ANTIHYPERTENSIVE AND VASODILATORY EFFECTS OF *VERNONIA AMYGDALINA* AND *SWIETENIA MACROPHYLLA* EXTRACTS

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by

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TABLES OF CONTENTS

CHAPTER 4 – SIGNAL TRANSDUCTION PATHWAYS OF *VERNONIA AMYGDALINA* ETHANOL EXTRACT IN VASORELAXATION

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVATIONS

2-APB	2-aminoethoxydiphenyl borate
2D-IR	Two-dimensional correlation infrared
4-AP	4-aminopyridine
5-HT	Serotonin
AA	Arachidonic acid
AC	Adenylyl cyclase
ACEIs	Angiotensin-converting enzyme inhibitors
ACh	Acetylcholine
A/G	Albumin/globulin
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
ARBs	Angiotensin receptor blockers
AST	Aspartate aminotransferase
AT	Angiotensin
ATP	Adenosine triphosphate
B_2	Bradykinin
$BaCl_2$	Barium chloride
BK_{Ca}	Big-conductance calcium-activated potassium channel
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine monophosphate
CBC	Complete blood count
CCBs	Calcium channel blockers

CE	Capillary electrophoresis
cGMP	Cyclic guanosine monophosphate
CMC	Carboxymethyl cellulose
CO_2	Carbon dioxide
COX	Cyclooxygenase
DAG	Diacylglycerol
DBil	Direct bilirubin
DBP	Diastolic blood pressure
DNA	Deoxyribonucleic acid
DRI	Direct rennin inhibitor
DTGS	Deuterated Tri-Glycine Sulfate
EC_{50}	Median effective concentration
EDHF	Endothelium-derived hyperpolarizing factor
EDRFs	Endothelium-dependent relaxing factors
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
eNOS	Endothelial nitric oxide synthase
ET	Endothelin
FTIR	Fourier transform infrared
GC	Gas chromatography
GGT	Gamma-glutamyl transferase
GI	Gastrointestinal
GPCR	G-protein-coupled receptor
GTP	Guanosine triphosphate
HCT	Hematocrit

HGB	Hemoglobin
HPLC	High performance liquid chromatography
IBil	Indirect bilirubin
IK_{Ca}	Intermediate-conductance calcium-activated potassium channel
IP	Prostaglandin I_2
IP_3	Inositol trisphosphate
IP_3R	Inositol trisphosphate receptor
IR	Infrared
K^+	Potassium ion
K_{ATP}	ATP-sensitive potassium channels
K_{Ca}	Calcium-activated potassium channels
K_{IR}	Inwardly rectifying potassium channels
K_V	Voltage-dependent potassium channels
KBr	Potassium bromide
KCl	Potassium chloride
Krebs’	Krebs-Henseleit
L-NAME	L-N^G-Nitroarginine methyl ester
L-NNA	Nω-nitro-L-arginine
LC/MS	Liquid chromatography with mass spectrometry
LD_{50}	Median lethal dose
LDCC	Ligand-dependent calcium channel
LFT	Liver function test
M_3	Muscarinic
MAP	Mean arterial pressure
MB	Methylene blue

MCH	Mean cell hemoglobin
MCHC	Mean cell hemoglobin concentration
MCV	Mean corpuscular volume
Mg^{2+}	Magnesium ion
$MgSO_4$	Magnesium sulfate
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphate
n	Number of determination
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaH_2PO_4	Sodium dihydrogen phosphate
NO	Nitric oxide
NO-cGMP	Nitric oxide-cyclic guanosine monophosphate
NSAIDs	Non-steroidal anti-inflammatory drugs
O_2	Oxygen
ODQ	1H-(1,2,4)Oxadiazolo(4,3-a)quinoxalin-1-one
PE	Phenylephrine
PGI_2	Prostacyclin
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PKG	cGMP-dependent protein kinase
PLC	Phospholipase C
PLT	Platelet
R_{max}	Maximum relaxation

RBC	Red blood cell
RFT	Renal function test
ROCC	Receptor-operated calcium channel
RyR	Ryanodine receptor
SBP	Systolic blood pressure
SD	Sprague-Dawley
SD-IR	Second derivative infrared
SEM	Standard error of mean
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
sGC	Soluble guanylyl cyclase
SHRs	Spontaneously hypertensive rats
SK_{Ca}	Small-conductance calcium-activated potassium channel
SM50	50% ethanol extract of *S. macrophylla* seed
SOCC	Store-operated calcium channel
SPSS	Statistical Package for Social Sciences
SR	Sarcoplasmic reticulum
TBil	Total bilirubin
TEA	Tetraethylammonium ion
TLC	Thin layer chromatography
TP	Total protein
TSMs	Traditional Systems of Medicine
TxA_2	Thromboxane
UK	United Kingdom
USA	United States of America
USM	Universiti Sains Malaysia

UV	Ultraviolet
VAE	*V. amygdalina* 95% ethanol extract
VOCC	Voltage-operated calcium channel
VSM	Vascular smooth muscle
VSMCs	Vascular smooth muscle cells
WBC	White blood cell

LIST OF SYMBOLS

%	Percentage
°C	Degree Celsius
°C/min	Degree Celsius per minute
10^9/L	10^9 cells per liter
10^{12}/L	10^{12} cells per liter
µL	Microliter
µM	Micromolar
µmol/L	Micromole per liter
cm^{-1}	Reciprocal centimeter
fL	Femtoliter
g	Gram
g/dL	Gram per deciliter
g/L	Gram per liter
h	Hour
mg	Milligram
mg/mL	Milligram per milliliter
min	Minute
mM	Millimolar
mm	Millimeter
mmHg	Millimeter of mercury
mmol/L	Millimole per liter
pg	Picogram
rpm	Revolution per minute
U/L	Units per liter

KESAN ANTIHIPERTENSI DAN VASODILATORI EKSTRAK *VERNONIA AMYGDALINA* DAN *SWIETENIA MACROPHYLLA*

ABSTRAK

Di Malaysia, hipertensi merupakan topik hangat dalam kesihatan awam dan perubatan, oleh demikian, cara penyelesaian amat diperlukan. Dalam kajian ini, herba tempatan Malaysia seperti *Clinacanthus nutans* Lindau, *Strobilanthes cripus, Murdannia bacteata, Elephantopus scaber Linn., Pereskia bleo, Pereskia grandifolia* Haw., *Vernonia amygdalina,* dan benih *Swietenia macrophylla* King telah dipilih dan ekstraknya (ekstrak air, ekstrak 50% etanol, dan ekstrak 95% etanol) telah digunakan untuk kajian permulaan awal bagi aktiviti antihipertensi dan pemvasokenduran. Keputusan menunjukkan bahawa benih *Swietenia macrophylla* King mempunyai potensi tertinggi dalam pemvasokenduran, diikuti oleh *Vernonia amygdalina*. Oleh itu, ekstrak 95% etanol daripada *V. amygdalina* (VAE) dan ekstrak 50% etanol daripada benih *S. macrophylla* King (SM50) yang menunjukkan potensi terbaik dalam pemvasokenduran telah disiasat selanjutnya mekanisme tindakan pemvasokenduran melalui kajian *in vitro* dengan perakaman tindak balas kontraksi cincin aorta yang terpencil terhadap ekstrak dengan kehadiran penyekat tertentu. Kajian telah menunjukkan VAE dan SM50 yang mengandungi kepelbagaian komponen kimia menunjuk kesan pemvasokenduran yang lebih kuat ke atas cincin aorta kerana mungkin melibatkan pelbagai mekanisme. Laluan utama yang menyebabkan kesan pemvasokenduran oleh VAE dalam *in vitro* cincin aorta terpencil adalah melalui EDRF yang merangkumi PGI_2 dan NO, diikuti oleh reseptor saluran berpaut (sebagai pembuka saluran K^+), dan seterusnya faktor relaksasi tanpa-

