ANTIHYPERTENSIVE PROPERTIES OF STANDARDISED *ORTHOSIPHON STAMINEUS* BENTH. LEAVES EXTRACTS AND ITS NANO LIPOSOMES IN SPONTANEOUS HYPERTENSIVE RATS

by

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"If you want your life to be a magnificent story, then begin by realizing that you are the author and everyday you have the opportunity to write a new page."

- Mark Houlahan

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LIST OF ABREVIATIONS

A	Pre-exponential factor
AAS	Atomic absorption spectroscopy
ACE	Angiotensin converting enzyme
ACE-I	Angiotensin converting enzyme inhibitor
$AlCl_3$	Aluminium chloride
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
A.P.T.T	Activated partial thromboplastin time
As	Arsenic
AST	Aspartate aminotransferase
ARBs	Angiotensin II receptor blockers
AT-I	Angiotensin I
AT-II	Angiotensin II
ATR	Attenuated total reflection
AT-R1	Angiotensin II type I receptor
ATRs	Angiotensin receptors
AUC	Area under plasma concentration-time curve
BSA	Bovine serum albumin
C	Concentration
Cd	Cadmium
CL	Clearance
C_{max}	Maximum concentration
CMC	Carboxymethyl cellulose
CO_2	Carbon dioxide

DAD	Diode array detector
DBP	Diastolic blood pressure
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1- picrylhydrazil
Ea	Activation energy
EA-1	RNA isolated from EAHY 926 cells treated with *Orthosiphon stamineus* ethanolic extract
EA-2	RNA isolated from EAHY 926 cells treated with nano liposomes of *Orthosiphon stamineus* ethanolic extract
EAHY 926	Human umbilical vein Cell
EA-NC	RNA isolated from EAHY 926 cells treated with PBS
EC_{50}	Half maximal effective concentration
ECGS	Endothelial cell growth supplement
ECM	Endothelial cell medium
EDTA	Ethylenediaminetetraacetic acid
EUP	Eupatorin
FBS	Fibrinogen and fetal bovine serum
FT-IR	Fourier transform infra-red
FT-NIR	Fourier transform near-infrared
g	Relative centrifugal force or g-force
g	Gram
g/kg	Gram per kilogram
GGT	Gamma-glutamyl transferase
h	Hour
H_3PO_4	Orthosphoshoric acid
HA	Hippuric acid

Hb	Hemoglobin
HCA	Hierarchical clustering analysis
HCl	Hydrochloric acid
HDL	High-density lipoproteins
Hg	Mercury
HHL	Hippuryl-histidyl-leucine
HI FBS	Heat inactivated foetal bovine serum
HL	Histidyl-leucine
HNO_3	Nitric acid
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
HR	Heart rate
HR-1	RNA isolated from HRGEC treated with *Orthosiphon stamineus* ethanolic extract
HR-2	RNA isolated from HRGEC treated with nano liposomes of *Orthosiphon stamineus* ethanolic extract
HRGEC	Human renal glomerular endothelial cell
HR-NC	RNA isolated from HRGEC treated with PBS
HUVEC	Human umbilical vein endothelial cell
IC_{50}	Half maximal inhibitory concentration
ICH	International conference on harmonization
IV	Intravenous
J	Joule
kb	kilobases
KBr	Potassium bromide
KCl	Potassium chloride
K_e	Elimination rate constant
L	Litre

LD_{50}	Lethal dose
LOD	Limit of detection
log	Logarithm
LOQ	Limit of quantification
LPS	Lipopolysaccharide
LSD	Least significant difference
M	Molar
MAP	Mean arterial pressure
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MDA	Malondialdehyde
MDA-MB-231	Human hormone resistant breast cancer cell line
mg	Milligram
mg/g	Milligram per gram
mg/kg	Milligram per kilogram
MIC	Minimum inhibitory concentration
min	Minutes
miRNA	Micro ribonucleic acid
mL	Millilitre
MLT	Microbial limit test
mm	Millimetre
mM	Mill molar
mm^3	Cubic millimetre
MRC	Methylripariochromene A
mRNA	Messenger ribonucleic acid

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
mU	Milli unit
mV	Milli volt
n	Number of sample
N	Normal
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
ng	Nanogram
nm	Nanometre
NO	Nitric oxide
OECD	Organisation for economic cooperation and development
OPA	*O*-Phthaladehyde
OS	*Orthosiphon stamineus*
OS-E	*Orthosiphon stamineus* ethanolic extract
OS-EL	Nano liposomes of *Orthosiphon stamineus* ethanolic extract
OS-EW	*Orthosiphon stamineus* 50% ethanolic extract
OS-M	*Orthosiphon stamineus* methanolic extract
OS-MW	*Orthosiphon stamineus* 50% methanolic extract
OS-W	*Orthosiphon stamineus* water extract
Pb	lead
PBS	Phosphate buffered saline
PCA	Principle component analysis
PCR	Polymerase chain reaction
PCV	Packed cell volume
PDI	polydispersity index
PE	Plating efficiency

pH	Power of hydrogen
Plt	Platelet count
PP	Pulse pressure
ppm	Part per million
PS	Penicillin/streptomycin
PT/I.N.R	Prothrombin time and international normalized ratio
R^2	Regression correlation coefficient
RA	Rosmarinic acid
RAAS	Rennin-angiotensin-aldosterone-system
RBC	Red blood cells
RDW	Red cell distribution width
RH	Relative humidity
RIN	RNA integrity number
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
rpm	Round per minutes
RSD	Relative standard deviation
RVSEB	Rappaport vassiliadis salmonella enrichment broth
S.E.M	Standard error of the mean
S/N	Signal to noise ratio
SbGTT	Subcutaneous glucose tolerance test
SBP	Systolic blood pressure
SD	Standard deviation
SD	Sprague–Dawley rats
Sec	Second
SGOT	Glutamate oxaloacetate transaminase
SGPT	Glutamate pyruvic transaminase

SHR	Spontaneously hypertensive rats
SHRSP	Stroke-prone spontaneously hypertensive rats
SIN	Sinensetin
SPL	Soybean phospholipid
$t_{1/2}$	Half-life
t_{90}	Shelf life
TEM	Transmission electron microscopy
T_{max}	Time to reach to maximum concentration
TMF	3'-hydroxy-5,6,7,4'-tetramethoxyflavone
TMM	Tetramethylmurexide
UV/Vis	Ultraviolet-visible
v/v	Volume per volume
V_d	Volume of distribution
WBC	White blood cells
WHO	Word health organization
WKY	Normotensive Wistar Kyoto
Zn	Zinc
$ZnCl_2$	Zinc chloride

