DEVELOPMENT OF NANO-FORMULATION USING PVP/P407 BASED ON SOLID DISPERSION FROM STANDARDISED ETHANOLIC EXTRACT OF *ORTHOSIPHON STAMINEUS* LEAF FOR SOLUBILITY AND ORAL BIOAVAILABILITY IMPROVEMENTS

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by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	Pre-exponential factor
AAE	Ascorbic acid equivalent
AAS	Atomic Absorption Spectroscopy
$AlCl_3$	Aluminium chloride
Ar	Arsenic
ATR	Attenuated Total Reflection
AUC	Area under the curve
BCF	Bio-pharmaceutics Classification System
BP	British Pharmacopoeia
BSA	Bovine serum albumin
C	Concentration
C_L	Clearance
C_{max}	Maximum plasma peak concentration
CO_2	Carbon dioxide
DAD	Diode array detector
DMSO	Dimethyl sulfoxide
DPPH	2, 2-diphenyl-1-picrylhydrazyl
Ea	Actvation energy
EA.hy926	Normal endothelial cell line
EDTA	Ethylenediamine tetraacetic acid
EF	Extraction factor
EMEA	European Agency for the Evaluation of Medicinal Products
ESD	Ethanolic solid dispersion
EtOH	Ethanol
EUP	Eupatorin
FBS	Fetal bovine serum
$FeCl_3{:}6H_2O$	Ferric chloride hexahydrate
FRAP	Ferric reducing antioxidant power
FTIR	Fourier transform infrared spectroscopy
GACP	Good agriculture and collection practice
GAE	Gallic acid equivalent
GAP	Good agriculture and practice

GCMS	Gass chromatography mass spectroscopy
GIT	Gastrointestinal tract
H_2SO_4	Sulphuric acid
HCA	Hierarchiral clustering analysis
HCl	Hydrochloric acid
HCT-116	Human colorectal cell line
Hg	Mercury
HNO_3	Nitric acid
HPLC	High performance liquid chromatography
HPTLC	High performance- thin layer chromatography
I.V	Intravenous
IC	Inhibitory concentration
ICH	International Conference on Harmonization
K	Rate constant
K_e	Elimination rate constant
LOD	Limit of detection
LOQ	Limit of quantification
MCF-7	Human hormone sensitive and invasive breast cancer cell line
MEM	Minimum essential medium
MeOH	Methanol
Mg	Magnesium
MHM	Malaysian Herbal Monograph
MOA	Ministry of Agriculture
MS	Malaysian Standard
MS	Mass spectroscopy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na_2CO_3	Sodium carbonate
NCI	National Cancer Institute
NMR	Nuclear Magnetic Resonance
P	Phosphorus
P188	Poloxamer 188
P407	Poloxamer 407
PA/W	Peak area per weight
Pb	Lead

PBS	Phosphate buffer saline
PCA	Principle component analysis
PCS	Photon correlation spectroscopy
PDI	Poly dispersity index
PS	Penicillin streptomycin
PVP	Polyvinylpyrrolidone
QE	Quercetin equivalent
R	Universal gas constant
R^2	Regression coefficient
RA	Rosmarinic acid
RH	Retardation humidity
RPMI	Roswell Park Media Institute
RSD	Relative standard deviation
RVSEB	*Rappaport Vassiliadis Salmonella enrichment broth*
SCF	Supercritical fluid technology
SD	Standard deviation
SD	Solid dispersion
SDA	Sabourand dextrose agar
SDC	Soybean-casein digest agar
SEM	Scanning electron spectroscopy
SEM	Standard error mean
SIM	Stability indicting method
SIN	Sinensetin
T	Temperature
$T_{1/2}$	Half life
TEM	Transmission electron spectroscopy
TFC	Total flavonoid content
TGA	Thermogravimetric Analyzer
T_{max}	Time of maximum plasma concentration
TMF	3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone
TPA	Total protein analysis
TPC	Total phenolic content
TPTZ	2, 4, 6- tripyridyl-s-triazine
UNIMAP	Universiti Malaysia Perlis

USFDA	United States Food and Drug Administration
USM	Universiti Sains Malaysia
USP	United States Pharmacopoeia
UV-Vis	Ultraviolet visible spectroscopy
V_d	Volume of distribution
WHO	World Health Organization
WPHMP	Working party of herbal medicinal products
XLD	Xylose, lysine, deoxycholate agar
Zn	Zinc
ZS	Zeta sizer
λ_{max}	Maximum absorption

LIST OF UNITS

µg/mL	Microgram per millilitre
µL	Microlitre
µm	Micrometre
µmol/L	Micromolar per litre
cfu/g	Colony-forming unit per gram
cm^{-1}	Per centimetre
g	Gram
g/mL	Gram per millilitre
h	Hour
h^{-1}	Per hour
$Kelvin^{-1}$	Per Kelvin
kg	Kilogram
$KJmol^{-1}$	Kilojoule per mole
L	Litre
L/kg.h	Litre per kilogram hour
M	Molar
mAU	Milliabsorbance units
mg	Milligram
mg/g	Milligram per gram
mg/kg	Milligram per kilogram
mg/mL	Milligram per mililitre
mL/min	Millilitre per minute
mL^{-1}	Per mililitre
mM	Millimolar
mV	Millivolt
mΩ	Milliohm
N	Normal
nm	Nanometre
ppm	Part per million
rpm	Revolution per minute
w/v	Weight per volume
w/w	Weight per weight