endotelium seperti laluan isyarat receptor-M_3 dan -β_2. Manakala laluan pemvasokenduran SM50 kebanyakannya melibatkan laluan reseptor saluran berpaut (penyumbatan VOCC, pembuka saluran K^+ dan penghambat reseptor IP_3) dan kurang terlibat dalam laluan β_2-adrenergik tanpa-endotelium dan laluan isyarat NO/sGC/cGMP. Selain itu, SM50 juga digunakan dalam kajian *in vivo* ke atas spontaneously hypertensive rats (SHRs) melalui penyuapan tiga dos SM50 yang berbeza (1000 mg/kg, 500 mg/kg dan 250 mg/kg) dengan intubasi gastrik sekali setiap hari selama empat minggu. Setelah empat minggu rawatan, penurunan nilai SBP, DBP, dan MAP yang ketara dapat dilihat dalam ketiga-tiga kumpulan SHRs dan tindak balas antihipertensi telah muncul walaupun hanya selepas mingggu pertama penyuapan. Ini membuktikan bahawa SM50 mempunyai kesan antihipertensi yang kuat dan aktiviti antihipertensinya adalah bergantung kepada kepekatan ekstrak. Dalam pada itu, kajian toksisiti menunjukkan bahawa SHRs yang dirawat 1000 mg/kg/hari SM50 selama 28 hari telah menunjukkan hepatotoksisiti. Ujian fungsi hati menunjukkan peningkatan tahap transferas gama-glutamil (GGT) yang ketara (35.00 ± 10.61 U/L) dan pengurangan yang ketara untuk tahap albumin (9.70 ± 0.72 g/L). SHRs juga mengalami penurunan berat badan (9.47% untuk 1000 mg/kg, 5.15% untuk 500 mg/kg, dan 7.46% untuk 250 mg/kg). Oleh itu, dapat disimpulkan bahawa SM50 mungkin menyebabkan hepatotoksik pada 1000 mg/kg dan dos yang selamat untuk tikus adalah disyorkan pada sekitar 250 mg/kg. Walau bagaimanapun, masih terlalu awal untuk menyimpulkan bahawa dos ini adalah selamat untuk digunakan pada manusia, lebih banyak kajian harus dijalankan sebelum membuat kesimpulan yang kukuh.

ANTIHYPERTENSIVE AND VASODILATORY EFFECTS OF *VERNONIA AMYGDALINA* AND *SWIETENIA MACROPHYLLA* EXTRACTS

ABSTRACT

In Malaysia, hypertension is an increasingly important medical and public health issue and there is an imminent need to decrease its prevalence. In this study, the local herbs in Malaysia such as *Clinacanthus nutans* Lindau, *Strobilanthes cripus, Murdannia bacteata, Elephantopus scaber Linn., Pereskia bleo, Pereskia grandifolia* Haw., *Vernonia amygdalina,* and *Swietenia macrophylla* King seeds were selected and their extracts (water extracts, 50% ethanol extracts, and 95 % ethanol extracts) underwent preliminary testing to screen for their antihypertensive activities and their vasorelaxation. The results showed that the seeds of *Swietenia macrophylla* King has the highest potential in vasorelaxation, followed by *Vernonia amygdalina*. Thus, the vasorelaxation mechanism of *V. amygdalina* and *S. macrophylla* seeds extracts with the best potential in vesorelaxation (95% ethanol extract of *V. amygdalina*, VAE and 50% ethanol extract of *S. macrophylla* seeds, SM50) were further investigated through *in vitro* study by recording the contractile responses of the isolated aortic rings to the extracts with the presence of selective blockers. This study found that, crude VAE and SM50, which contain multiple chemical components, exhibited a stronger vasorelaxant effect on isolated aortic rings due to their possible involvement of multiple mechanisms. The major route for inducing the vasorelaxant effect by VAE in *in vitro* isolated aortic rings was via EDRFs which includes PGI_2 and NO, followed by channel-linked receptors (as K^+ channels opener), and subsequently endothelium-independent relaxing factors such as M_3- and β_2-receptors signaling

pathways. While the vasorelaxation pathways employed by SM50 were mostly attributed to channel-linked receptors pathways (blockage of VOCC, K^+ channels opener, and IP_3 receptor inhibitor), with smaller contributions from the endothelium-independent β_2-adrenergic pathways, and the NO/sGC/cGMP signalling pathways. Besides, SM50 also underwent an *in vivo* study through orally administered spontaneously hypertensive rats (SHRs) with three different doses of SM50 (1000 mg/kg, 500 mg/kg, and 250 mg/kg) by gastric intubation once a day for four weeks. After four weeks of treatment, a clear decrease in the SBP, DBP, and MAP values could be observed in the three groups of SHRs and it only took one week for the antihypertensive action to manifest. This indicated that SM50 possess strong antihypertensive effect and its antihypertensive activity is strongly concentration dependent. However, toxicity study showed that SHRs which were treated with 1000 mg/kg/days of SM50 for 28 days exhibited hepatotoxicity. The liver function tests showed significant elevation of gamma-glutamyl transferase (GGT) level (35.00 ± 10.61 U/L) and significant reduction in albumin level (9.70 ± 0.72 g/L). The SHRs also experienced decrease in body weights (9.47% for 1000 mg/kg, 5.15% for 500 mg/kg, and 7.46% for 250 mg/kg). Therefore, it can be concluded that SM50 might cause hepatotoxic effect at 1000 mg/kg and the suggested safety doses for rat is 250 mg/kg. However, it is too early to conclude that this dosage is safe for consumption in human, more studies should be conducted before drawing a firm conclusion.

CHAPTER 1

INTRODUCTION

1.1 Hypertension

Hypertension is a chronic medical condition in which the blood pressure is elevated (a sustained systolic blood pressure greater than 140 mmHg and/or a sustained diastolic blood pressure greater than 90 mmHg). This may lead to stroke, heart disease, and heart failure. Besides, the other organs such as kidneys, limbs, and eyes may also suffer damage. Typically, hypertension has been classified into four classes (see Table 1.1) by the seventh report of Joint National Committee (Lenfant et al., 2003). In Malaysia, hypertension is an increasingly important medical and public health issue. According to Hypertension Clinical Practice Guidelines of Malaysia on 2013, the prevalence of hypertension in Malaysia for adults of more than 18 years old has increased from 32.2% in 2006 to 32.7% in 2011, whereas for those adults of more than 30 years old, the prevalence has increased from 42.6% to 43.5%. Therefore, there is an imminent need to decrease the prevalence of hypertension.

