The pharmacological evaluation on the effects of *S. polyanthum* leaves extracts was carried out using *in vivo* and *in vitro* methods. One of the *in vivo* approaches includes determination on the effects of orally-administered *S. polyanthum* leaves extracts on the blood pressure of conscious rats by using non-

invasive tail-cuff method. This study mimicked the traditional oral route of administration in human, and the method was devoid of anaesthetic effect. Only the systolic blood pressure (SBP) was determined in this study while the mean arterial blood pressure (MAP) and diastolic blood pressure (DBP) readings were omitted since the point of readings are often indistinguishable due to the distraction that results from the motion and breathing artifacts. This predicament may explain the scarcity of data on mean arterial and diastolic blood pressures in other studies with a comparable design (Eddouks *et al.*, 2005; Maghrani *et al.*, 2005; Ichimura *et al.*, 2006; Ryu *et al.*, 2008; Jaffri *et al.*, 2011; Ng *et al.*, 2011; Azizan *et al.*, 2012; Thaweekhotr *et al.*, 2012; Kaur *et al.*, 2013; Tom *et al.*, 2013).

In addition, investigation on the hypotensive effect of plant extracts was usually employed on hypertensive rats models, such as Spontaneously Hypertensive rats (SHR) (Eddouks *et al.*, 2005; Ichimura *et al.*, 2006; Lima-Landman *et al.*, 2007; Ryu *et al.*, 2008; Ng *et al.*, 2011; Azizan *et al.*, 2012; Tom *et al.*, 2013) or N_{ω}-Nitro-L-arginine methyl ester (L-NAME)-induced hypertensive rats (Lima-Landman *et al.*, 2007; Jaffri *et al.*, 2011; Thaweekhotr *et al.*, 2012). The normotensive Sprague-Dawley rats (Kaur *et al.*, 2013) or Wistar rats (Eddouks *et al.*, 2005; Maghrani *et al.*, 2005; Ichimura *et al.*, 2006; Lima-Landman *et al.*, 2007; Jaffri *et al.*, 2011; Ng *et al.*, 2011; Tom *et al.*, 2013) were regularly used as the control group. By taking into account that hypertensive models should mimic the condition of hypertensive human beings, this study utilized the SHR model, which is the inbred of WKY with high blood pressure. The hypertensive state in SHR was constitutively imprinted in their genetic traits, and so, the high blood pressure has been maintained over generation (Okamoto and Aoki, 1963). In the present study, SHR and WKY as the

normotensive control, were treated with a single dose of extracts and the changes in SBP were monitored within 24 hours. These was followed by a 3-week study, where the SHR were treated with repeated-dose of extracts daily, and the changes in SBP were monitored over the 3-week period.

On the other hand, another *in vivo* approach in the present study were to determine the direct effects of intravenously-administered *S. polyanthum* leaves extracts on blood pressure and heart rate of anaesthetized rats, *via* invasive arterial and venous cannulation. The blood pressure was measured *via* invasive method using blood pressure transducer that measured direct arterial pressure in the unit of voltage (Parasuraman and Raveendran, 2012). The blood pressure transducer allowed recordings of the direct blood pressure and calculated other parameters, such as MAP, SBP, DBP, and heart rate (HR). These parameters were utilized in various *in vivo* studies with comparable study design (Adeneye *et al.*, 2006; Shih *et al.*, 2008; Senejoux *et al.*, 2011).

Since the measurement method is invasive, rats were anaesthetized prior to experiment. Anaesthesia provides a calm resting condition to rats, and at the same time, evades unnecessary stress. Previous invasive blood pressure measurement methods utilized barbiturates such as sodium pentobarbital (Testai *et al.*, 2002; Lima-Landman *et al.*, 2007; Shih *et al.*, 2008; Kamkaew *et al.*, 2011), thiopental (Adaramoye *et al.*, 2009); thiobutabarbitural or inactin (Kamadyaapa *et al.*, 2009); or a combination of inactin and sodium pentobarbital (Zhang and Tan, 1997). Other researchers opted for the use of urethane (Eno and Owo, 1999; El Tahir Kamal and

Al-Ajmi, 2003; Adeneye *et al.*, 2006) or ketamine in combination with xylazine (Phillips *et al.*, 2006; de Souza *et al.*, 2011).