LIST OF SYMBOLS

%	Percent
°C	celcius
λmax	Maximum wavelength
δ	Delta
ζ	Zeta
Da	Dalton

PEMBANGUNAN NANO-FORMULASI PVP/P407 BERASASKAN SERAKAN PEPEJAL EKSTRAK ETANOL TERPIAWAI DAUN *ORTHOSIPHON STAMINEUS* UNTUK PENAMBAHBAIKAN KETERLARUTAN DAN BIOPEROLEHAN ORAL

ABSTRAK

Kajian ini melibatkan penilaian kualiti bahan mentah *Orthosiphon stamineus*, pemiawaian ekstrak yang berbeza, pembangunan nano-formulasi menggunakan ekstrak etanol maserasi dan penilaian aktiviti biologi terpilih (antioksidan dan sitotoksisiti). Daun kering *O. stamineus* memenuhi sifat fizikokimia, logam berat dan had mikrob yang digunakan untuk kajian selanjutnya. Lima ekstrak yang berbeza iaitu metanol, metanol (50%), etanol, etanol (50%) dan air diperolehi dengan menggunakan tiga teknik yang berbeza (Soxhlet, refluk dan maserasi). Analisis spektroskopi (FTIR dan UV) dan kromatografi (HPLC) telah dijalankan untuk pemiawaian ekstrak mentah. Keputusan menunjukkan flavonoid dan asid fenolik adalah bahan kimia utama dalam ekstrak *O. stamineus*. Kaedah RP-HPLC/DAD yang lebih baik telah disahkan untuk analisis kuantitatif asid rosmarinik (RA), 3-hidroksi-5, 6, 7, 4-metoksiflavon (TMF), sinensetin (SIN) dan eupatorin (EUP) dalam ekstrak mentah. Kaedah ini digunakan dalam kajian farmakokinetik dan kestabilan dalam ekstrak dan ekstrak yang diformulasi. Pengesahan kaedah RP-HPLC/DAD termasuk kekhususan, kelinearan ($R^2 \geq 0.999$), kepersisan (intra- dan antara hari) dan perolehan (90.2-104%). LOD dan LOQ sebatian yang dipilih masing-masing adalah dalam julat 0.17 ± 0.01 hingga 0.24 ± 0.03 μg/mL dan 0.53 ± 0.03 hingga 0.73 ± 0.09 μg/mL. Cap jari FTIR dan data HPLC dianalisis menggunakan alat kemometrik (PCA dan HCA) untuk kandungan metabolit (primer dan sekunder) dan

aktiviti terpilih. Perubahan dalam peratusan metabolit ini (flavonoid, fenolik, polisakarida, protein dan glikosaponin) dalam setiap ekstrak telah ditentukan. Ekstrak etanol termaserat telah dibangunkan dengan lebih lanjut menggunakan pelbagai polimer menggunakan serakan pepejal bagi meningkatkan keterlarutan dan bioperolehan oral dari flavonoid dan asid fenolik dalam ekstrak tersebut. Penyebaran pepejal yang dioptimumkan dianalisis menggunakan HPLC dan dicirikan dengan menggunakan kaedah cap jari FTIR dan kaedah fizikokimia (saiz zarah, potensi zeta, TEM dan SEM). Kesan pH pada kestabilan dan keterlarutan dalam penimbal dan air, pembebasan *in-vitro*, analisis metabolit total, dan aktiviti biologinya menunjukkan bahawa rumusan nano ekstrak etanol (ESD) yang menggunakan polimer (PVP/P407) dengan nisbah ekstrak kepada polimer (1.0: 1.1: 0.3) meningkatkan kandungan flavonoid (TMF = 3.56 ± 0.01% w/w, SIN = 2.46 ± 0.01% w/w dan EUP = 7.87 ± 0.01% w/w) dan RA (20.66 ± 0.01% w/w) berbanding ekstrak etanol (P <0.0001) dengan saiz zarah kurang daripada 200 nm. Berdasarkan kajian kestabilan dipercepatkan pada tiga keadaan penyimpanan (30, 40 dan 60 °C), jangka hayat RA, TMF, SIN dan EUP dalam ESD meningkat dengan signifikan (P <0.001) berbanding ekstrak etanol pada suhu di bawah 30 °C. Farrmakokinetik dan bioperolehan oral ESD dan ekstrak etanol yang dilakukan pada tikus Sprague-Dawley (SD) menunjukkan peningkatan signifikan (P <0.05) sebatian penanda (RA = 15.12 ± 0.92%, TMF = 29.82 ± 3.31% SIN = 38.76 ± 4.03% dan EUP = 34.60 ± 3.74%). Kajian ini memberikan maklumat mengenai penilaian kualiti bahan mentah dan ekstrak *O. stamineus* dan kejayaan pembangunan penyebaran pepejal etanol (ESD) menggunakan PVP/P407 bagi meningkatkan keterlarutan, kestabilan dan bioperolehan oral bagi flavonoid serta sebatian lain.

DEVELOPMENT OF NANO-FORMULATION USING PVP/P407 BASED ON SOLID DISPERSION FROM STANDARDISED ETHANOLIC EXTRACT OF *ORTHOSIPHON STAMINEUS* LEAF FOR SOLUBILITY AND ORAL BIOAVAILABILITY IMPROVEMENTS