It is known that lowering blood pressure can greatly reduce hypertension complication such as cerebrovascular, cardiovascular, and renal disease (Wang et al., 2014). An impressive evolution of antihypertensive agents have been developed over the few past decades. In early, a *Rauwolfia* alkaloids, reserpine was widely used as antihypertensive drugs, however it has been replaces by other drugs due to its drastic side effects including depression and cancer (Lobay, 2015). In 1950s, thiazide diuretics had discovered helpful in lowering blood pressure, followed by β-blockers, calcium channel blockers (CCBs), α-blockers, angiotensin-converting enzyme

inhibitors (ACEIs), angiotensin receptor blockers (ARBs), and direct renin inhibitor (DRI) Aliskiren in subsequent years for antihypertensive drugs development (Düsing, 2010). However, there are five of the aforementioned antihypertensive drugs (thiazide diuretics, β-blockers, CCBs, ACEIs, and ARBs) have been stated in the Clinical Practice Guidelines in Management of Hypertension as the current most frequently choices of first line mono-therapy drugs for treating hypertension. Nonetheless, combination drugs often being preferred since they exert a better antihypertensive effect compared to single drug therapy (MOH, 2013). At this point, crude extract of local herbs could achieve the holistic therapeutic criteria whereby treating the hypertension by using multiple signaling mechanism pathways instead of either one of the mono-pathway. Native plant remedies are readily available, low cost and are less likely to cause safety concern (Taiwo et al., 2010). Therefore, awareness about its importance in health care delivery system is growing tremendously. These benefits also reinforces the use of local herbs in this current study.

Table 1.1 Classification of blood pressure

Classification	**Systolic Blood Pressure (mmHg)**	**Diastolic Blood Pressure (mmHg)**
Normal	≤ 120	≤ 80
Prehypertension	120 – 139	80 – 89
Stage 1 hypertension	140 – 159	90 – 99
Stage 2 hypertension	≥ 160	≥ 100

1.2 Herbal remedies in Malaysia

In Traditional Systems of Medicine (TSMs), medicines from natural origin have been used as a source of remedy for the prevention, cure, and treatment of different ailments (Rates, 2001). Herbal medicine has always been an asset to the people of Malaysia due to our country's rich herbal resources. Therapeutically important plants in TSMs have been extensively explored recently, for their benefits and various applications in herbal health supplements (Borris, 1996, Gurib-Fakim, 2006, Katiyar et al., 2012). Furthermore, traditional medicine strategy published recently states that traditional and complementary medicines have gained a growing economic importance worldwide (Qi and Kelley, 2014). Therefore, bringing the consumption and use of such remedies into a valid framework for the rational scientific use of such medicines is imperative.

Malaysia is a tropical country with plentiful rainforests, a lot of herbs could grow throughout the year. Most of the local herbs in Malaysia such as *Clinacanthus nutans* Lindau, *Strobilanthes cripus*, *Murdannia bacteata*, *Elephantopus scaber* Linn., *Pereskia bleo*, *Pereskia grandifolia* Haw., *Vernonia amygdalina*, and *Swietenia macrophylla* King (Figure 1.1) are widely distributed plants used for dietary and medicinal purposes due to they are safe to consume and less information showed they are toxic (Chen et al., 2010, Goh, 2000, Quasie et al., 2016, Tan et al., 2005).

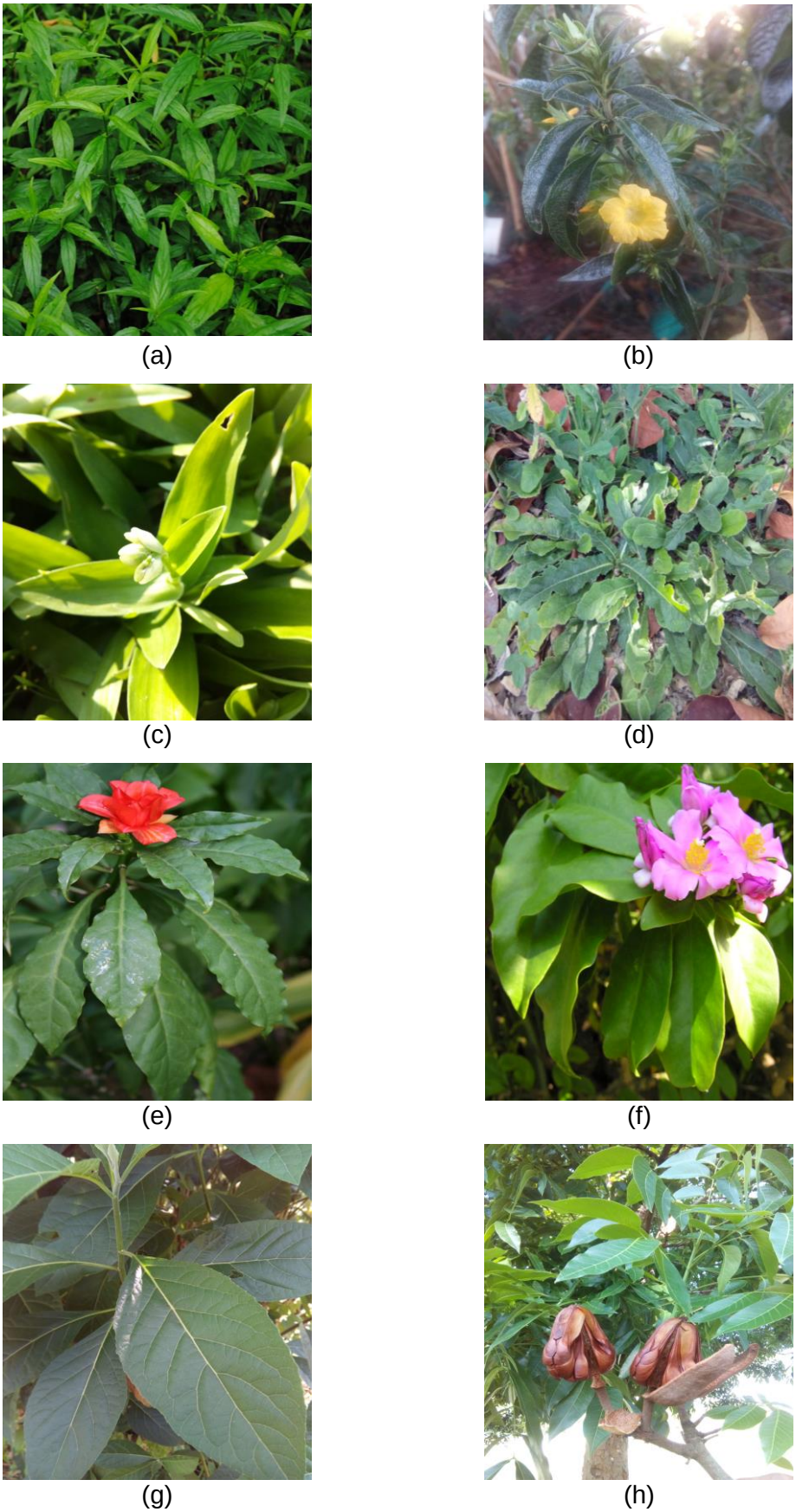

Figure 1.1 Pictures of (a) *C. nutans*, (b) *S. cripus*, (c) *M. bacteata*, (d) *E. scaber*, (e) *P. bleo*, (f) *P. grandifolia*, (g) *V. amygdalina*, and (h) *S. macrophylla*.

According to Goh (2001), *Clinacanthus nutans* Lindau, *Strobilanthes cripus*, *Murdannia bacteata*, *Elephantopus scaber* Linn., *Pereskia bleo*, *Pereskia grandifolia* Haw., *Vernonia amygdalina*, and *Swietenia macrophylla* King are traditionally used in Malaysia to treat cancer, inflammation, diabetes, hypertension, and act as antioxidant agents.