According to the "Guidelines for the Use of Anesthetics, Analgesics and Tranquilizers in Laboratory Mammals", provided by the University of Minnesota Board of Regents (2009), barbiturates are amongst the commonly used anaesthetics in laboratory animals for the relative ease of use. In fact, intraperitoneal injection of sodium pentobarbital at 50 mg/kg was reported to produce an anaesthetic effect on Crl: CD(BR) rats after 3.5 ± 1.4 min and the effect lasted for 95.0 ± 4.8 min (Field *et al.*, 1993). Even though Field and colleagues (1993) have claimed that sodium pentobarbital caused a moderate to severe respiratory and cardiovascular depression, however, it was demonstrated in another study that the use of 50 mg/kg sodium pentobarbital did not significantly affect the baselines blood pressure of the anaesthetized SHR as compared to the conscious SHR (Chiueh and Kopin, 1978). In addition, the use of 50 mg/kg sodium pentobarbital did not significantly affect the DBP of normotensive Sprague-Dawley rats but significantly increased the HR in comparison with conscious rats (Fluckiger *et al.*, 1985).

Most importantly, it was demonstrated in some previous studies that the use of sodium pentobarbital at 50 mg/kg did not cause any significant difference in the magnitude of blood pressure and HR reductions by plant extracts in both anaesthetized and in conscious Wistar rats (Lahlou *et al.*, 2002b; Lahlou *et al.*, 2004a). A more recent study has shown that sodium pentobarbital has provided the most stable hemodynamic values as compared to thiopental and urethane, and the

values are similar to the conscious rats (Zorniak *et al.*, 2010). For the stated reasons, sodium pentobarbital was used in this study at a dose of 50 mg/kg body weight.

Among the usually employed rat models using the intravenous route of administration includes anaesthetized normotensive rats such as Sprague-Dawley rats (Zhang and Tan, 1997; Shih *et al.*, 2008; Ameer *et al.*, 2010b) and Wistar rats (Lahlou *et al.*, 2004a; Kamkaew *et al.*, 2011; Senejoux *et al.*, 2011; Soncini *et al.*, 2011), as well as on the anaesthetized hypertensive rats including of one-kidney, one-clip hypertensive rats (Nworgu *et al.*, 2008); deoxycorticosterone acetate (DOCA)-salt-hypertensive rats (Lahlou *et al.*, 2002a); L-NAME-induced hypertensive rats (Soncini *et al.*, 2011); and SHR rats (Senejoux *et al.*, 2011).

The *in vitro* functional vascular study approach were used in the present study to determine the direct effects of *S. polyanthum* leaves extracts on the isolated rat's thoracic aorta rings. The usage of isolated thoracic aorta rings in this set-up enabled the elucidation on the effect of *S. polyanthum* leaves extracts without the influence of intricate homeostatic control that usually exists in the body. Myograph was used to measure the force generated by a contracting muscle, such as trachea, gut, small, and large blood vessels. The generated force was converted into an electrical output that can be displayed and analyzed on a computer monitor in the unit of gram of contraction. Based on the changes of the force of contraction, the ability of drugs or plant extracts to relax or constrict the isolated blood vessel was evaluated.

Some of the previous reports on the vasorelaxation ability of plants utilized various types of blood vessels which includes mesenteric, basilar, renal, tail, and

femoral arteries (Adaramoye *et al.*, 2009; Kamkaew *et al.*, 2011). However, the commonly used prototype for blood vessel in vasorelaxation studies of plant extracts is the thoracic aorta (Leeya *et al.*, 2010; Kamkaew *et al.*, 2011; Ouedraogo *et al.*, 2011). This study also utilized the rat's thoracic aorta, specifically, the descending part that is located posteriorly from ascending thoracic aorta, and is adjacent to the vertebral column before it extends below the diaphragm and becomes the abdominal aorta (Figure 1.1).