ABSTRACT

This study involved the quality assessment of *Orthosiphon stamineus* raw materials, standardisation of different extracts, development of nano-formulation using macerated ethanolic extract and evaluated for selected biological activities (antioxidant and cytotoxicity). The dried leaves of *O. stamineus* fulfilled the physicochemical properties, heavy metals and microbial limits to be used for further study. Five different extracts namely methanolic, methanolic (50%), ethanolic, ethanolic (50%) and water were obtained using three different techniques (Soxhlet, reflux and maceration). The spectroscopic (FTIR and UV) and chromatographic (HPLC) analysis were carried out for standardisation of the crude extracts. The results revealed the flavonoids and phenolic acids were the major chemical constituents in *O. stamineus* extracts. An improved RP-HPLC/DAD method was validated for the quantitative analysis of rosmarinic acid (RA), 3-hydroxy-5, 6, 7, 4-methoxyflavone (TMF), sinensetin (SIN) and eupatorin (EUP) in the crude extracts. This method was applied in the pharmacokinetic and stability studies in the extract and formulated extract. RP-HPLC/DAD method validation including specificity, linearity ($R2 \geq 0.999$), precision (intra- and inter-day) and recoveries (90.2-104%). The LOD and LOQ of this selected compounds were in the range of 0.17 ± 0.01 to 0.24 ± 0.03 μg/mL and 0.53 ± 0.03 to 0.73 ± 0.09 μg/mL, respectively. The FTIR fingerprint and HPLC data set were analysed using chemometric tools (PCA and HCA)

for its metabolite contents (primary and secondary) and selected activities. The variation in the percentage of these metabolites (flavonoids, phenolics, polysaccharides, proteins and glycosaponins) in each extract was determined. The macerated ethanolic extract was further developed using various polymers for solid dispersion to enhance solubility and oral bioavailability of the flavonoids and phenolic acids in the extract. The optimised solid dispersion was analysed using HPLC and was further characterised using on FTIR fingerprints and physicochemical methods (particles size, zeta potential, TEM and SEM). The effect of pH on stability and solubility in buffer and water, *in-vitro* release, total metabolite analysis, and its biological activities indicated that the nano-formulation of the macerated ethanolic extract (ESD) using polymers (PVP/P407) with a ratio of extract to polymers (1.0:1.1:0.3) enhanced the flavonoids contents (TMF=3.56 ± 0.01% w/w, SIN=2.46 ± 0.01% w/w and EUP=7.87 ± 0.01% w/w) and RA (20.66 ± 0.01% w/w) compared to the ethanolic extract (P <0.0001) with particles size less than 200 nm. Based on accelerated stability study at three storage conditions (30, 40 and 60°C), the shelf life of RA, TMF, SIN and EUP in ESD was significantly enhanced (P <0.001) compared to the ethanolic extract at temperature below 30 °C. The pharmacokinetic and oral bioavailability of ESD and ethanolic extract performed on Sprague-Dawley (SD) rat indicated significant enhancement (P <0.05) of mean absolute oral bioavailability of marker compounds (RA= 15.12 ± 0.92%, TMF= 29.82 ± 3.31%, SIN= 38.76 ± 4.03% and EUP= 34.60 ± 3.74%). The present study provides information on the quality assessment of *O. stamineus* raw and extract materials and the successful development of ethanolic solid dispersion (ESD) using PVP/P407 that enhanced the solubility, stability and oral bioavailability of flavonoids as well as other compounds.

CHAPTER 1 INTRODUCTION

1.1 Background of study

Orthosiphon stamineus Benth. (Cat's Whiskers) is one of the popular medicinal plants in Southeast Asia particularly in Malaysia, Indonesia and Thailand. It is popularly consumed as herbal tea for the general health and to treat a wide range of diseases related to kidney and urinary disorders, diabetes, high blood pressure and bone or muscular pain (Adam et al., 2009; Indariani et al., 2014; Sumaryono et al., 1991; Tezuka et al., 2000). Three types of phytochemicals were identified in various extracts of *O. stamineus* including polymethoxylated flavonoids (Akowuah et al, 2004; Lyckander & Maltreud, 1996), phenylpropanoids (caffeic acid derivatives) (Olah et al., 2007; Olah et al., 2003) and terpenoids (diterpenes and triterpenes) (Masuda et al., 1992; Masuda et al., 1992a, 1992b). Among these compounds, 3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone (TMF), sinensetin (SIN) eupatorin (EUP) and caffeic acid derivatives (rosmarinic acid) were found to possess potential therapeutic properties, as they were shown to exert antioxidant properties (Akowuah et al., 2005; Akowuah et al., 2004), diuretic and uricosuric actions in rats (Olah et al., 2003) and anticancer properties (Movahedi et al., 2015). Moreover, SIN and EUP were reported to have anti-inflammatory properties (Yam et al., 2010) and SIN has been reported to reverse the P- glycoprotein-mediated multidrug resistance in the absorption of drugs (Choi et al., 2002). Eupatorin has been reported to have antiproliferative activity against selective cancer cell lines, but it has no cytostatic effects in normal human cell line (Androutsopoulo et al., 2008; Dolečková et al., 2012; Tezuka et al., 2000).

Studies have been performed to identify and quantify the phytochemical contents in *O. stamineus* extracts particularly using high performance liquid chromatography (HPLC) (Akowuah et al., 2004; Loon et al., 2005; Siddiqui & Ismail, 2011; Yam et al., 2012). However, very few methods can detect simultaneously four markers (RA, TMF, SIN and EUP) (Akowuah et al., 2005; Akowuah et al., 2004) and most of it suffered from drawbacks including time consuming for HPLC separation used of buffer solution which may deteriorate efficiency of column and used of unsuitable solvent system. There is a great need for a faster, reliable and reproducible method for routine standardisation work of *O. stamineus* extract, raw material and commercial products for quality assessment of this herb. Despite the growing interest in these flavonoids in *O. stamineus* concerning of their routine standardisation work, there is a paucity of information regarding to their solubility, dissolution rate, stability and bioavailability of these compounds. Previous study was reported that the oral bioavailability of three lipophilic flavonoids (TMF, SIN and EUP) in *O. stamineus* extract was very poor and incomplete absorption, hence, it limited the therapeutic properties of *O. stamineus* (Loon et al., 2005). To overcome the issues, there are many formulation strategies including nanoparticles, liposomes, complex with phospholipids, cyclodextrins and solid dispersions which appear to provide longer circulation, better permeability, and resistance to metabolic processes (Anand et al., 2007; Hou et al., 2013; Kaur & Kaur, 2014). Among these approaches, solid dispersion is the most promising method due to the ease of preparation, ease of optimization and reproducibility of the manufacturing method (Chiou & Riegelmant, 1971). In comparison with other techniques, solid dispersion has shown many important advantages to become one of the most promising strategies for solubility enhancement including reduction of particle size to molecular level, reduce the agglomeration of