Their uses in traditional medicine have been scientifically supported by numerous studies. For example, *Clinacanthus nutans* Lindau has been reported to possess antioxidant, anti-inflammation, antimicrobial, and anti-viral activity (Mustapa et al., 2015, P'ng et al., 2013). Preclinical studies have shown that the *Strobilanthes cripus* possess antioxidant, free radical-scavenging, anticancer, antidiabetic, antimicrobial, wound healing, and antiulcerogenic activities (Nurraihana and Norfarizan-Hanoon, 2013). The extracts of *Murdannia bracteata* have been reported to have hepatoprotective effects and antioxidant activity (Ooi et al., 2015, Yam et al., 2010). The whole plant of *Elephantopus scaber* Linn. had been tested for acute toxicity, and has analgesic, antipyretic, antimicrobial, antioxidant, antidiabetic, anti-inflammatory, cardiovascular, hepatoprotective, diuretic, and laxative activities (Hiradeve and Rangari, 2014, Poli et al., 1992). There are several pharmacological reports on the extracts of *Pereskia bleo*, mainly on cytotoxic, anti-proliferative and anti-nociceptive activities (Abdul-Wahab et al., 2012, Sri Nurestri et al., 2008). Some experimental pharmacological studies with *Pereskia grandifolia* Haw. have reported the anti-tumor, antioxidant potential, diuretic activity, and hypotensive effect its extracts (Kazama et al., 2012, Nurestri et al., 2009, Sim et al., 2010). A number of studies have shown that *Vernonia amygdalina* possesses antioxidant, anti-hyperlipidemic, and antidiabetic properties (Adaramoye et al., 2008, Akah and Okafor, 1992, Atangwho et al., 2013, Ijeh and Ejike, 2011). Based on the previous

research, various pharmacological activities of *Swietenia macrophylla* King leaves had been established, which include antimicrobial, anti-inflammatory, antioxidant effects, anticancer, antitumor, and anti-diabetic activities (Moghadamtousi et al., 2013), while the seeds of *Swietenia macrophylla* King have the effect against hypertension and diabetes (Ch'ng et al., 2016, Hashim et al., 2013).

The knowledge of using the above mentioned herbal remedies to treat hypertension had been passed down for generations. However, these concepts have yet to be proven scientifically and there was no evidence to prove that these herbs possess antihypertensive activity. Thus, it is important to do a preliminary test to screen for the antihypertensive activities of these herbs.

1.3 Signaling mechanisms involved in vasorelaxation study

Blood vessels isolated from living organisms are commonly used for the *in vitro* studies on anti-hypertensive drugs research (Yildiz et al., 2013). Blood pressure are regulated within a narrow range where the blood could convey sufficient perfusion for tissue but not cause any harm on the vascular system, especially the endothelium and vascular smooth muscle cells (Loh et al., 2016). Therefore, it is important to study and focus on the signaling mechanism pathways involved in vascular tone regulation to keep the blood pressure in the optimal range, including signaling amplification from second messengers and the interaction between enzyme-linked, channel-linked and G-protein coupled receptors.

There are several signal transduction mechanisms that modulate intracellular calcium concentration and so for the vascular tone. The primary pathways involved in the vasorelaxation of blood vessels are nitric oxide-cyclic guanosine

monophosphate (NO-cGMP) pathway and G-protein-coupled pathway. They are subsequently followed by the potassium channels, which can be subdivided into calcium-activated potassium channels (K_{Ca}), ATP-sensitive potassium channels (K_{ATP}), voltage-dependent potassium channels (K_V), and inwardly rectifying potassium channels (K_{IR}). Calcium channels are essentially involved in the regulation of vascular tone and they are also divided into a few groups, such as voltage-operated calcium channel (VOCC), receptor-operated calcium channel (ROCC), store-operated calcium channel (SOCC), as well as the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) signaling pathways (Loh et al., 2016).

1.3.1 cGMP-coupled signal transduction

NO-cGMP system is very important in regulating vascular smooth muscle tone. Vascular endothelial cells normally produce NO by endothelial nitric oxide synthase (eNOS) from the breakdown of L-arginine. NO diffuses from endothelial cells to adjacent vascular smooth muscle cells (VSMCs) where it activates soluble guanylyl cyclase (sGC) leading to increased formation of cGMP second messenger from guanosine triphosphate (GTP) and cause vasorelaxation (Jakala et al., 2009, Moncada, 2007). The cGMP relaxes vascular smooth muscle (VSM) through several ways, such as stimulates cGMP-dependent protein kinase (PKG) that activates myosin light chain phosphate (MLCP) which dephosphorylates myosin light chain (MLC), inhibits calcium entry into the VSM which decreases intracellular calcium concentrations, activates K^+ channels which leads to hyperpolarization, and decreases inositol trisphosphate (IP_3) (Horowitz et al., 1996, Klabunde, 2011, Ko et al., 2008).

1.3.2 G-protein coupled signal transduction

G-protein-coupled receptor (GPCR) will responds to ligands through the activation of the G-proteins, which are located on the intracellular surface of the cell membrane. In general, G-protein can be categorized into α, β, and γ types. Once GTP is bound to the G-protein and activates it, the G-protein is cleaved into Gα and Gβγ dimmers. Typically, Gα-proteins can be classified into $G_s\alpha$, $G_i\alpha$, and $G_q\alpha$ which are functionally responsible for different roles in signaling transduction of blood vessels (Walaas et al., 1992).

The $G_s\alpha$-protein coupled pathway in VSM stimulates adenylyl cyclase (AC), which catalyses the formation of cyclic adenosine monophosphate (cAMP) second messenger from adenosine triphosphate (ATP) (Berumen et al., 2012, Nichols and Nichols, 2008). An increase in cAMP in VSM causes relaxation or reduced contraction. The reason for this opposite effect is that calcium-calmodulin activates myosin light chain kinase (MLCK), which phosphorylates MLC and causes contraction, however, MLCK is inhibited by cAMP. The $G_s\alpha$-protein is coupled to several important receptors that bind vasorelaxant substances, among which are β_2-adrenoceptors (bind to β_2-agonists such as epinephrine and isoproterenol), A_2 purinergic receptors (bind to adenosine), and prostaglandin I_2 (IP) receptors (bind prostacyclin, PGI_2, which is produced from arachidonic acid, AA and catalysed by cyclooxygenase, COX in endothelial cell) (Bonaventura et al., 2011, Jakala et al., 2009, Klabunde, 2011).

Whereas, $G_i\alpha$-protein is functionally opposed to $G_s\alpha$-protein. $G_i\alpha$-protein in VSM is coupled to α_2-adrenoceptor. Binding of this receptor to an agonist such as norepinephrine causes a reduction in cAMP, which leads to VSM contraction (Klabunde, 2011, Qin et al., 2008).

In VSMC, there are two signal transduction pathways linked to $G_q\alpha$-protein which are phospholipase C (PLC) pathway and Rho-kinase pathway. In PLC pathway, the activated $G_q\alpha$-protein stimulates PLC to form IP_3 and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP_2), IP_3 bound to the IP_3 receptor which located in the membrane of sarcoplasmic reticulum (SR) and stimulates SR release of calcium to the cytoplasm, while DAG activates protein kinase C (PKC) and stimulates contraction. The Rho-kinase pathway inhibits MLCP, which enhances contraction (Jakala et al., 2009, Klabunde, 2011, Yildiz et al., 2013).