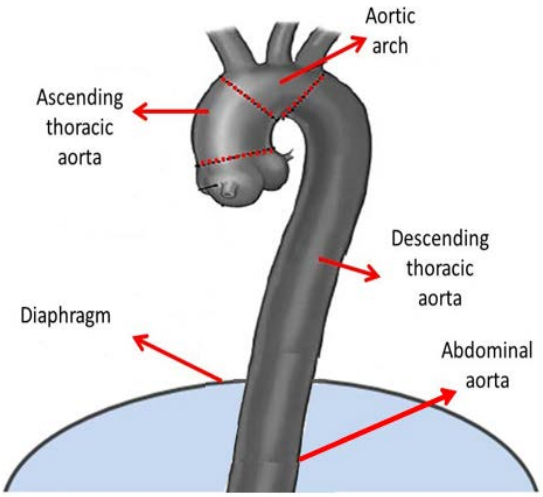

Figure 1.1: Thoracic aorta. Figure is adapted from Isselbacher (2007)

In fact, the ability of a plant extract to reduce rat's blood pressure is often being associated with the vasorelaxation potential (Kamadyaapa *et al.*, 2009; Ameer *et al.*, 2010b; Sanchez-Salgado *et al.*, 2010; Taiwo and Odeigah, 2010; Vergara-Galicia *et al.*, 2010; Soncini *et al.*, 2011). Even the isolated compounds, such as eugenol (Lahlou *et al.*, 2004b; Interaminense *et al.*, 2007), methyleugenol (Lahlou *et al.*, 2004a), dodoneine (Ouedraogo *et al.*, 2011), eucalyptol (Lahlou *et al.*, 2002b), and artemetin (de Souza *et al.*, 2011), have shown both abilities to cause reduction in blood pressure *in vivo* and to cause vasorelaxation *in vitro*.

Apart from investigation on the effects of *S. polyanthum* leaves extracts, studies on mechanism of actions are similarly important. Elucidation on the mechanism of reduction in blood pressure can be determined by *in vivo* method while the mechanism of vasorelaxation can be determined by *in vitro* method. Various antagonistic studies on specific receptors, or targeted to any systems that possibly mediate the reduction in blood pressure and vasorelaxation effects of the extract may be used in order to elucidate the pharmacodynamics of plant extracts. This study focused on the possible involvement of autonomic receptors. The autonomic receptors have long been held as one of the components that were responsible in the regulation and control of blood pressure (Purves *et al.*, 2001; Franchini and Cowley, 2004; Jones *et al.*, 2004).

Nicotinic- and muscarinic-acetylcholine receptors, and α-and β-adrenergic receptors are among the autonomic receptors investigated in the present study. In conjunction, the respective receptor blockers such as hexamethonium, atropine, phentolamine, and propranolol, were employed to block the nicotinic- and

muscarinic-acetylcholine receptors, and α-and β-adrenergic receptors, respectively, according to a study protocol by Zhang & Tan (1997). A few of the subtype-specific autonomic receptors, which includes the M_3 subtype of muscarinic acetylcholine receptors; the α_2 subtype of α-adrenergic receptors; and the β_2 subtype of β-adrenergic receptors; they are known to cause blood pressure reduction through vasorelaxation, mediated by the endothelium-derived relaxing factor, known as nitric oxide. Hence, this study was briefly extended into discovering any possible involvement of nitric oxide by using N-ω-nitro-l arginine methyl ester or L-NAME as a blocker according to a study protocol previously reported by Lahlou and co-workers (2004a). The L-NAME blocks the endothelial nitric oxide synthase (eNOS), an enzyme used in the production of nitric oxide, a potent vasodilator.