drug particles in the formulation, enhancing wettability and porosity, as well as changing drug crystalline state to amorphous which leads to faster dissolution for *in vivo* study (Vo et al., 2013). Another crucial aspect that can reduce the potency of herbal product is the stability of the phytonutrients which can be oxidised due to exposure to heat and moisture. Product shelf-life can be improved by altering the product formulation and enhancing the primary andsecondary packaging. Product formulation can be improved by adding preservatives, altering the biophysical nature of the active pharmaceutical ingredient or adding carrier molecules such as whey protein and maltodextrin *via* spray drying technique (Pang et al., 2014).

In the present study, this work has been carried out with the aim to establish the quality assessment of *O. stamineus* raw materials and to perform and improve the standardisation of *O. stamineus* raw materials, extracts and nano-formulated extract using HPLC technique with reference to RA, SIN, TMF and EUP as marker compounds before undertaking any study. The fingerprint of *O. stamineus* extracts was further analysed using spectroscopic technique combined with principle component analysis (PCA and HCA) and analysis of primary and secondary metabolites were also evaluated. Next, this work focused on solid dispersion of standardised macerated ethanolic extract prepared in a variety of polymers (PVP, P407 and P188) *via* solvent evaporation method for the enhancement of its solubility and dissolution rate. The optimized formulation was further analysed for solubility, physicochemical properties, stability under various pH conditions, and analysis of primary and secondary metabolites along with HPLC and FTIR profiles, morphologies, *in-vitro* release, antioxidant and anticancer properties. The nano-formulation ethanolic solid dispersion (ESD) was further evaluated to determine the enhancement of oral bioavailability of

lipophilic flavonoids (TMF, SIN and EUP) and RA in rat plasma. A comparison of accelerated stability study has been performed for the ethanolic extract and nano-formulated ESD to determine the shelf life of marker compounds (RA, TMF, SIN and EUP).

1.2 Problem statements

Orthosiphon stamineus leaves has been selected throughout the study due to its pharmacological properties as antioxidant, diuretic and anticancer activities. It is popularly consumed as herbal tea either in sachet form or as a raw leaves. There are a number of products derived from *O. stamineus* leaves available in the market. However, many products are not registered with regulatory such as National Pharmaceutical Regulatory Agency (NPRA). Moreover, the herbal industries are still lacking or do not adhere to the quality assessment and standardisation guidelines due to the inadequancy of knowledge, financial support and modern technologies. The quality assessment and standardisation of herbal product are necessary to ensure the quality, efficacy and safety of the finish product. In addition, it also can ensure the reproducibility of raw materials that produces a high quality of herbal products.

Despite the growing interest in these flavonoids in *O. stamineus* leaves concerning of their routine standardisation work, there is a lack of information regarding solubility, dissolution rate, stability and oral bioavailability of these compounds. So, in order to overcome these problems, present work was focused on the development of nano-formulation of standardised ethanolic extract (macerated) using solid dispersion *via* solvent evaporation method to enhance the solubility and oral bioavailability of lipophilic flavonoids. In addition, the current study conducted

and performed the pharmacokinetic and oral bioavailability of ethanolic extract and nano-formulated ESD using intravenous and oral absorption with reference to RA, SIN, TMF and EUP as marker compounds.

Another problem with *O. stamineus* extracts which will limit its therapeutic properties was the stability of extract and it products with reference to marker compounds (RA, TMF, SIN and EUP). The ethanolic extract specifically is highly hygroscopic in nature. Therefore, the physical appearance, texture and colour get easily oxidised and the chemical components particularly the bioactive markers such as rosmarinic acid and eupatorin degraded overtime. So, with the help of encapsulation process of macerated ethanolic extract with selected polymers, the present study provides the analysis of accelerated stability of ethanolic extract and it nano-formulated ethanolic extract (ESD) with reference to RA, SIN, TMF and EUP in order to compare the quantity, quality and shelf life of marker compounds in both samples under variable conditions (elevated temperatures).

1.3 Significance of study

From this study, all the information gathered can contribute to the knowledge and give better understanding especially to researchers, farmers, manufacturer as well as consumers on the importance of quality assessment of herbs in order to produce a high quality and reproducibility of batch to batch of raw materials as well as to produce a high value extract. This study also showed a development of new formulation using water-soluble co-polymers (PVP/P407) *via* solid dispersion technique that can enhance the solubility, stability and oral bioavailability of bioactive compounds (RA, TMF, SIN and EUP) as well as improved delivery of the *O. stamineus* pharmacological properties.

1.4 General objectives

Present study focused on the quality assessment of *O. stamineus* leaves following Malaysian Herbal Monograph (MHM) as guidelines. *O. stamineus* leaves were extracted using different solvents; methanol, ethanol and water *via* three extraction methods including maceration, Soxhlet and reflux. The extracts were standardised based on the selected markers (RA, TMF, SIN and EUP) using HPLC analysis. The fingerprint analysis was done using FTIR and combined with chemometric tools (PCA and HCA). Furthermore, the extracts were analysed for the selected primary and secondary metabolites as well as the antioxidant and cytotoxic activities. Moreover, the selected extract (macerated ethanolic extract) was further prepared for the new nano-formulation in order to enhance the solubility and oral bioavailability of the lipophilic flavonoids (TMF, SIN and EUP) and RA with therapeutic effects. In addition, the stability of nano-formulated ESD was also

performed using accelerated stability study in order to determine the shelf life of the nano-formulated ESD based on the marker compounds.