Most of the $G_q\alpha$-protein-coupled receptors such as angiotensin (AT) receptor, serotonin (5-HT) receptor, muscarinic (M_3) receptor, and endothelin (ET) receptor are present in both endothelium and VSMC. However, AT receptor, 5-HT receptor, and ET receptor are functionally predominated in VSMC, while M_3 receptor is functionally predominated in endothelium. In addition of this, the other $G_q\alpha$-protein-coupled receptors such as α_1-adrenergic receptor and thromboxane (TxA_2) receptor exist only in VSMC, and bradykinin (B_2) receptors exist only in the endothelium. In the endothelium, the $G_q\alpha$-protein-coupled receptors produce NO by increasing the intracellular concentration of calcium in endothelium and subsequent eNOS activation. Whereas in the VSMC, the $G_q\alpha$-protein-coupled receptors produce a direct effect in the increase of intracellular concentration of calcium by passing through the PLC-dependent pathway (Bockaert et al., 2006, Chen et al., 2000, de Gasparo et al., 2000, Goodman et al., 2001, Ishii and Kurachi, 2006, Jakala et al., 2009, Klabunde, 2011, Loh et al., 2016, Yildiz et al., 2013).

1.3.3 Calcium channels

There are two main groups of calcium channels in VSMCs, namely voltage-operated calcium channel (VOCC, also called L-type calcium channels) and ligand-gated calcium channels (such as ROCC and SOCC). IP_3 receptor (IP_3R) and Ryanodine receptor (RyR) are also classified as ligand-gated calcium channels which locate in the membrane of SR (Landsberg and Yuan, 2004, McFadzean and Gibson, 2002). When an agonist binds to a $G_q\alpha$-protein-coupled receptor, it trigger the formation of DAG and IP_3. DAG activates ROCC mediated calcium influx and IP_3 activates IP_3R mediated calcium release from SR and cause membrane depolarization (Putney et al., 2001). The subsequent depletion of calcium from SR in turn activates SOCC mediated calcium influx and the membrane depolarization leads to VOCC mediated large amount of calcium influx and cause vasoconstriction (Feletou and Vanhoutte, 2005, McFadzean and Gibson, 2002). The high level of cytoplasmic calcium concentration also triggering RyR mediated calcium release from SR. In order to lowering and maintain the calcium concentration in cytoplasmic under resting conditions, calcium are sequestrated into SR by SERCA and extruded by Ca^{2+}-Mg^{2+}-ATPase in the plasma membrane (Landsberg and Yuan, 2004).

1.3.4 Potassium channels

Potassium channels control the frequency and shape of action potentials and establish cell membrane potential. This large family is classified into four groups, which are K_{Ca}, K_{ATP}, K_V, and K_{IR} channels. K_{Ca} channels are activated by intracellular calcium, they consist of small-conductance (SK_{Ca}), intermediate-conductance (IK_{Ca}), and big-conductance calcium-activated potassium channels (BK_{Ca}). SK_{Ca} and IK_{Ca} channels are more abundantly expressed in the endothelium,

but expressed poorly in the VSMCs, hence it has been claimed they are able to contribute to the endothelium-derived hyperpolarizing factor (EDHF) signals and react in the adjacent VSMCs, whereas the BK_{Ca} channels are expressed in virtually all VSMCs (Archer and Rusch, 2001, Ding et al., 2002, Eichler et al., 2003, Jakala et al., 2009, Marchenko and Sage, 1996). In VSMCs, the elevated levels of intracellular calcium cause the opening of BK_{Ca} channels which in turn allow potassium to flow out of the cell. This cause further hyperpolarization and closing of the voltage gated calcium channels (Schumacher et al., 2001).

K_{ATP} channels are inhibited by intracellular ATP and are therefore likely to have a very low open probability in VSMCs under normal conditions, however the increasing intracellular cAMP levels may activate K_{ATP} channels (Sobey, 2001). The opening of these channels can cause membrane hyperpolarization and lead to vasorelaxation (Jakala et al., 2009, Standen et al., 1989).

There are two more potassium channels which are important based on their functional roles in the regulation of membrane potential, which are K_V channels and K_{IR} channels. They are less significant when compared to K_{CA} channels and K_{ATP} channels because their functional roles are strictly regulated through the membrane potential (Loh et al., 2016). K_V channels are sensitive to voltage changes in the cell's membrane potential and serve an important buffering function against depolarization and vasoconstriction (Nelson and Quayle, 1995), whereas K_{IR} channels are activated when hyperpolarization occur, they conduct potassium current into cells and push the membrane potential back to the resting potential (Sobey, 2001).

The overall signaling pathways that mentioned above are summarized in Figure 1.2.