LIST OF SYMBOLS

ΔG	Gibbs free energy
λ_{max}	Lambda max or maximum absorption
µg	Micro gram
µg/mL	Micro gram per millilitre
µL	Microliter
µm	Micrometre
^{o}C	Degree Celsius
^{o}T	Temperature in kelvin
%	Percent

CIRI-CIRI ANTIHYPERTENSIF EKSTRAK TERPIAWAI DAUN *ORTHOSIPHON STAMINEUS* BENTH. DAN NANO LIPOSOMNYA TERHADAP TIKUS HYPERTENSIF SPONTAN

ABSTRAK

Kajian ini dijalankan untuk memenuhi jurang antara amalan herba peribumi dan sains perubatan kontemporari ke atas kesan antihipertensi daun *Orthosiphon stamineus* (OS). Kajian kualiti dan keselamatan bahan mentah tumbuhan diperiksa menggunakan analisis gravimetrik dan ujian had mikrob (MLT). Daun OS didapati memenuhi kualiti dari segi fizikokimia serta pencemaran mikrob. Analisis spektroskopi kualitatif (UV, FT-IR, FT-NIR), kromatografi (HPTLC) dan kromatografi kuantitatif (HPLC) telah dijalankan terhadap ekstrak OS yang berlainan untuk pemiawaian. Hasil kajian menunjukkan bahawa bahan kimia utama dalam ekstrak OS adalah fenolik dan flavonoid seperti asid rosmarinik (RA), 3-hidroksi-5,6,7,4-metoksiflavon (TMF), sinensetin (SIN) dan eupatorin (EUP). Tambahan lagi, teknik HPLC-DAD gradien yang digabungkan dengan pengekstrakan fasa pepejal telah dibangunkan dan disahkan untuk pengenalpastian dan pengkuantitian 17 asid amino bebas dalam ekstrak OS. Hasil kajian menunjukkan bahawa asid L-aspartik dan asid L-glutamik adalah asid amino bebas utama dalam ekstrak OS dengan 0.93 ± 0.01 nmol/mg dan 4.01 ± 0.12 nmol/mg. Metabolit primer dan sekunder ekstrak OS telah dianalisis untuk menentukan jumlah flavonoid, polifenol, fosfolipid, protein, polisakarida dan glikosaponin. Perbezaan peratusan metabolit ini dalam setiap ekstrak telah ditunjukkan. Ekstrak OS yang berlainan dan sebatian piawai (RA, TMF, SIN dan EUP) telah dinilai dalam assai perencatan enzim penukaran angiotensin (ACE-I) secara *in vitro*. Hasil kajian menunjukkan bahawa OS-E dan EUP, pada kepekatan 50

µg/mL mempamerkan perencatan tertinggi (masing-masing pada 52.67 ± 0.89 dan 73.11 ± 2.39%) terhadap ACE berbanding dengan ekstrak dan sebatian piawai lain. Captopril telah digunakan sebagai kawalan positif dan menunjukkan perencatan sebanyak 86.14 ± 2.98% pada kepekatan 6.8 ng/mL. Keupayaan mengkelat Zn^{2+} oleh RA, TMF, SIN, EUP, captopril dan OS-E telah dijalankan menggunakan reagen tetrametilmureksida (TMM). Hasil kajian menunjukkan OS-E dan captopril mempunyai keupayaan tinggi (79.42 ± 1.91 dan 100 ± 1.59) untuk mengikat dengan Zn^{2+} pada kepekatan 5 mg/mL. Antara sebatian piawai yang diuji, EUP menunjukkan keupayaan tertinggi dalam mengikat Zn^{2+} (56.03 ± 1.26%) pada kepekatan 5 mg/mL. Tambahan lagi, skor cantuman dan afiniti ikatan bagi sebatian penanda Zn^{2+} pada ACE telah dinilai. Hasil kajian menunjukkan bahawa EUP mempunyai tenaga afiniti pengikatan (ΔG) dan kecekapan ligan tertinggi dengan -6.93 kcal/mol. Seterusnya, ekstrak OS-E telah disediakan dalam formulasi liposom menggunakan lesitin soya nyah minyak (OS-EL) dan dicirikan melawan parameter berbeza. OS-EL dicirikan melawan parameter yang berbeza. Hasil kajian menunjukkan penghasilan liposom nano daripada OS-E. Farmakokinetik dan bioavailabiliti oral bagi RA, TMF, SIN dan EUP dalam OS-E dan OS-EL dikaji ke atas tikus Sprague Dawley (SD) menunjukkan peningkatan signifikan dalam keterlarutan akueus dan bioavailabiliti oral bagi RA, TMF, SIN dan EUP dengan masing-masing pada 70.64 ± 3.87, 66.26 ± 5.95, 76.61 ± 3.99 and 81.39 ± 2.46% dalam OS-EL berbanding OS-E. OS-E dan OS-EL kemudiannya dikaji untuk aktiviti antihipertensi *in vivo* ke atas tikus SHR pada dos 250 mg/kg/hari selama 28 hari. Captopril digunakan sebagai kawalan positif pada dos 5 mg/kg/hari. Selain itu, aktiviti ACE-I ekstrak-ekstrak ini dalam plasma dan tisu SHR yang berbeza juga diukur selepas 28 hari rawatan melalui kaedah HPLC-UV yang dibangunkan. Keputusan menunjukkan bahawa, kedua-dua OS-E dan OS-EL mampu