1.5 Specific objectives of the study

The objectives of present study are as follows:

1. To improve analytical method using chromatographic and spectroscopic analysis for standardisation of *O. stamineus* leaves extracts based on selected marker compounds.
2. To develop and optimised nano-formulation of standardised ethanolic extract using PVP/P407 *via* solid dispersion method based on aqueous solubility enhancement of the selected marker compounds.
3. To evaluate the physicochemical properties, *in-vitro* activities and comparison study of accelerated stability of nano-formulated ESD and ethanolic extact.
4. To determine the pharmacokinetic profile (*in-vivo*) of the nano-formulated ESD and ethanolic extract.

1.6 Summary of research work

The summary of present work is summarized in Figure 1.1. The overall methodology consists of many steps, which includes procurement of *O. stamineus* leaves, quality assessment following Malaysian Herbal Monongraph (Monograph committee, 2009) using gravimetric analysis, extractive value, heavy metals and microbial limit test. The *O. stamineus* leaves were extracted by three different methods (maceration, reflux and Soxhlet) using methanol, methanol (50%), ethanol, ethanol (50%) and water. All extracts were standardised using different spectroscopic and chromatographic techniques (UV-Vis, FT-IR, FT-NIR, HPTLC and HPLC). Moreover, the contents of total metabolites (primary and secondary) and it biological activities (antioxidant and cytotoxicity) were also quantified in the extracts. The best standardised extract (macerated ethanolic extract) was selected for further development of nano-formulated solid dispersion using selected polymers in order to improve the solubility, stability and oral bioavailability of the selected constituents with it therapeutic properties. The optimised solid dispersion was further characterised for it physicochemical properties, stability under various pH conditions, analysis of total metabolites along with HPLC and FTIR profiles, morphologies, *in-vitro* release, stability of prepared nano-formulation and pharmacokinetic study.

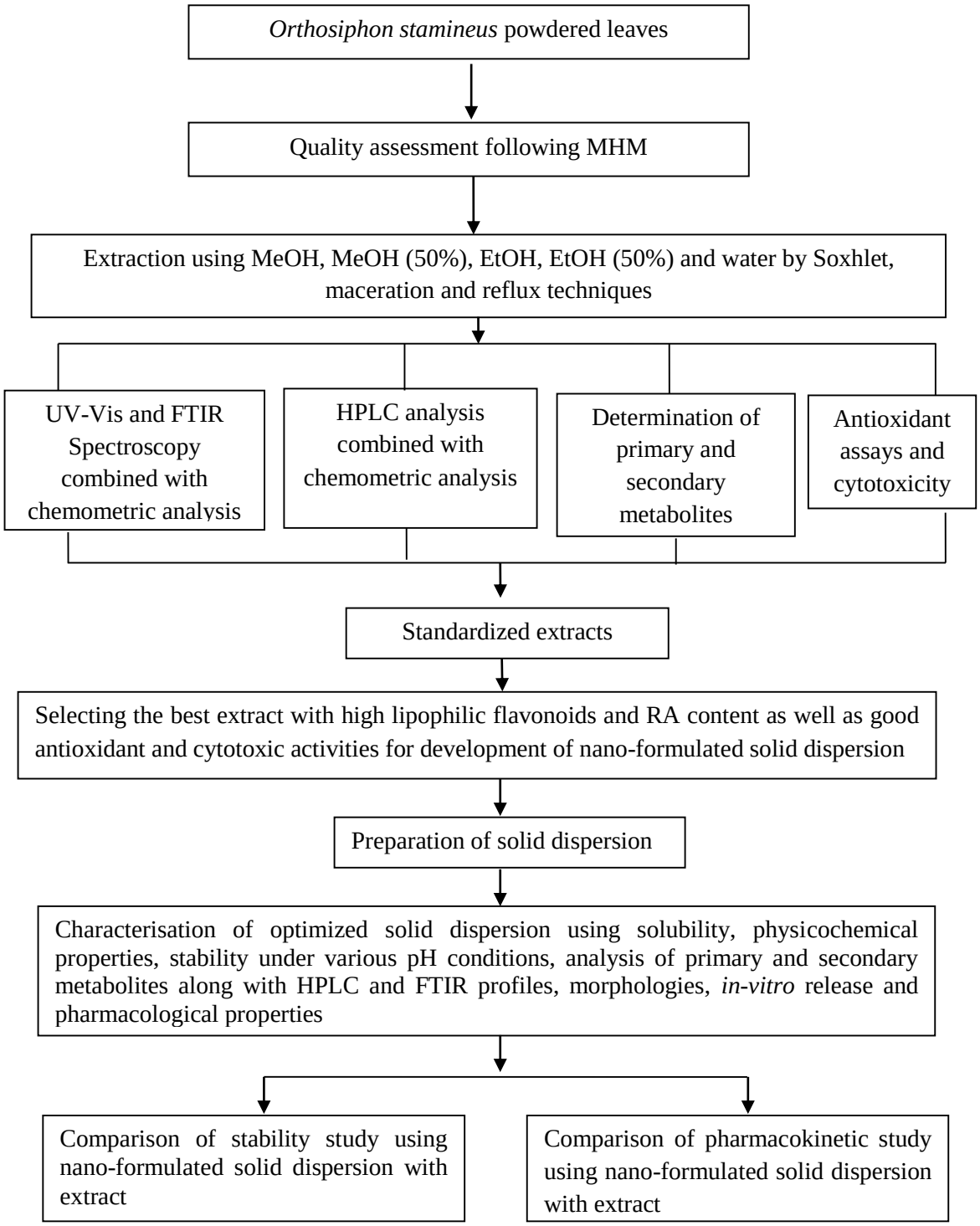

Figure 1.1 Flow chart of research work

CHAPTER 2 LITERATURE REVIEW

2.1 *Orthosiphon stamineus* Benth.