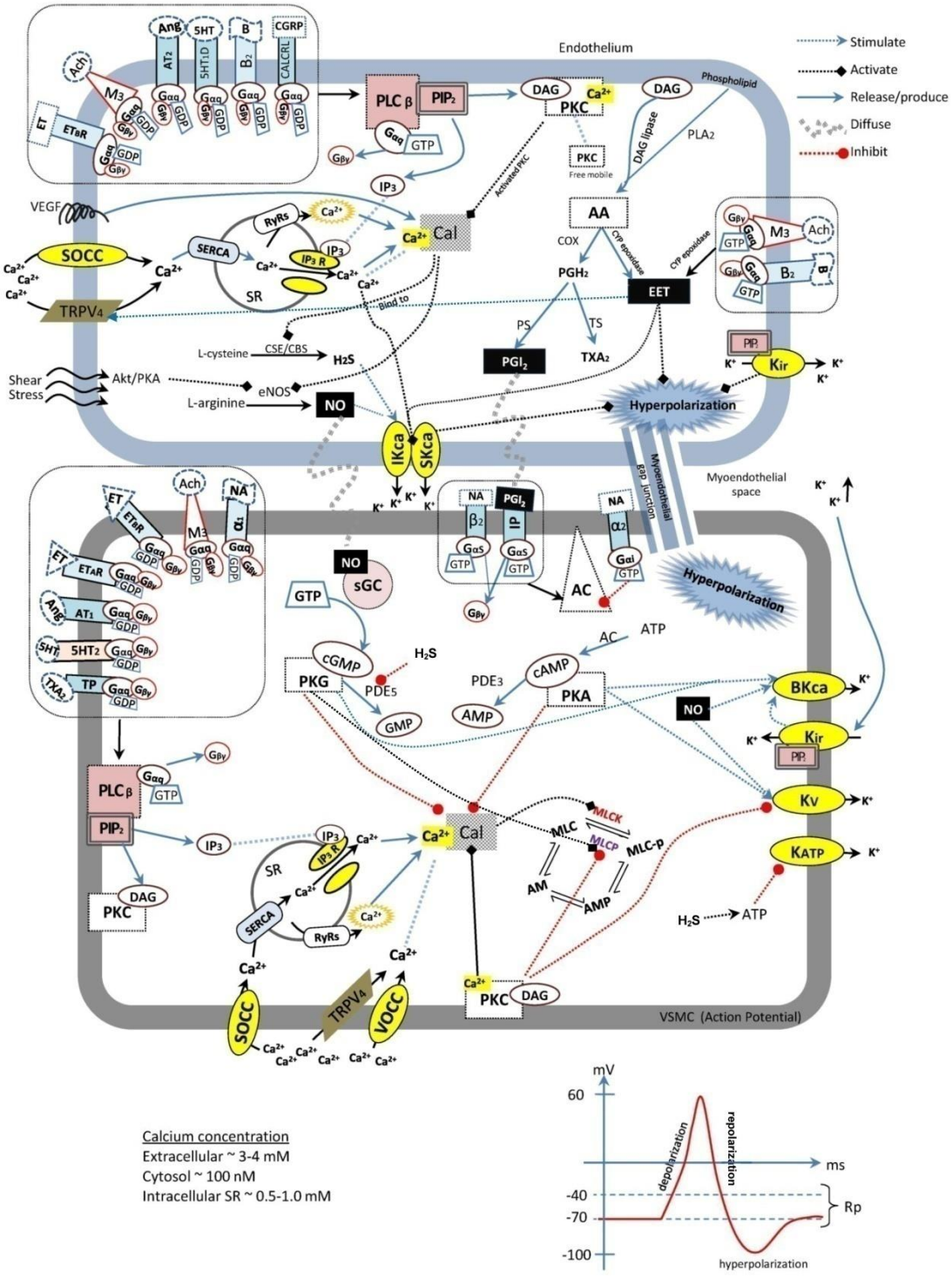

Figure 1.2 Signaling pathways involved in vascular tone regulation.

1.4 Problem statement

According to Hypertension Clinical Practice Guidelines of Malaysia, thiazide diuretics, β-blockers, CCBs, ACEIs, and ARBs were selected as first line monotherapy agents, but their adverse effects (constipation, dehydration, erection problem, dizziness, fatigue, joint pain, and stomach upset) and low effectivity were always being reported (Grimm et al., 1997, Husserl and Messerli, 1981, MOH, 2013). Therefore, the discovery of new drugs of employing multiple vasorelaxation-mediated signaling mechanism pathways is highly desired. Thus, the vasorelaxant effect of the potential antihypertensive herbal extracts were investigated in this study, since the extracts may contain a lot of active ingredients which can undergo multiple vasorelaxation-mediated signaling pathways and decrease the time taken to achieve the targeted blood pressure with less concomitant adverse effects.

In this study, the pharmacological activities of the above-mentioned herbal extracts (water extract, 50% ethanol extract, and 95% ethanol extract) were investigated in terms of their vasorelaxant activities through *in vitro* aortic ring assay. After that, the medicinal potential and mechanism pathways utilized by the potential herbs or extracts to induce vasorelaxant effect were also investigated through *in vitro* aortic ring assay, followed by *in vivo* antihypertensive study by oral administration to the spontaneous hypertensive rats (SHRs) for 28 days consecutively in different dosages along with toxicology study. The crude herbal extracts were expected to exert stronger vasorelaxant effect using holistic mechanism of actions compared to the monotherapy agents, as well as to exhibit a strong antihypertensive effect in *in vivo* whilst without causing adverse effect in animal models.

1.5 Objectives

i. To evaluate the vasorelaxant effect of *Clinacanthus nutans* Lindau, *Strobilanthes cripus*, *Murdannia bacteata*, *Elephantopus scaber* Linn., *Pereskia bleo*, *Pereskia grandifolia* Haw., *Vernonia amygdalina*, and *Swietenia macrophylla* King seeds extracts using *in vitro* isolated rat aortic ring model.

ii. To determine the potential extracts through *in vitro* isolated rat aortic ring model.

iii. To investigate the mechanism actions of potential extracts using *in vitro* isolated rat aortic ring models.

iv. To determine the antihypertensive and toxicity effects of the most potential extract in SHRs using *in vivo* model.

CHAPTER 2

TRI-STEP FTIR ANALYSIS OF RAW HERBS

2.1 Introduction

Presently, the fingerprint of herbs or their extracts can theoretically be developed using ultraviolet (UV), thin layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC), tandem instrumentation of liquid chromatography with mass spectrometry (LC/MS), capillary electrophoresis (CE), and deoxyribonucleic acid (DNA) chip technology (Li et al., 2010, Liang et al., 2004, Zhang et al., 2010). Nevertheless, each of them has advantages and limitations. For example, TLC is normally developed in an open system or in open condition hence results may be affected by environment factors such as humidity and temperature, which may have low reproducibility, and may be repeated only if all the factors are well standardized (Lei et al., 2010, Liang et al., 2004). Although, the above-mentioned methods can give the accurate quantity of some chemical components, but the content can only be identified with matching marker compounds (Liang et al., 2004). Furthermore, in current approaches, active or analytical markers are used for determination of specific constituents but not holistically applicable for authentication or as fingerprint. It is obviously necessary to find an analytical method that can account for thousands of components which defines the whole fingerprint of a herb or extract.

In recent years, a tri-step infrared spectroscopy method consisting of conventional Fourier transform infrared (FTIR), second derivative infrared spectroscopy (SD-IR) and two-dimensional correlation infrared spectroscopy (2D-IR)

was proposed and applied specifically for analyzing complex mixture systems (Liu et al., 2008). FTIR spectroscopy is a simple, rapid, low cost, repeatable and reproducible methodology, and is non-destructive, preserving precious samples. Moreover, various types of samples such as herbs pieces, powder, and extracts can be directly analyzed through FTIR, and the full chemical property in the analyte can be revealed and shown on FTIR spectrum. Although herbs contain large number of chemical constituents, their FTIR spectra are distinguishably different owing to the variety in the volume and variety of chemical components (Li et al., 2014).

In this study, all the collected raw herbs were identified through both biological and chemical ways. In biological way, the raw herbs were identified and authorized by a plant taxonomist, while in chemical way, the fingerprints of raw herbs were determined and characterized through tri-step FTIR method. The tri-step FTIR identification method by means of spectral resolution enhancement is used to progressively amplify the differences between the spectra in order to achieve a distinct identification of the sample. Conventional FTIR is used for a basic discrimination between the herbs and constitutes as a “primary identification”. SD-IR is used to amplify the differences obtained by using conventional FTIR spectroscopy with higher resolving power and constitutes a “secondary identification”. The 2D-IR method can obtain information on the dynamics of the sample under external perturbation and will develop the classic spectrum in the second dimension. It has a higher resolution capability and constitutes a “tertiary identification” (Sun et al., 2010).

By using the tri-step FTIR identification method, a high-quality IR spectra was obtained based on the use of the SD-IR method to increase the apparent resolution of the spectrum. The enhanced expression of sample information obtained

by using the 2D-IR method was used to distinguish between similar samples. This method was also able to verify the presence of selected components within the sample herbs.

2.2 Materials and methods

2.2.1 Materials

Potassium bromide (KBr) was purchased from Merck (Darmstadt, Germany).

2.2.2 Biological identification of herbs

The raw aerial parts (including leaves, young stems, twigs, and flowers or fruits) of *Clinacanthus nutans* Lindau, *Strobilanthes cripus*, *Murdannia bacteata*, *Elephantopus scaber Linn.*, *Pereskia bleo*, *Pereskia grandifolia* Haw., *Vernonia amygdalina*, and *Swietenia macrophylla* King were collected from the area of Paya Terubong, Penang, Malaysia and were identified by Mr. Vellosamy Shunmugam, a plant taxonomist from the School of Biological Sciences, Universiti Sains Malaysia (Zakaria et al., 2016).