menurunkan SBP (-23.08 ± 5.16 dan -28.96 ± 6.65) dan aktiviti ACE dalam plasma, aorta, jantung, paru-paru dan buah pinggang SHR secara signifikan. Walaubagaimanapun, OS-EL menunjukkan kesan antihipertensi yang lebih kuat (dan aktiviti penindasan ACE) berbanding OS-E. Kajian kestabilan dipercepatkan bagi OS-E dan OS-EL yang disimpan pada empat suhu yang berbeza (30, 40, 50 dan 60 ° C) selama enam bulan telah dilakukan dengan menggunakan HPLC dan spektroskopi FT-IR yang digabungkan dengan pendekatan kemometrik. Berdasarkan hasil kajian, sebatian penanda (RA, TMF, SIN dan EUP) dalam OS-E dan OS-EL adalah lebih stabil pada suhu rendah (30°C dan ke bawah). Kajian genotoksisiti akut dan ketoksikan subkronik OS-EL menunjukkan pengambilan OS-EL tidak menyebabkan kematian, tanda-tanda keracunan atau fungsi fisiologi dalam kedua-dua jantina haiwan. Data yang diperolehi daripada analisis pengekspresan miRNA menunjukkan bahawa dua miRNA; hsa-miR-149-3p dan hsa-miR-21-3p telah diekspres secara berbeza (kawal selia menaik) dalam HRGEC yang dirawat dengan OS-E dan OS-EL. miR-149 dan miR-21 mampu mensasarkan gen ACE dan AGTR1 secara langsung dan bertindak sebagai pengatur gen negatif. Oleh itu, boleh dicadangkan bahawa, OS boleh bertindak sebagai perencat ACE (ACE-I) dan penghalang reseptor angiotensin (ARBs). Sebagai kesimpulan, kajian ini memberikan bukti ke atas aktiviti antihipertensi, hubungan struktur aktiviti bagi sebatian penanda, kualiti dan keselamatan ekstrak terpiawai OS.

ANTIHYPERTENSIVE PROPERTIES OF STANDARDISED *ORTHOSIPHON STAMINEUS* BENTH. LEAVES EXTRACTS AND ITS NANO LIPOSOMES IN SPONTANEOUS HYPERTENSIVE RATS

ABSTRACT

This study was conducted to fulfill gaps between indigenous herbal practices and contemporary medicinal sciences on antihypertensive effect of *Orthosiphon stamineus* (OS) leaves. Quality and safety of the plant raw material were examined using gravimetric analysis and microbial limit test (MLT). OS leaves were found to be qualified in terms of physicochemical properties as well as microbial contamination. Spectroscopic (UV, FT-IR, FT-NIR) and chromatographic (HPTLC and HPLC) analyses were carried out on different extracts of OS for standardisation. The results showed that the major chemical constituents in OS extracts are phenolics and flavonoids such as rosmarininc acid (RA), 3-hydroxy-5, 6, 7, 4-methoxyflavone (TMF), sinensetin (SIN) and eupatorin (EUP). Furthermore, a gradient HPLC-DAD combined with solid-phase extraction technique was developed and validated for identification and quantification of 17 free amino acids in OS extracts. The results demonstrated that L-aspartic acid with 0.93 ± 0.01 nmol/mg and L-glutamic acid with 4.01 ± 0.12 nmol/mg are the major free amino acid in OS extracts. Primary and secondary metabolites of OS extracts were analysed for their total flavonoids, polyphenols, phospholipids, proteins, polysaccharides and glycosaponins. The variation in the percentage of these metabolites in each extracts was indicated. Different extracts of OS and standard compounds (RA, TMF, SIN and EUP) were evaluated for *in vitro* assay of angiotensin converting enzyme inhibition (ACE-I). The results showed that, at final concentration of 50 µg/mL OS-E and EUP exhibit the

highest inhibition (52.67 ± 0.89 and 73.11 ± 2.39%, respectively) against ACE among extracts and tested standard compounds. Captopril was used as positive control and showed 86.14 ± 2.98% inhibition at concentration of 6.8 ng/mL. Chelation ability of Zn^{2+} by RA, TMF, SIN, EUP, captopril and OS-E was conducted using tetramethylmurexide (TMM) reagent. The results demonstrated that OS-E and captopril have a high ability (79.42 ± 1.91 and 100 ± 1.59%, respectively) to bind with Zn^{2+} at concentration of 5 mg/mL. Among the standard compounds, EUP shows the highest binding ability with Zn^{2+} (56.03 ± 1.26 %) at concentration of 5 mg/mL. In addition, the docking scores and binding affinities of marker compounds with Zn^{2+} in ACE were evaluated. The results showed that EUP has the highest binding affinity energy (ΔG) and ligand efficiency with -6.93 kcal/mol. Subsequently, OS-E extract was prepared in liposomal formulation using deoiled soya lecithin (OS-EL) and characterized versus different parameters. The results revealed production of nano liposomes of OS-E. The pharmacokinetics and oral bioavailability of RA, TMF, SIN and EUP in OS-E and OS-EL on Sprague Dawley (SD) rats indicated significant improvement in aqueous solubility and oral bioavailability of RA, TMF, SIN and EUP with 70.64 ± 3.87, 66.26 ± 5.95, 76.61 ± 3.99 and 81.39 ± 2.46%, respectively in OS-EL compared to OS-E. OS-E and OS-EL were then studied for their *in vivo* antihypertensive activity on Spontaneous Hypertensive Rats (SHR) at dose of 250 mg/kg/day for 28 days. Captopril was used as positive control at dose of 5 mg/kg/day. Moreover, ACE-I activity of these extracts in plasma and different tissues of SHR after 28 days treatment was also measured using developed HPLC-UV method. The results demonstrated that both OS-E and OS-EL were able to reduce SBP (-23.08 ± 5.16 and -28.96 ± 6.65) and ACE activity in plasma, aorta, heart, lung and kidney of SHR significantly. However, OS-EL showed stronger antihypertensive effect (and

suppression of ACE activity) than OS-E. The accelerated stability studies of OS-E and OS-EL stored at four different temperatures (30, 40, 50 and 60°C) for six months were performed using HPLC and FT-IR spectroscopy combined with chemometric approach. Based on the study findings, the marker compounds (RA, TMF, SIN and EUP) in both OS-E and OS-EL were more stable at lower temperature (30°C and below). The genotoxicity, acute and repeated dose oral toxicity study of OS-EL showed that administration of OS-EL does not cause death, visible signs of toxicity or other physiological functions in any animals of both sexes. The data obtained from miRNA expression analysis revealed that two miRNA; hsa-miR-149-3p and hsa-miR-21-3p are expressed differently (up regulated) in HRGEC treated with OS-E and OS-EL. miR-149 and miR-21 are able to directly target ACE and AGTR1 genes, respectively and act as negative gene regulators. It is suggested that OS could act ac ACE inhibitor (ACE-I) and angiotensin receptor blockers (ARBs). In conclusion, the current study provides evidence on antihypertensive activity, structure activity relationship of the active marker compounds, quality and safety of the standardised OS extract.