Orthosiphon stamineus Benth is one of the valuable medicinal plants in Lamiaceae family due to its therapeutic effects. Synonym names of *O. stamineus* were known as *O. aristatus, O. spiralis* (Lour) Merr., *O. aristatum* Blume Bijdr., *Clerodendranthus spicatum* (Thunb.), *C. stamineus* (Bentham) Kudo and *Clerodendrum spicatus* Thunberg (Merrill, 1935). In Malaysia, it is known as *'misai kucing*' due to the uniqueness of its flower resembling a cat's whiskers. Other vernacular names were given to this herb including Java tea (English), Remujung and Kumis kucing (Indonesia), Balbas-pusa (Phillipines) and Yaa nuat maeo (Thailand) (Himani et al., 2013). In Malaysia, there are two varieties that have been identified based on the colour of the flowers; white and purple flowers. These varieties can be differentiated based on the colour of the corolla and calyx and the leaves characteristics where the leaves of the purple flower are broader and shorter than the white flower (Chan & Loo, 2006).

2.1.1 Plant description

O. stamineus Benth (Lamiaceae) or locally known as '*misai kucing*' in Malaysia is a herbaceous shrub which can grow up to a height of 1-1.5 m (Figure 2.1). This herb originated from Southeast Asia countries including Malaysia, Indonesia, Thailand, Vietnam and neighbouring countries (Truong et al., 2010). Two different varieties were found in Malaysia based on the colour of the flower; purple and white colours. Both varieties produced petioles of dark green leaves. The leaves were placed opposite to each other on the stem. However, there were differences on the leaves for

both varieties. The purple variety has shorter and broader leaves than the white variety. The white variety produces a rhomboid shape with acuminate apex, obtuse based leaves without coloured spot. The stem morphology for both varieties are similar except the colour of the stem where the white variety has green stem while the purple variety has greenish maroon colour stem. With respect to the morphology of flower, there are differences in term of colour and size of corolla and calyx. The purple variety produces light purple colour at the lobes of the white corolla while white variety produced totally white colour without having purple colour at the edge. The calyx colour is also different between two varieties where the purple variety produces maroon colour calyx while the white variety produces green calyx. In terms of size of the corolla and calyx, there were also differences between both varieties where the white variety has a longer corolla and calyx tubes compared to the purple variety. The seed of both varieties is oval in shape with hard and rough surface testa and each fruit produces four seeds. The colour of the fruit after maturityis also different where the purple variety produces purplish red fruits while the white variety produces greenish red in colour (Chan & Loo, 2006).

Figure 2.1 Picture of *O. stamineus* Benth. (a) whole plant (b) flower

2.1.2 Traditional uses of *O. stamineus*

O. stamineus can be found in South East Asian countries especially in Malaysia, Indonesia, Thailand, Vietnam and Myanmar. This herb is popular and recognized in European countries including England, Holland and France as a herbal product (Adnyana et al., 2013) and it is also called as "Java tea". Traditionally, *O. stamineus* leaves has been used for treating a range of diseases such as a remedy for kidney stones and nephritis, edema, inflammation, urinary, lithiasis, hepatitis, rheumatism, eruptive fever, diabetes, influenza, jaundice, 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).

2.1.3 Phytochemicals review of *O. stamineus*

O. stamineus contains important class of compounds including polyphenols (lipophilic flavonoids and phenolic acids), terpenoids (diterpenes and triterpenes), sterols and essential oils. Polyphenols are very important due to its antioxidant properties and prevention of various diseases associated with cancer, cardiovascular and neurodegenerative diseases (Manach et al., 2004). Polyphenols are naturally occurring in vegetables, fruits, beverages and cereals. It influences the taste of food including bitterness, colour, flavour, astringency, odour and oxidative stability (Pandey & Rizvi, 2009). So, due to the increasing demand of polyphenols consumption nowadays, the research on *O. stamineus* has also increased in many aspects including chemical constituents as well as biological activities. The phytochemicals study of *O. stamineus* has been conducted since 1930's (Tezuka et al., 2000). Extensive study on chemical compounds in different parts of *O. stamineus* was reported previously and summarized in Table 2.1. Among the reported compounds, polymethoxylated flavonoids such as sinensetin and eupatorin and caffeic acid derivatives, which include rosmarinic acid, cichoric acid, and caffeic acid were the most important components of *O. stamineus* leaves (Olah et al., 2003).