1.5 Objectives of study

This study was aimed to achieve the following general and specific objectives.

1.5.1 General objectives

The general objective of the present study was to evaluate the effects of *S. polyanthum* leaves extracts on rat's blood pressure and related parameters, and the possible mechanism of actions.

1.5.2 Specific objectives

1. To determine the phytochemical composition in *S. polyanthum* leaves extracts (aqueous and methanolic).
2. To determine the effects of orally-administered *S. polyanthum* leaves extracts on the systolic blood pressure of conscious Wistar-Kyoto and Spontaneously Hypertensive rats.
3. To determine the effects of intravenously-administered *S. polyanthum* leaves extracts on the mean arterial, systolic, and diastolic blood pressures, and the heart rate of anaesthetized Wistar-Kyoto and Spontaneously Hypertensive rats.
4. To determine the effects of *S. polyanthum* leaves extracts on the isolated thoracic aorta rings of Wistar-Kyoto and Spontaneously Hypertensive rats.
5. To investigate the involvement of autonomic receptors in mediating the blood pressure reduction and the vasorelaxation effect by *S. polyanthum* leaves extracts.

1.6 Research hypotheses

1. *S. polyanthum* leaves extracts (aqueous and methanolic) consist of different phytochemical compositions.
2. The orally-administered *S. polyanthum* leaves extracts cause reduction in systolic blood pressure of conscious Wistar-Kyoto and Spontaneously Hypertensive rats.

3. The intravenously-administered *S. polyanthum* leaves extracts cause reductions in mean arterial, systolic and diastolic blood pressures of anaesthetized Wistar-Kyoto and Spontaneously Hypertensive rats.
4. *S. polyanthum* leaves extracts relaxed the isolated thoracic aorta rings from Wistar-Kyoto and Spontaneously Hypertensive rats.
5. Autonomic receptors mediate the blood pressure reduction and the vasorelaxation effect by *S. polyanthum* leaves extracts.

1.7 Justification of study

The ability of *S. polyanthum* leaves extracts to reduce blood pressure was previously investigated in the normotensive, and in the induced-type of hypertensive rats. Hajar (1996), as cited in Sundari and co-authors (2000) showed that the intravenous administration of *S. polyanthum* leaves infusion caused significant reduction in blood pressure of Wistar rats which were induced hypertensive using a high-sodium diet. Sukrasno and colleagues (2010) reported that the oral administration of the aqueous extract of *S. polyanthum* leaves caused a significant reduction in mean arterial blood pressure of conscious normotensive Wistar rats. Recently, Ismiyati (2013) reported that the oral administration of ethanolic extract of *S. polyanthum* leaves caused a significant reduction in the increment of blood pressure by phenylephrine in conscious Wistar rats. Despite these studies, there was no reported study yet on the SHR, a chronic and stable model of hypertension that mimics the human essential hypertension (Doggrell and Brown, 1998). Therefore, the present study evaluated on the effects of *S. polyanthum* leaves extracts on few

blood pressure parameters such as the mean arterial, systolic, and diastolic blood pressures and the heart rate of SHR, with WKY as the normotensive control.

In addition, there was no reported investigation yet on the mechanism of blood pressure reduction by *S. polyanthum* leaves extracts. In order to address this gap of information, the present study was the first report that elucidated on the vasorelaxation ability of the extracts, and also investigated on the involvement of autonomic receptors in mediating the reduction of blood pressure and the vasorelaxation effects by *S. polyanthum* leaves extracts. The in-depth understanding on the mechanism of actions is important in order to avoid the concomitant side effects following drugs or herbal interactions.

Besides the lack of scientific investigation on the hypotensive effect by *S. polyanthum* leaves extracts, the phytochemical composition of *S. polyanthum* leaves extracts was also not extensively studied. Thus, the present study was extended to screen for the phytohemical constituents in *S. polyanthum* leaves extracts. The plant's phytochemical profile is important to improve the quality of herbal products in future, and that should be based on consideration of any synergistic, additive, and/or nullifying effects of the bioactive constituent(s) in the herbal preparations. In the long-run, the quality of traditional medicine will be enhanced as emphasized by the WHO (2013) in its report, "WHO Traditional Medicine Strategy: 2013-2023".