CHAPTER 1
INTRODUCTION

1.1 Herbal Medicines and Hypertension

Herbal products as sources of medicine have been used for many years practically in all cultures. Consumption of herbal products has been increased significantly over the last decade (Ekor, 2014). This is probably because herbs are remarked as natural and therefore they are safe to use. Plants as an important source of new drugs development are still used, despite the extensive developments in synthetic chemistry. Several well-known medicines derived from plants include L-hyoscyamine, morphine, colchincine, taxol anddigitoxin. Traditional medicines and herbal products for the primary health care are used by more than 80% of world population and mostly in developing countries (Hussain et al., 2009). In a period of 1983-1994 in North America, about 40% of the new drugs were derived from natural compounds (Simmonds, 2003). Interestingly, over 70% of the new reported chemical in 1981-2006 were from the study of natural products (Newman and Cragg, 2007). Promoting and encouraging of the use of herbal products and remedies are recommended by World Health Organization (WHO) in the National Health Care Program (Hussain et al., 2009).

Herbal products refer to organic chemicals that might come from a single plant or combination of more than one plant, from any raw or processed part of plant such as stems, leaves, roots, flowers and seeds. However, if they are combined with synthetic chemicals or other active substances, they are not considered as herbal medicines (WHO, 1996). Due to the presence of molecules and products that combat diseases in plants, they have played an important role in maintaining health. Many

products derived from plants in the form of dried plant materials, fresh or extracts are used as folk remedies. Different compounds derived from plant can be used as starting material for preparation of novel synthetic drugs. The potential of herbs as a source of new drugs has not been explored yet and only a limited number of plant species among 250,000 have been investigated for their bioactive compounds (Borchardt, 2002)

Traditional medicine has a number of proven benefits for prevention and cure of different ailments. Application of modern medicines along with traditional medicines made a strong comeback of herbs in many countries in the last decade (WHO, 1998). Appreciation of natural remedies might be due to the increment of the cost of treatment with modern medicines and fear of their side effects, which represents alternative healthcare movement. Due to this reason, the demand for herbal products has increased tremendously in the world market especially among young generation.

The development of spectroscopic methods for the elucidation of natural compound structure together with development of biological sciences have opened a new era to study structure activity relationship. These developments have allowed for preparing derivatives or synthetic analogues using natural compounds as model.

Hypertension or high blood pressure is one of the most important concerns in developed countries. It causes heart to work harder to maintain high blood pressure. Moreover, it contributes to atherosclerosis (hardening of arteries), besides increasing the risk of heart disease and stroke. Local plants, from different countries such as *Agathosma betulina* from Rutaceae family, *Allium sativum* from Alliaceae or Liliaceae family, *Annona muricata* from Annonaceae family, *Apium graveolens* from Apiaceae family, *Aristolochia manshuriensis* from Aristolochiaceae family, *Artocarpus altilis*

from Moraceae family, *Coleus forskohlii* from Lamiaceae family, have been widely used with hypotensive and antihypertensive therapeutic values to reduce high blood pressure (Tabassum and Ahmad, 2011). In Malaysia, *Orthosiphon stamineus* plant from Lamiaceae family have been used traditionally to cure hypertension (Perry, 1998). However, scientific data to support its effect is not available. Therefore, more research coupled with modern medicine needs to be carried out to verify its effectiveness, and elucidate the safety profile of such herbal remedies for their hypotensive/antihypertensive potential.

1.2 Standardisation of Herbal Medicine

Standardisation is prescribing a set of standards or inherent characteristics, constant parameters, definitive qualitative and quantitative values that carry an assurance of quality, efficacy, safety and reproducibility. It is the process of developing and agreeing upon technical standards. Specific standards work out by experimentation and observations, which would lead to the process of prescribing a set of characteristics exhibited by a particular herbal medicine. Hence, standardisation is a tool in the quality control process.

Several problems that are not applicable to synthetic drugs often influence the quality of herbal drugs. For instance: 1) herbal drugs are usually mixture of many constituents, 2) the active principle(s) is (are), in most cases unknown, 3) selective analytical methods or reference compounds may not be available commercially, 4) plant materials are chemically and naturally variable, 5) Chemo-varieties and chemo cultivars exist, 6) the source and quality of the raw materials are variable. Moreover, the light exposure, temperature, nutrients, use of fresh plants, age, part of the collected plant, water availability, period and time of collection, method of collecting, drying,

packing, storage, transportation, contamination with microorganism, heavy metals or pesticides, and processing (for example, mode of extraction and polarity of the extracting solvent, instability of constituents) might impact the quality, safety and efficacy of the herbal drugs (Calixto, 2000). In spite of many proven benefits of natural products, they cannot be widely accepted in the main stream of pharmaceuticals due to lack of standardisation. Therefore, it is necessary to provide scientific evidence on standardisation to support their efficacy and bring these remedies into the mainstream pharmaceutical market (Barnes, 2003).

Due to the long-history of the use of herbal products in various cultures, they are usually considered safe. However, series of harmful effects after consumption of herbal products have been reported including direct toxic effect especially because of presence of heavy metal, allergic reactions, and mutagenic effects. In addition, the toxic effects of herbal preparation may be attributed to inherent toxicity of plant constituents, ingredients, manufacturing malpractice, and contamination (Mosihuzzaman and Choudhary, 2008). Therefore, standardisation and quality control of the raw materials and herbal preparation are necessary to be carried out.

Numbers of International Pharmacopoeia such as British Pharmacopoeia and United States Pharmacopeia, which contained collection of recommended procedures such as macroscopic and microscopic examination, determination of total ash, acid-insoluble ash and water-soluble ash, determination of pesticide residue, determination of swelling and foaming index, limit test for heavy metals, limit test for microorganisms, and test for determination of extractable matter, water and volatile matterfor specifications and quality control of plant raw materials, have been conducted by WHO (WHO, 1973). Moreover, many international authorities and

agencies including the European Agency for Evaluation of Medicinal Products and the European Scientific Cooperation of Phytomedicine, The US Agency for Healthcare Policy, and Research the European Pharmacopoeia Commission have started creating a new mechanism for quality control and standardisation of botanical medicines (Sharma et al., 2010).