After identification, the specimens for each herb were ready for preparation. Firstly the fresh aerial part of herbs were sandwiched in between newspaper sheets and dried in an oven at 38°C for one week. Next, each dried specimen was sewn onto pieces of white paper and notes about the plant was written on the lower right of the paper. After that, the specimens of *C. nutans*, *S. cripus*, *M. bacteata*, *E. scaber*, *P. bleo*, *P. grandifolia*, *V. amygdalina*, and *S. macrophylla* were authenticated by Dr. Rahmad Zakaria and the voucher specimens of these herbs with the references no. of 11700, 11701, 11702, 11703, 11704, 11705, 11706, and 11707, respectively were

deposited in the Herbarium of the School of Biological Sciences, USM (Appendix A – H).

2.2.3 Tri-step FTIR identification method

A spectrum 400 FTIR spectrometer (Perkin-Elmer) equipped with a Deuterated Tri-Glycine Sulfate (DTGS) detector was used to characterize the herbs. A tablet of KBr was used as the background. The dried leaves of *C. nutans*, *S. cripus*, *M. bacteata*, *E. scaber*, *P. bleo*, *P. grandifolia*, *V. amygdalina*, and dried seeds of *S. macrophylla* were pulverized and passed through a 200-mesh sieve. Each sample powder (about 1 – 2 mg) was mixed evenly with 100 mg of crystalline KBr. The mixture was ground and pressed into a tablet at a pressure of not more than 10 psi for two minutes. After that, the spectra were calculated from a total of 16 co-added scans in the range of 400 – 4000 cm^{-1} with a resolution of 4 cm^{-1}. The interferences of water and carbon dioxide were eliminated online when scanning. The raw FTIR data were then processed by using the spectrum software of the Perkin-Elmer FTIR spectrometer (version 6.3.5). The FTIR spectrum was generally accepted when a transmission higher than 60% was achieved. Otherwise, the test was repeated with either the sample powder or KBr added (Choong et al., 2014).

All the SD-IR spectra were obtained after Savitzky-Golay polynomial fitting (13-point smoothing) of the original IR spectra taken at room temperature. To obtain the 2D-IR spectrum, the sample tablet was placed into the sample pool with a programmable heated jacket controller (Model GS20730, Specac). In order to avoid the loss of or change in some of the unstable compositions, the dynamic spectra were collected at different temperatures ranging from room temperature to 120°C at intervals of 10 ± 2°C (with a rate of increase of 2°C/min). The full temperature scan

took a total time of 50 minutes for each sample. Then, the 2D-IR correlation spectra were obtained by treating the series of dynamic spectra with the 2D-IR correlation analysis software developed by Tsinghua University, Beijing, China (Li et al., 2014, Li et al., 2010).

2.3 Results and discussion

The conventional FTIR spectra of the herbs are shown in Figure 2.1, and their characteristic absorption peaks are assigned in Table 2.1. Their SD-IR spectra and 2D-IR spectra are shown in Figure 2.2 and Figure 2.3, respectively.

2.3.1 Assignments and comparison by using conventional FTIR spectra

Figure 2.1 shows the conventional FTIR spectra of the leaves of *C. nutans*, *S. cripus*, *M. bacteata*, *E. scaber*, *P. bleo*, *P. grandifolia*, *V. amygdalina*, and seeds of *S. macrophylla*. The peaks that appeared at 3400 – 3300 cm^{-1} are O-H stretching vibration absorption peaks. The peaks nearby at 2921 cm^{-1} and 2851 cm^{-1}, are methylene C-H asymmetric and symmetric stretching vibration absorption peaks, respectively. The peak at 1735 cm^{-1} is the carbonyl group C=O stretching vibration absorption peak. The peaks nearby at 1640 cm^{-1} contain O-H bending vibration absorption and conjugated carbonyl stretching vibration absorption peaks. The absorption peaks within the 1430 – 1400 cm^{-1} region contain a variety of ingredients, such as C-H bending vibration absorption and (O)C-H stretching vibration peaks, while the peaks in the range of 1300 – 950 cm^{-1} are the absorption peaks for all types of C-O stretching (see Table 2.1).

In Figure 2.1, all the spectra of the herbs, except that for *S. macrophylla* seed, are very similar. An obvious difference between them lies in the intensities of the absorption peaks in the range of 1200 – 950 cm^{-1}, corresponding to the peak intensity nearby at 1640 cm^{-1}. The absorption peaks of *S. cripus*, *M. bacteata*, and *E. scaber* (Figure 2.1b, 2.1c, and 2.1d) in the range of 1200 – 950 cm^{-1} have higher intensities than the absorption peaks at 1640 cm^{-1}, in contrast to the peak intensities of other herbs. This indicates that *S. cripus*, *M. bacteata*, and *E. scaber* have a higher content of saccharides or sugar than the other herbs. In the spectra of *C. nutans* and *P. bleo* (Figure 2.1a and 2.1e), both the 1649 cm^{-1} and 1549 cm^{-1} absorption peaks appears simultaneously in their spectra. The shapes of these two absorption peaks correspond to those of the amide I and amide II proteins, indicating that these two herbs contain a certain amount of protein. In addition, the spectra of *P. bleo* and *P. grandifolia* (Figure 2.1e and 2.1f) record the appearance of some absorption peaks at 1318 cm^{-1}, 780 cm^{-1}, and 514(515) cm^{-1}, which are due to the symmetrical stretching vibration and bending vibration absorption of the C-O bond of oxalate ions. This indicates that *P. bleo* and *P. grandifolia* contain a certain amount of calcium oxalate.

The spectrum of *S. macrophylla* seed is completely different from the spectra of the other herbs. This is because seed has higher content of plant oil and show obvious characteristic peaks in its IR spectrum. The molecule of oil is a long-chain fatty acid ester. The main functional groups contained in the oil are a methylene group and an ester carbonyl. The spectrum of *S. macrophylla* seed (Figure 2.1h) shows more C=C double bonds due to the fact that the plant oil contains more unsaturated fatty acid. Therefore, a significant alkene C-H stretching absorption peak (3009 cm^{-1}) is observed in its spectrum. The peak at 2925 cm^{-1} is the absorption peak due to the methylene C-H asymmetric stretching vibration, and the peak at 2854 cm^{-1}

is the absorption peak due to the methylene C-H symmetric stretching vibration. In addition, the peak at 1746 cm^{-1} is the absorption peak for the ester carbonyl C=O stretching vibration while the peak appearing at 722 cm^{-1} (C-H bending vibration) indicates that *S. macrophylla* seed contains long-chain carbons ($^{n}C > 4$).