CHAPTER 2

LITERATURE REVIEW

2.1 Hypertension

Hypertension is defined as the chronic elevation of blood pressure. It is a multi-factorial disease involving complex interactions between genetic and environmental factors (Lerman *et al.*, 2005; Guyenet, 2006; Dugdale, 2011). According to the United States National Heart, Lung, and Blood Institute (NHLBI) (2012) and the American Heart Association (2013), a person with a systolic blood pressure (SBP) of less than 120 mmHg and a diastolic blood pressure (DBP) of less than 80 mmHg is classified as "normal". Further increment of blood pressure between 120 to 139 mmHg for SBP and between 80 to 89 mmHg for DBP is classified under the "Pre-Hypertension" category. Further increment of blood pressure between 140 to 159 mmHg for SBP and between 90 to 99 mmHg for DBP is classified under "Stage I Hypertension" category. When the blood pressure rises above or equal to 160 mmHg for SBP and above or equal to 100 mmHg for DBP, that person is classified under "Stage II Hypertension" category. If blood pressure rises above 180 mmHg for SBP and 110 mmHg for DBP, the situation is termed as "hypertension crisis" and an emergency treatment is necessary.

In general, hypertension can be classified into primary and secondary hypertension. In primary hypertension, or also known as essential or idiopathic hypertension, no known cause can be identified. However, the risk for cardiovascular and renal diseases is increased (Houston, 2009b). Kaplan and Opie

(2006) stated that primary hypertension accounts for, as many as 95.0 % of all cases of hypertension. In contrast, the secondary hypertension results from various medical conditions such as chronic kidney disease, eclampsia, thyroid disease, coarctation of aorta, aldosteronism, pheochromocytoma, brain lesion, Cushing syndrome, and sleep apnoea (Lyerly and Goodfriend, 2009).

In certain cases that usually affect elder people, only the systolic blood pressure is high. This condition is known as isolated systolic hypertension. In addition, the NHLBI (2012) indicated that pregnancy and a few medicines, such as corticosteroids for asthmatic patients, birth control pills, cold-relief products or hormone therapy for reducing menopause symptoms, may also raise the systolic blood pressure.

2.1.1 Prevalence

Hypertension is on the rise globally and locally. It is a global dilemma with a worldwide prevalence of 40.0 % amongst those aged 25 and above, in 2008 as reported by the WHO (2014). In Malaysia, the National Health and Morbidity Survey II (NHMS) conducted in 1996, have shown that 32.9 % or an estimated of 2.6 million Malaysian adults, aged 30 and above were affected with hypertension (Lim *et al.*, 2004). In accordance, NHMS III that was carried out in 2006, has revealed that the prevalence of hypertension among the similar age group had increased to 40.5 % (Rampal *et al.*, 2008). Similarly, the high prevalence of hypertension between 26.8 % and 35.6 % was also recorded by smaller scale studies

conducted in Malaysia (Mohd Yunus *et al.*, 2004; Narayan and Rashid, 2007; Akter *et al.*, 2010).

2.1.2 Risk factors

There are a few interplayed risk factors for hypertension which include age, race or ethnicity, obesity, gender, level of education, and family history of hypertension. According to the NHLBI (2012), blood pressure tends to rise with age. About 65.0 % of Americans, aged 60 and above was affected with hypertension. Isolated systolic hypertension is the most common form of hypertension that affects the elderly, where about 2 out of 3 people, aged 60 and above were classified as having isolated systolic hypertension. Likewise, some local studies conducted in Malaysia also revealed that the potential to get hypertension also increases steadily with increasing age (Mafauzy *et al.*, 2003; Mohd Yunus *et al.*, 2004; Narayan and Rashid, 2007; Rampal *et al.*, 2008; Akter *et al.*, 2010). With increased age, the large arteries stiffen while the plaque builds-up, and in long-term, it increases the incidence of cardiac and vascular diseases.