1.3 Justification of the Research

In this study, *Orthosiphon stamineus* (OS) a local plant was selected for studying its antihypertensive effect. *O. stamineus* known as misai kucing is a medicinal plant grown in South East Asia and currently cultivated in Indonesia and Malaysia. In Malaysia, the leaves of this plant (misai kucing) have been used traditionally in treating angiogenesis related diseases, urinary lithiasis, edema, inflammation, eruptive fever, influenza, hepatitis, jaundice, rheumatism, diabetes and hypertension (Mukesh et al., 2015; Perry, 1998). Recent scientific findings also showed that *O. stamineus* have the potential for different pharmacological properties. Although a number of products manufactured from *O. stamineus* are available in the market, there is still lack of information in terms of chemical components related to primary metabolites such as content of specific amino acids. The basis for the traditional use of this herb as antihypertensive and structure activity relationship has not yet been scientifically verified. Furthermore, a new step in development of new generation of standardised herbal medicine is preparation of botanical formulation to increase the solubility and bioavailability of the active constituents with therapeutic activity. In addition, microRNAs, as candidates for diagnostic and prognostic biomarkers and predictors of this herb response, have not yet been investigated. Therefore, this research aims to fulfil the gaps between indigenous herbal practices and contemporary medicinal sciences.

1.4 General Objectives

This study generally seeks to standardise *O. stamineus* leaves extracts by developing new analytical techniques to measure the content of primary and secondary metabolites in *O. stamineus* extracts. Moreover, it aims to demonstrate the *in vitro* and *in vivo* antihypertensive properties of standardised *O. stamineus* extracts based on structure activity relationship of marker compounds. In addition, it seeks to prepare new formulation from standardised *O. stamineus* extract using soy bean phospholipids in order to improve the solubility and bioavailability of the active constituents with therapeutic activity. It also aims to utilize expression of miRNA subsets as a new tool to elucidate the mechanism of plant in treatment of hypertension.

1.5 Specific Objectives

1) To standardise *Orthosiphon stamineus* extracts using selected markers by developing new analytical methods.
2) To evaluate antihypertensive properties of the various standardised extracts of *Orthosiphon stamineus* based on structure activity relationship of marker compounds.
3) To prepare nano liposomes of the most active extract in order to improve antihypertensive activity, solubility and bioavailability of the active marker compounds.
4) To determine pharmacokinetic, stability, acute and sub chronic toxicity studies of the nano liposomes of *Orthosiphon stamineus* extract.
5) To investigate the expression of miRNA subsets as potential biomarkers for antihypertensive activities.

1.6 Hypotheses

O. stamineus has been used in traditional medicine to treat hypertension. Subsequently, presence of high content of phenolics and flavonoids compounds such as sinensetin (SIN), eupatorin (EUP), 3'-hydroxy-5, 6, 7, 4'-tetramethoxy flavone (TMF) and rosmarinic acid (RA) were detected in *O. stamineus* extract in previous studies. Whereas, inhibition activity of angiotensin converting enzyme (ACE) has been attributed to the presence of flavonoids in the plant extract, due to the generation of chelate complexes within the active centre of ACE. Therefore, possible research hypothesis would be that there is a correlation between the presence of these compounds and antihypertensive properties of the plant.

1.7 Significance of Study

The findings of this study provide knowledge on application of analytical methods for standardisation of plant materials and extract to produce, safe and high quality herbal medicinal products for manufacturers and consumers. Moreover, this research fills the gaps between indigenous herbal practices and contemporary medicinal sciences on antihypertensive effect of medicinal plant. Additionally, this study is a significant endeavour in promoting the use of natural phospholipid bilayer obtained from food grade soybean lecithin to prepare new botanical formulation to improve the extract's solubility and permeability as the major factors for improving oral bioavailability.

1.8 Methodology Flowchart

The overall methodology consists of many steps, which includes quality test of raw material by gravimetric analysis, extractive value, heavy metals and microbial limit test. The plant raw materials will be extracted by maceration method to prepare

water (OS-W), ethanolic (OS-E), methanolic (OS-M), 50% ethanolic (OS-EW) and 50% methanolic (OS-MW) extracts. All extracts will be standardised by different spectroscopic and chromatographic techniques (UV-Vis, FT-IR, FT-NIR, HPTLC and HPLC). Moreover, the contents of primary and secondary metabolites also will be quantified in the extracts. The molecular docking study will be carried out on the marker compounds of extracts to determine the docking scores and binding affinities of marker compounds with Zn^{2+} in angiotensin converting enzyme. Then *in vitro* angiotensin converting enzyme inhibitory (ACE-I) assays including enzymatic ACE-I assay and chelating activity of Zn^{+2} will be studied on extract and marker compounds. Two extracts with highest ACE-I activity will be screened for *in vivo* antihypertensive properties on Spontaneous Hypertensive Rats (SHR) in order to select the best extract with highest antihypertensive activity. The most active extract will be formulated using soy been phospholipids in order to improve the solubility and bioavailability of the active constituents with therapeutic activity. Subsequently, the nano formulated extract will be characterized for different parameters. Moreover, pharmacokinetic, stability and toxicology studies will be done on nano formulated extract. Then, *in vivo* anti-hypertension activity of nano formulated extract will be studied on SHR. At the end, miRNA expression study will be done to investigate the expression of miRNA subsets as potential biomarkers for antihypertensive activities. The methodology of this study is summarized in the following flowchart (Figure 1.1).