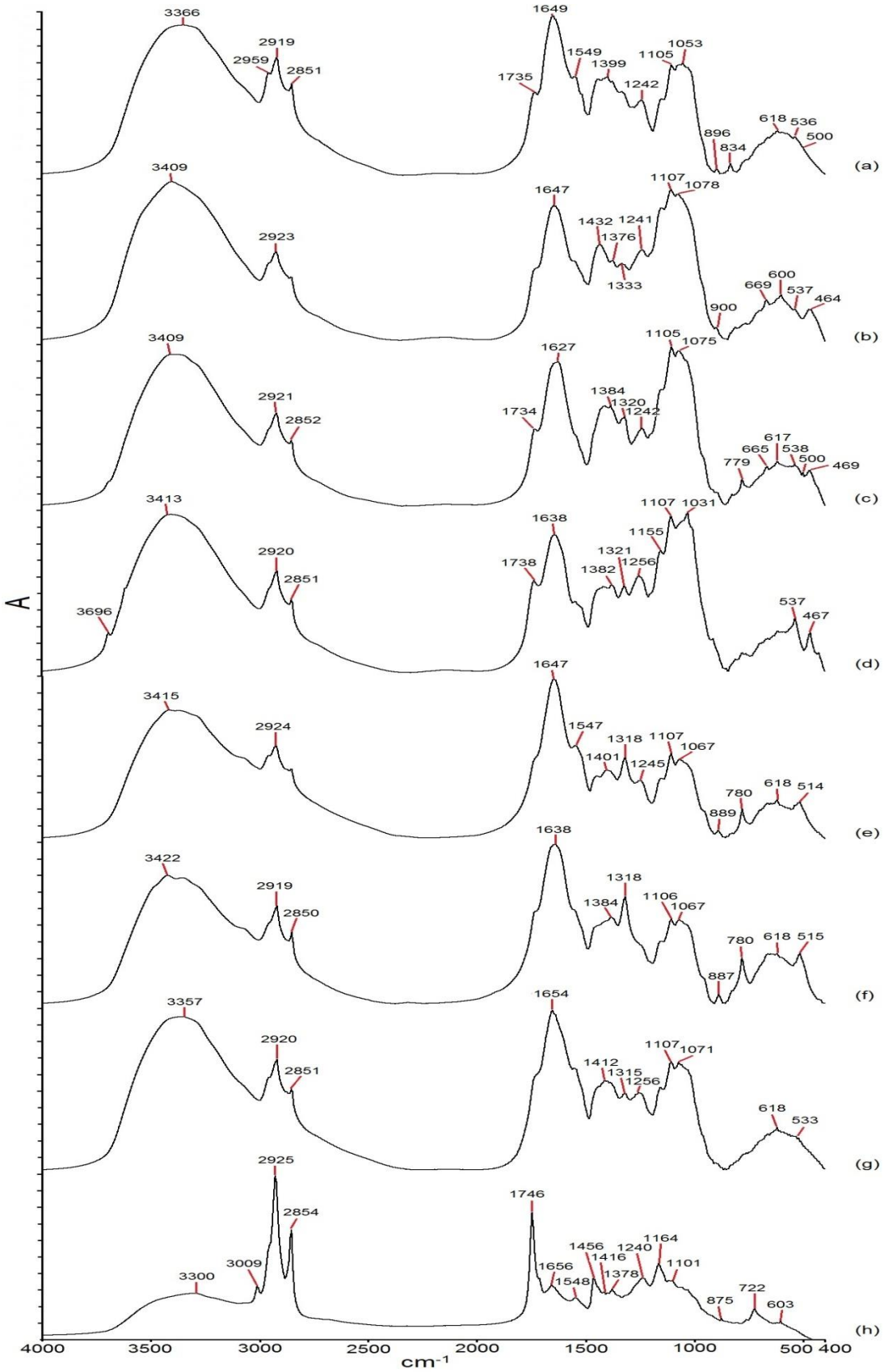

Figure 2.1 Conventional FTIR spectra of (a) *C. nutans*, (b) *S. cripus*, (c) *M. bacteata*, (d) *E. scaber*, (e) *P. bleo*, (f) *P. grandifolia*, (g) *V. amygdalina*, and (h) *S. macrophylla* seed. Notes: A, absorbance

Table2.1 Peak assignments on the conventional FTIR spectra of herbs

Peak (cm^{-1})								**Primary assignment**	**Possible compounds**
C. nutans	***S. crispus***	***M. bracteata***	***E. scaber***	***P. bleo***	***P. grandifolia***	***V. amygdalina***	***S. macrophylla***		
-	-	-	3696	-	-	-	-	O-H, ν	Various
3366	3409	3409	3413	3415	3422	3357	3300	O-H, ν	Various
-	-	-	-	-	-	-	3009	C-H, ν	Alkene
2959	-	-	-	-	-	-	-	C-H, ν_{as}	Various
2919	2923	2921	2920	2924	2919	2920	2925	C-H, ν_{as}	Various
2851	-	2852	2851	-	2850	2851	2854	C-H, ν_{s}	Various
1735	1734	1738	-	-	-	-	1746	C=O, ν	Ester
1649	-	-	-	1647	-	-	1656	N-H, δ	Amide I
-	1647	1627	1638	-	1638	1654	-	O-H, δ	Saccharides
1549	-	-	-	1547	-	-	1548	N-H, δ	Amide II
-	-	-	-	-	-	-	1465	Ring	Aromatic
-	1432	-	-	-	-	1412	1416	C-H, δ	Saccharides
1399	-	-	-	1401	-	-	-	(O) C-H, ν	Saccharides
-	1376	1384	1382	-	1384	-	1378	(O) C-H, δ	Ester
-	1333	1320	1321	-	-	1315	-	(O) C-H, δ	Saccharides
-	-	-	-	1318	1318	-	-	C-O, ν_{s}	Calcium oxalate
1242	1241	1242	1256	1245	-	1256	1240	C-O, ν	Ester, Glycoside
-	-	-	1155	-	-	-	1164	C-O, ν	Saccharides
1105	1107	1105	1107	1107	1106	1107	1101	C-O, ν	Saccharides
1053	1078	1075	1031	1067	1067	1071	-	C-O, ν	Saccharides

Notes: ν, stretching; ν_{s}, symmetrical stretching; ν_{as}, asymmetrical stretching; δ, bending.

2.3.2 Comparison and discussion of the SD-IR spectra

The SD-IR spectra can resolve the problem of overlapping peaks, enhance the spectral characteristics, and improve the apparent resolution in the IR spectrum. Figure 2.2 shows the SD-IR spectra of the herbs within the wave-number range of 1800 – 800 cm^{-1}, which contains the main absorption bands of the chemical constituents of the herbs. As shown in the figure, some relatively weak absorption peaks in the conventional IR spectra can be seen more clearly in the SD-IR spectra (Sun et al., 2010).

In Figure 2.2, the intensities of the peak around 1740 cm^{-1} (carbonyl C=O stretching vibration) among these eight herbs are not the same. The strongest peak is from *S. macrophylla* seed (Figure 2.2h), indicating that it has the highest content of ester compounds. The appearance of the absorption peaks at 1165, 1078, 989, 952, 890, and 826 cm^{-1} in the spectrum of *M. bacteata* (Figure 2.2c) can be ascribed to the herb contains more stachyose. In the SD-IR spectra of *P. bleo* and *P. grandifolia* (Figure 2.2e and 2.2f), the absorption peaks at 1317 cm^{-1} are very strong, once again showing that they have a high content of calcium oxalate.

In the SD-IR spectrum of *C. nutans* (Figure 2.2a), the absorption peaks at 1739, 1715, 1635 (C=O stretching vibration), 1577, 1540, 1516, 1496 (aromatic ring skeleton vibration), 1472, 1375 (C-H bending vibration), and 1282 cm^{-1} (C-O stretching vibration) are stronger, which indicates that it contains more flavonoids. *S. crispus*, *V. amygdalina*, and *S. macrophylla* seed (Figure 2.2b, 2.2g, and 2.2h) also have absorption peaks due to C=O stretching (around 1740 cm^{-1} and 1660 cm^{-1}), aromatic ring skeleton vibration (around 1545 cm^{-1}), and C-H bending vibration (around 1470 cm^{-1}) in their spectra; therefore, they also contain flavonoids. The