Although hypertension can affect anyone, however, race may play a role and it has been considered as one of the main factors for hypertension as stated by the NHLBI (2012). According to this statement, hypertension is more common among African American adults than in Caucasian or Hispanic American adults. In relation to these groups, African Americans tend to be affected with hypertension earlier; are often to have more severe hypertension; and are less likely than Caucasians to achieve target control levels with hypertension treatment. African Americans also

have higher rates than Caucasians of early death from hypertension-related problems, such as coronary heart disease, stroke, and kidney failure. On the other hand, the NHMS II reported that the prevalence of hypertension was high across the ethnic groups of Malay, Chinese, Indian and indigenous people (Lim *et al.*, 2004). However, the prevalence of hypertension among the indigenous (34.3 %) and the Malays (33.5 %) slightly outnumbered the Chinese (33.1 %) and the Indians (30.8 %), but were not statistically significant. Similar findings were reported in the subsequent NHMS III. The findings showed that Malays (41.3 %) had the highest prevalence of hypertension, followed by the Chinese (40.0 %) and the Indians (37.7 %), but these percentages were not statistically significant (Rampal *et al.*, 2008). In agreement, a smaller scale study conducted in a rural community in Mukim Dengkil, Selangor pointed out that there was no significant association between race and hypertension (Mohd Yunus *et al.*, 2004).

Overweight or obesity is also identified as a risk factor according to the NHLBI (2012). This is supported by a few other studies conducted in Malaysia that reported a significant association between obesity and hypertension (Narayan and Rashid, 2007; Latiffah *et al.*, 2008). The NHMS III also revealed that hypertension increased with the increase of body mass index, whereby obese individuals were eight times more likely to develop hypertension (Rampal *et al.*, 2008). Obesity increases with increasing age, and so, increases the chance of getting hypertension (Akter *et al.*, 2010). However, a study by Mohd Yunus and colleagues (2004) in a rural community in Mukim Dengkil, Selangor found that there was no significant association between hypertension and obesity.

Meanwhile, it is debatable if gender is one of the risk factors for hypertension. A few studies conducted in Malaysia reported that there was no significant difference between male and female in getting hypertension (Mafauzy *et al.*, 2003; Latiffah *et al.*, 2008). However, in the NHMS II, female had higher prevalence of hypertension as compared to male (Lim *et al.*, 2004). This finding is in agreement with the smaller scale studies conducted in Malaysia (Mohd Yunus *et al.*, 2004; Narayan and Rashid, 2007). On contrary, in the NHMS III, the male Malaysians aged 15 to 49 years old, had higher prevalence of hypertension as compared to female of similar range of age; meanwhile, female Malaysians, aged 50 years old and above had higher prevalence of hypertension as compared to male of similar range of age (Rampal *et al.*, 2008). This finding has indicated that, with the increment of age in female, their chances to develop hypertension increases. In fact, this finding was in corroboration with the report by the US NHLBI (2012) which pointed for men before the age of 45 are more likely to have hypertension than women. Interestingly, the condition is more likely to affect women than men after the age of 65. This is supported by Hart and Charkoudian (2014) who suggested that young women are relatively protected against the risk of hypertension due to greater peripheral vasodilator influences compared to young men and older people; however, this protective effect eventually is lost at menopause.

Interestingly, education level is also identified as a risk factor. There is an inverse relationship between hypertension with an increased level of education (Narayan and Rashid, 2007; Latiffah *et al.*, 2008; Rampal *et al.*, 2008). Besides education, family history of hypertension also increases the prevalence of hypertension. Individuals with family history of hypertension were twice likely to

develop hypertension, as compared to those who were not (Rampal *et al.*, 2008). Other than that, unhealthy lifestyle habits, such as excessive salt consumption, alcohol consumption, lack of potassium in diet, lack of physical activity, and smoking, may also raise the risk for hypertension (Rampal *et al.*, 2008).