Table 2.1 Chemicals constituents in different parts of *O. stamineus*

Compounds	Class of compounds	Locality	Part of plants	Extracts	References
Methylripariochromene A	Benzopyrene	Indonesia	Leaves	Chloroform and water fractions from water extract	(Matsubara et al., 1999)
Orthosiphol A & B	Oxygenated pimarane diterpenes	Myanmar	Leaves		(Masuda et al., 1992)
Orthosiphol C	Diterpene	Taiwan	Aerial	Methanol extract	(Nguyen et al., 2004)
Orthosiphol D & E	Diterpene	Japan	Aerial	Ethanol fraction of methanol extract	(Takeda et al., 1993)
		Japan	Aerial	Methanol extract	(Awale et al., 2002a)
Orthosiphol F-J	Diterpene	Vietnam	Aerial	Methanol extract	(Tezuka et al., 2000)
Orthosiphol K-N	Diterpene	Myanmar	Aerial	Methanol extract	(Awale et al., 2001)
		Indonesia	Aerial	Methanol extract	(Awale et al., 2003)
Orthosiphol O	Diterpene	Taiwan	Aerial	Methanol extract	(Nguyen et al., 2004)
Orthosiphol P & Q	Diterpene	Myanmar	Aerial	Methanol extract	(Awale et al., 2001)
Orthosiphol R-T	Diterpene	Japan	Aerial	Methanol extract	(Awale et al., 2002a)
Orthosiphol U-Z	Diterpene	Indonesia	Aerial	Methanol extract	(Awale et al., 2003)
Orthosiphonone A-D	Isopimarane diterpene	Indonesia	Aerial	Methanol extract	(Awale et al., 2003)
Hexanal	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
trans-2-Hexanal	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
cis-3-Hexen-l-ol	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Hexan-1-ol	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
4-Heptenal	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)

Table 2.1 Cont.

Heptenal	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Benzaldehyde	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
α-Pinene	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Camphene	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
l-Octen-3-ol	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
β-Pinene	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
3-Octanol	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
2-Pentenyl furane	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
2-Amylfurane	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
p-Cymene	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
1,8-Cineol	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Limonene	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Acetophenone	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
cis-2-Octenal	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)

Table 2.1 Cont.

Phenylacetaldehyde	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
trans,cis-Octa-3,5-dien-2-one	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
cis-Linalooloxide	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
trans,trans-Octa-3,5-dien-2-one	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Linalool	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
trans-Linalooloxide	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Undecan	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
2,6,6-Trimethyl-2-cyclohexe-l,4-dione	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Perillen	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Camphor	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
δ-Terpineol	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
trans -2-(cis)-6-Nonadienale	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Menthone	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Isomenthone	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Methylchavicol	monoterpene and sesquiterpene	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)

Table 2.1 Cont.

Borneol	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Decanal	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Naphthalene	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Dodecane	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Cittonellol	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Carvone	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
β-Cyclocitral	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
trans-Anethol	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Isobornylacetat	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Safranal	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
1-Methylnaphthalene	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Bornyl acetate	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Tridecan	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
2-methylnaphthalene	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
trans, trans-Deca-2,4-dienal	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)

Table 2.1 Cont.

γ-Elemene	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
α-Cubebene	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Damascenone	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
α-Copaene	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
β-Bourbonene	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Eugenol	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
β-Elemene	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Methyleugenol	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
cis-Caryophyllene	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
β-Carryophyllene	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Geranylacetone	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
α-Humulene	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
β-Ionone	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Germacrene D	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
α-Muuiolene	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)

Table 2.1 Cont.

δ-Cadinene	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Germacrene B	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Dehydroionone	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Caryophyllene oxide	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Hexahydrofamesylacetone	monoterpene and sesquiterpene hydrocarbons	Malaysia	Leaves and stems	essential oils	(Hossain et al., 2008)
Sinensetin	Flavonoid	Vietnam	Aerial	Methanol extract	(Tezuka et al., 2000)
		Malaysia	Leaves	Methanol extract	(Loon et al., 2005)
		Malaysia	Leaves	Chloroform fraction	(Yam et al., 2010)
		Romania	Leaves	Ethanol-water extracts (50 and 70%)	(Olah et al., 2003)
Eupatorin	Flavonoid	Malaysia	Leaves	Methanol extract	(Akowuah et al., 2004)
		Malaysia	Leaves	Standardised extract	(Loon et al., 2005)
		Malaysia	Leaves	Chloroform fraction	(Yam et al., 2010)
3'hydroxy-5, 6, 7, 4'-tetramethoxyflavone (TMF)	Flavonoid	Malaysia	Leaves	Methanol, 25-75% methanol extracts and water extracts	(Siddiqui & Ismail, 2011)
		Malaysia	Leaves	Methanol extract	(Akowuah et al., 2004)

Table 2.1 Cont.

Tetramethylscutellarein	Flavonoid	Vietnam	Aerial	Methanol extract	(Tezuka et al., 2000)
Salvigenin	Flavonoid	Vietnam	Aerial	Methanol extract	(Tezuka et al., 2000)
Ladanein	Flavonoid	Vietnam	Aerial	Methanol extract	(Tezuka et al., 2000)
vomifoliol	Flavonoid	Vietnam	Aerial	Methanol extract	(Tezuka et al., 2000)
5-hydroxy-6,7,3',4'-tetra-methoxyflavone	Flavonoid	Vietnam	Aerial	Methanol extract	(Tezuka et al., 2000)
7,3',4'-tri-O-methylluteolin	Flavonoid	Vietnam	Aerial	Methanol extract	(Tezuka et al., 2000)
6-hydroxy-5,7,4'-trimethoxy-flavone	Flavonoid	Vietnam	Aerial	Methanol extract	(Tezuka et al., 2000)
Tetramethylether-scutellarein	Flavonoid	East Asean	Leaves	Methanol extract	(Lyckander & Maltreud, 1996)
Caffeic acid	Polyphenol	Indonesia	Leaves	50% methanol extract	(Sumaryono et al., 1991)
Cichoric acid	Polyphenol	Romania	Leaves	Ethanol-water extracts (50 and 70%)	(Olah et al., 2003)
Rosmarinic acid	Polyphenol	Malaysia	Leaves	Methanol extract	(Akowuah et al., 2004)
	Polyphenol	Indonesia	Leaves	50% methanol extract	(Sumaryono et al., 1991)
		Malaysia	Leaves	Methanol, 25-75% methanol extracts and water extracts	(Siddiqui & Ismail, 2011)
		Malaysia	Leaves	Methanol extract	(Akowuah et al., 2004)

Table 2.1 Cont.