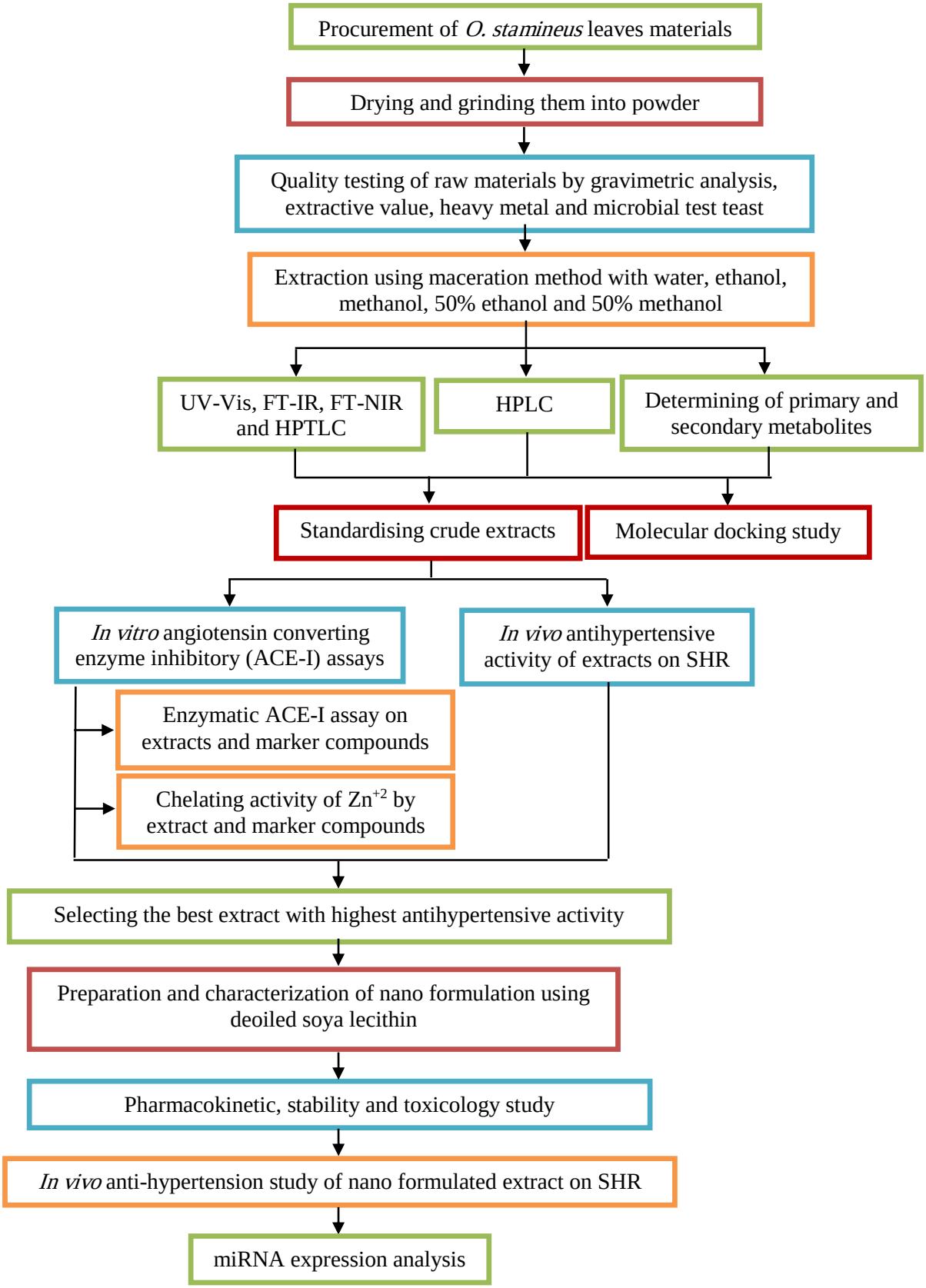


Figure 1.1: Methodology flowchart

CHAPTER 2
LITERATURE REVIEW

2.1 *Orthosiphon stamineus*

2.1.1 Taxonomy

Taxonomically, this plant is classified as the following scheme:

Family	Lamiaceae
Genus	*Orthosiphon*
Scientific name	*Orthosiphon stamineus*. (Benth)
Local name	Misai Kucing
Synonyms	*O. aristatus* (Bl.); *O. grandiflorus*, Bold., *O. spicatus*
Common name	Java tea, cat's whiskers

(Comprehensive information about *Orthosiphon stamineus* plant can be found at http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?423487)

Orthosiphon stamineus Benth is a well-known medicinal plant belonging to Lamiaceae family. This plant is native in Southeast Asian countries. It is a perennial herb, 25-200 cm tall with quadrangular, poorly ramified and ascending stem. The plant is herbaceous shrub and it can be found in tropical and sub tropical regions. The stem is acutely quadrangular, reddish in colour, erect and branches profusely. The leaves are simple, green and glabrous with a lanceolate leaf blade and serrate margin. The leaves are arranged in opposite pairs and the petiole is short about 1.3 cm in length and reddish purple in colour. The flowers are hermaphrodite, about 6.2 cm in length including the staments, with very irregular flower symmetry (Almatar et al., 2013). In South East Asia, this plant is known as *misai kucing* (Malaysia), *kumis kucing* and

remujung (Indonesia) and *yaa nuat maeo* (Thailand). In Malaysia, the local name of this plant refers to the white or blue colour of flowers with long filaments over mid-green foliage which makes the flower look like cat's whiskers in Malay *Misai* (whiskers) *kucing* (cat) (Figure 2.1).

2.1.2 Ethnopharmacology

O. stamineus has been traditionally used for treating ranges of diseases such as edema, inflammation, urinary, lithiasis, hepatitis, rheumatism, eruptive fever, diabetes, influenza, jaundice, as a remedy for kidney stones and nephritis, pain in the bladder with frequent urination, diuretic, biliary and hypertension (Awale et al., 2001; Dat et al., 1992; Goh et al., 1995; Tezuka et al., 2000). Leaves of this plant in Southeast Asian and European countries are used popularly as herbal tea, known as "Java tea".

Figure 2.1: Pictures of *Orthosiphon stamineus* leaves and flower

2.2 Review of Chemical Constituents of *Orthosiphon stamineus*

Phytochemically, the plant is rich in flavonoids especially polymethoxylated flavone, terpenes, diterpenoids and triterpenes such as hydroxyl betulinic acid, betulinic acid, oleanolic acid, ursolic acid and caffeic acid derivatives like rosmarinic acid (Sumaryono et al., 1991). The chemical constituents and chemical structure identified in the aerial parts of *O. stamineus* are illustrated in Table 2.1 and Table 2.2.