2.1.3 Pathogenesis

There are various pathophysiologic factors that contribute to the development of hypertension, including of genetics, environmental, neural, renin-angiotensin-aldosterone system (RAAS), and altered vascular-related functions (Oparil *et al.*, 2003; Gavras, 2009). Nevertheless, the relative roles of each of these factors in contributing to the development of hypertension may differ between individuals.

2.1.3 (a) Genetics

Several population-based studies have evidenced that genetics is one of the key factors for the genesis of hypertension (Hamet *et al.*, 2002; Oparil *et al.*, 2003). Among the important findings in a few early epidemiological studies include: i) a highly significant correlation in blood pressure scores between parents and natural children (Biron *et al.*, 1976), ii) a greater concordance of blood pressures in monozygotic identical twins than dizygotic twins (Feinleib *et al.*, 1977), and iii) a greater similarity of blood pressure within families than between families (Longini *et al.*, 1984). Likewise, recent findings further supported the role of genetics. It is widely accepted now that hypertension occurs more frequently in individuals with a positive family history of hypertension (Hamet *et al.*, 2002), and, it was reported that

both paternal and maternal histories of hypertension were determinants of systolic and diastolic blood pressures of the offspring (Mitsumata *et al.*, 2012).

In some cases, hypertension may be caused by genetic mutations such as observed in patients with Liddle's and Gordon's syndromes (Hamet *et al.*, 2002). The former syndrome is characterized by a defect in sodium channel, whereby it is continually activated, resulting in an increased sodium re-absorption. The latter syndrome is characterized by a defect in renal ion transport. These established forms of hypertension, even though are found to be rare in populations, has further affirmed the influence of genetics on the development of hypertension (Hamet *et al.*, 2002).

Even though the development of hypertension is greatly influenced by genetics, it is also strongly affected by environmental factors (Hamet *et al.*, 2002; Weder, 2005). Indeed, the interaction between gene-environmental factors determines the severity of blood pressure elevation and the timing of hypertension onset (Oparil *et al.*, 2003; Weder, 2005; Adrogué and Madias, 2007).

2.1.3 (b) Environment

A few environmental factors, such as high level of stress, high alcohol consumption, and high intake of sodium with low intake of potassium have been identified to contribute to the genesis of hypertension (Hamet *et al.*, 2002; Oparil *et al.*, 2003; Weder, 2005). High level of stress has partly reasoned the great incidence of hypertension in low socio-economic groups, as demonstrated by Klag and co-

workers (1991) in their study among the blacks of the United States of America. In addition, Calhoun and colleagues (1993) further demonstrated that normotensive blacks had a heightened sympathetic response to cold pressor test, a cardiovascular test performed by immersing the hand into an ice water container. By measuring the changes in blood pressure and heart rate, it was noticed that this repeated exposure to stress had increased the sympathetic outflow which led to the elevation of blood pressure.

High alcohol intake also contributed to hypertension. A study conducted by Criqui and colleagues (1989) revealed that a high alcohol intake above the threshold level of 20 milliliters per day was associated with higher systolic and diastolic pressures. The finding indicated that there was a linear relationship of both systolic and diastolic blood pressures with alcohol consumption.

Another pivotal environmental factors that contribute to pathogenesis of hypertension is the long-term dietary intakes of high sodium and low potassium (Weder, 2005; Adrogué and Madias, 2007). A worldwide epidemiological study, known as 'The International Study of Salt and Blood Pressure' or INTERSALT, which was conducted across 52 populations in year 1988 demonstrated a positive and linear relationship between 24-hour sodium excretion with blood pressure in 48 populations (Stamler, 1997). The INTERSALT study also showed that, in 4 remote areas where the populations consumed relatively low sodium and high potassium intakes, and with little, if any, alcohol consumption; these populations were virtually free of hypertension.