Table 2.1: Chemical constituents of *Orthosiphon stamineus*

Class of compounds	**Part of plant**	**Chemical constituents**	**Reference**
Diterpenes	Aerial	Orthisiphols F [1], orthosiphols G [2], orthosiphols H [3], orthosiphol I [4], orthosiphol J [5], orthosiphol S [6], staminols A [7], staminols B [8], staminolactones A [9], staminolactones B [10], norstaminol A [11], orthosiphonone A [12], orthosiphonone B [13]	(Awale et al., 2001; Tezuka et al., 2000)
Triterpenes	Aerial	Oleanolic acid [14], ursolic acid [15], betulinic acid [16], β-sitosterol [17]	(Tezuka et al., 2000)
Flavones	Aerial	7,3',4'-tri-O-methylluteolin [18], eupatorin [19], sinensetin [20], 3'-hydroxy-5,6,7,4'-tetramethoxyflavone [21], salvigenin [22], ladanein [23], scutellarein tetramethyl ether [24], 6-hydroxy-5,7,4'-trimethoxyflavone [25], kaempferol-3-O-β-glucoside [26], quercetin-3-O-β-glucoside [27]	(Sumaryono et al., 1991; Tezuka et al., 2000)
Phenolic acids	Leaves	Caffeoyl tartrate [28], rosmarinic acid [29], aurantiamide acetate [30], vomifoliol [31], caffeic acid [32], 2,3-dicaffeoyl tartrate [33]	(Sumaryono et al., 1991)

Table 2.1: Continued

Benzochromene	Leaves	Methylripariochromene A [34], acetovanillochromene [35], orthochromene A [36]	(Shibuya et al., 1999)

Number in brackets indicate the number of the structure

Table 2.2: Chemical structures of *Orthosiphon stamineus*

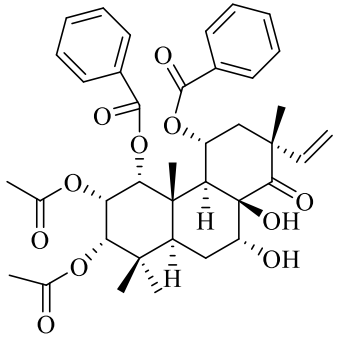
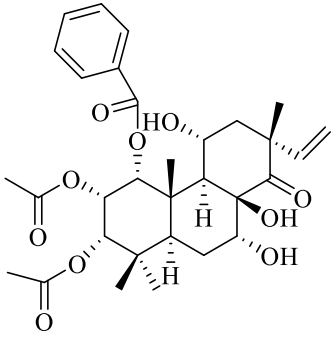
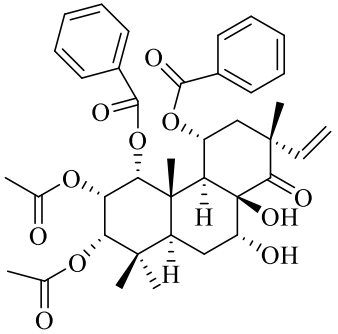
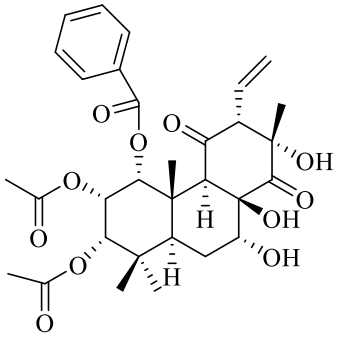
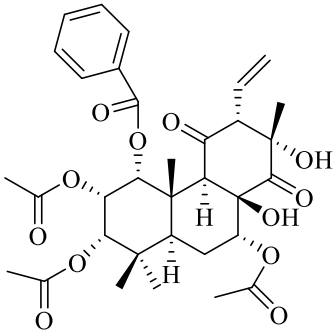
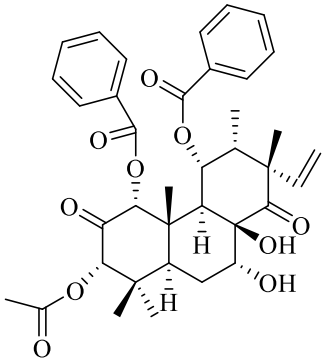
 Orthosiphol F [1]	 Orthosiphol G [2]	 Orthosiphol H [3]
 Orthosiphol I [4]	 Orthosiphol J [5]	 Orthosiphol S [6]

Table 2.2: Continued

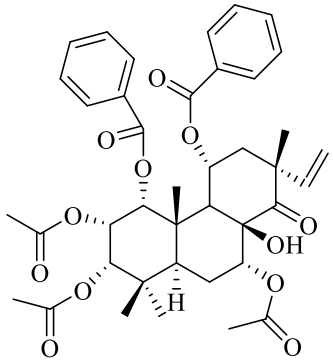
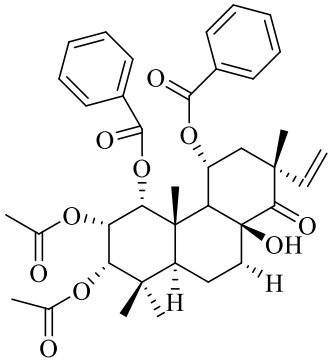
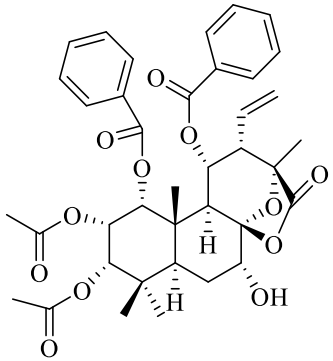
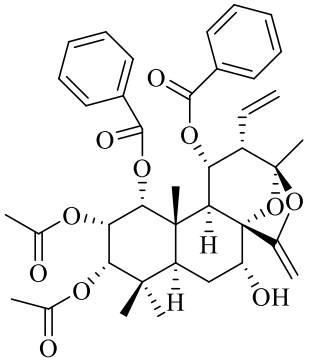
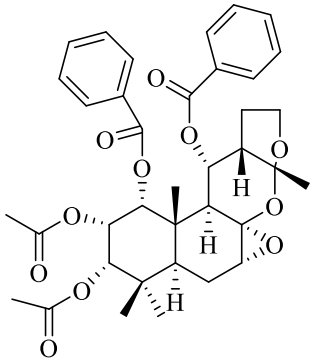
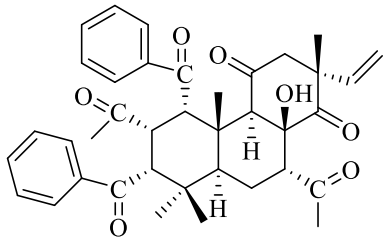
 Staminol A [7]	 Staminol B [8]	 Staminolactone A [9]
 Staminolactone B [10]	 Norstaminol A [11]	 Orthosiphonone A [12]

Table 2.2: Continued

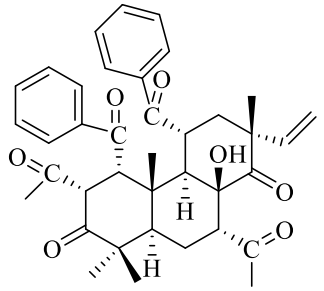
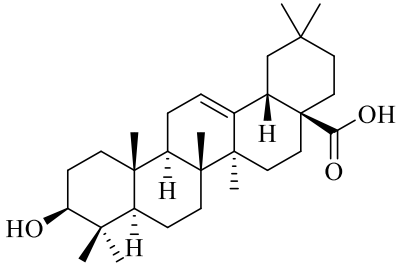
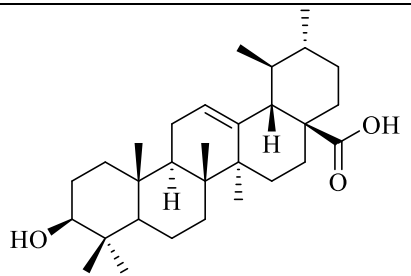
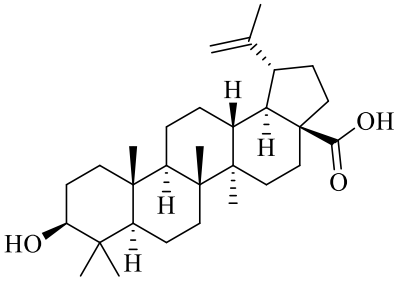
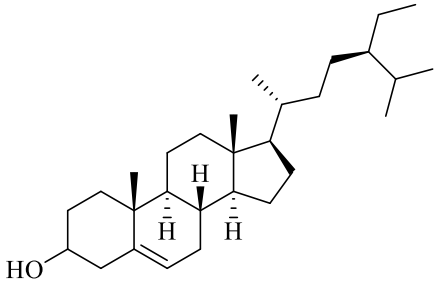
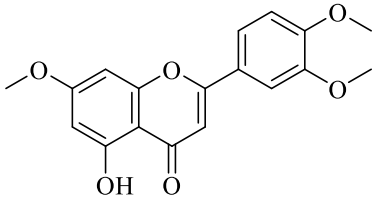
 Orthosiphonone B [13]	 Oleanolic acid [14]	 Ursolic acid [15]
 Betulinic acid [16]	 β-sitosterol [17]	 7,3’,4’-tri-*O*-methylluteolin [18]

Table 2.2: Continued

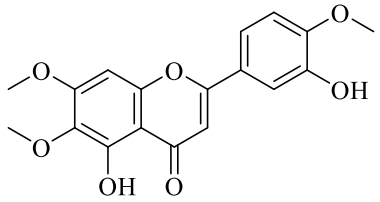
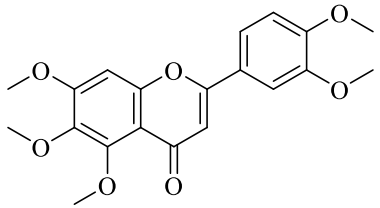
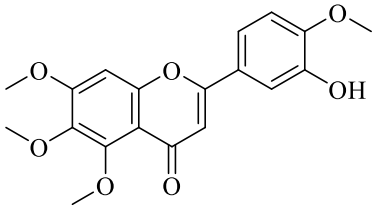
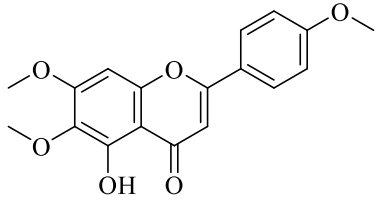
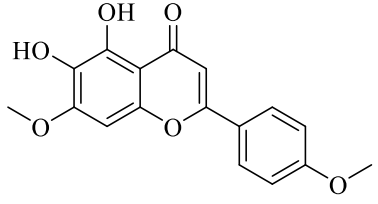
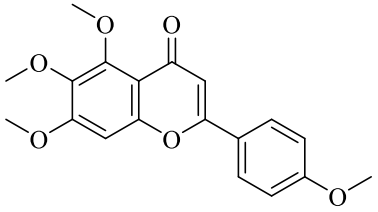
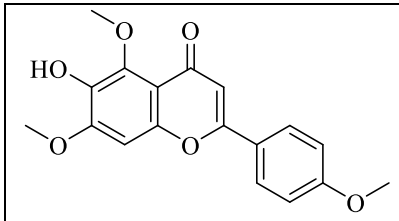
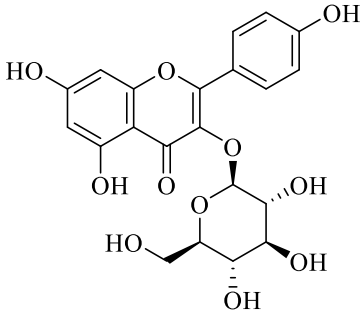
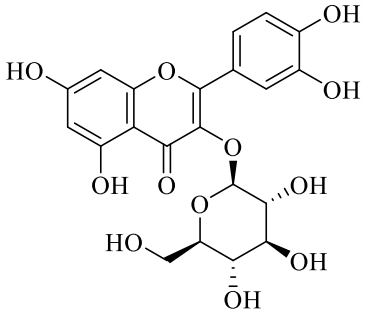
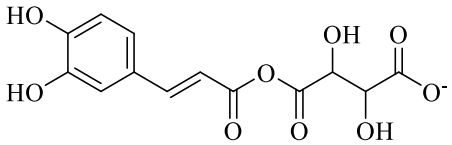
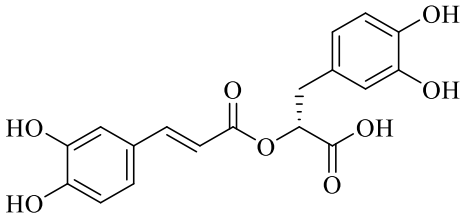
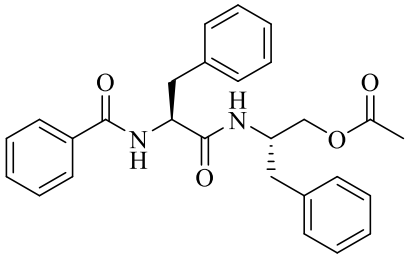
 Eupatorin [19]	 Sinensetin [20]	 3′-hydroxy-5,6,7,4′-tetramethoxyflavone [21]
 Salvigenin [22]	 Ladanein [23]	 Scutellarein tetramethyl ether [24]

Table 2.2: Continued

 6-Hydroxy-5,7,4′-trimethoxyflavone [25]	 Kaempferol-3-O-β-glucoside [26]	 Quercetin-3-*O*-β-glucoside [27]
 Caffeoyl tartrate [28]	 Rosmarinic acid [29]	 Aurantiamide acetate [30]