	Polyphenol	Indonesia	Leaves	50% methanol extract	(Sumaryono et al., 1991)
		Malaysia	Leaves	Methanol, 25-75% methanol extracts and water extracts	(Siddiqui & Ismail, 2011)
2,3-Dicaffeoyl-tartaric acid	Polyphenol	Indonesia	Leaves	50% methanol extract	(Sumaryono et al., 1991)
Aurantiamide acetate	Dipeptide	Vietnam	Aerial	Methanol extract	(Tezuka et al., 2000)
Oleanolic acid	Triterpene	Vietnam	Aerial	Methanol extract	(Tezuka et al., 2000)
Ursolic acid	Triterpene	Vietnam	Aerial	Methanol extract	(Tezuka et al., 2000)
Betulinic acid	Triterpene	Vietnam	Aerial	Methanol extract	(Tezuka et al., 2000)
Hydroxybetulinic acid	Triterpene	Vietnam	Aerial	Methanol extract	(Tezuka et al., 2000)
Staminol A & B	Diterpene	Vietnam	Aerial	Methanol extract	(Tezuka et al., 2000)
Staminols C & D	Diterpene	Indonesia	Aerial	Methanol extract	(Stampoulis et al., 1999)
Secoorthosiphol A-C	Diterpene	Taiwan	Aerial	Methanol extract	(Nguyen et al., 2004)
		Japan	Aerial	Methanol extract	(Awale et al., 2002b)
Norstaminol A-C	Diterpene	Vietnam	Aerial	Methanol extract	(Tezuka et al., 2000)
Norstaminone A	Diterpene	Myanmar	Aerial	Methanol extract	(Awale et al., 2003)
Neoorthosiphone A	Diterpene	China	Aerial	Methanol extract	(Awale et al., 2004)

2.1.4 Quality assessment and standardisation of *O. stamineus*

Due to increasing demand on the use of *O. stamineus* as food supplement and botanical products, the quality assessment and standardisation of this herb should be conducted to ensure the safety and efficacy of these products in the market. The process should be conducted at the first stage of cultivation to the production for clinical formulation. World Health Organization (WHO) established guidelines for good agricultural practice (GACP) for medicinal plants to ensure reproducible quality of herbal medicinal products (WHO, 2003; Yadav & Dixit, 2008). According to WHO, quality assessment and standardisation of herbal medicines is a process involved in the physicochemical properties of raw materials covering various aspects including micro and macroscopic examination for adulteration, foreign organic matter, ash values, moisture content, extractive values, crude fibre, qualitative and quantitative chemical evaluation, chromatographic examination and toxicology studies (pesticide residues, heavy metals and microbial limit test) (WHO, 1998). The process involved scientific investigations including physical, chemical and biological evaluations with the aim to ensure the quality, safety and efficacy of herbal products in the market. In this study, the quality assessment of *O. stamineus* raw leaves was evaluated following the standard in Malaysian Herbal Monograph (MHM) (Monograph committee, 2009) before undertaking this study. Moreover, the standardisation of different extracts was further analysed using new developed and validated method using RA, TMF, SIN and EUP as marker compounds.

2.1.5 Pharmacological review of *O. stamineus*

Many studies have been conducted in order to justify the traditional uses of *O. stamineus*. Some of studies were conducted in different extracts of *O. stamineus* and some in selected fractions and pure compounds (Adnyana et al., 2013). It was reported as good anti-oxidative (Awale et al., 2001), diuretic and nephroprotective (Arafat et al., 2008; Awale et al., 2003a; Madhukar et al., 2010), anti-inflammatory (Ohashi et al., 1999), anti-bacterial and antimicrobial (Tezuka et al., 2000), cytotoxic, anti-proliferative and antiangiogenic (Adnyana et al., 2013), anti-hypertensive (Ohashi., 2000; Matsubara et al., 1999), antidiabetic (Indariani et al., 2014; Rao et al., 2014), anti-pyretic (Yam et al., 2009) and hepatoprotective (Chin et., 2009; Yam et al., 2007). The summary of the previous pharmacological studies of *O. stamineus*is shown in Table 2.2. In present study, ten standardised extracts from three extraction techniques and nano-formulated extract of *O. stamineus* leaves were subjected to evaluation of their antioxidant and cytotoxic activities.

Previous studies have analysed the antioxidant potential of *O. stamineus*. Akowuah et al., (2004) reported the antioxidant properties of methanolic extract of *O. stamineus* leaves from various locations. The evaluation of antioxidant properties was based on the *in vitro* β-carotene-linoleic acid system assay. In addition, the content of three flavonoids (sinensetin, eupatorin and 3'-hydroxy-5, 6, 7, 4’-tetramethoxyflavone) and a caffeic acid derivative (rosmarinic acid) were measured using a developed HPLC method. The results demonstrated significant variation in antioxidant activities related to variation for compounds. Therefore, it was concluded that presence of flavonoids and phenolic acids in *O. stamineus* extract are implicated to antioxidant activity.

Subsequently, study by Akowuah et al. (2005) reported the effect of different extraction solvents on free radical scavenging activity of leaves of *O. stamineus* using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay. All extracts showed significant free radical scavenging activity. In addition, contents of SIN, EUP, TMF and RA were analysed by HPLC method. The results demonstrated that free radical scavenging activity (DPPH assay) was poorly correlated with the concentration of the flavonoids and phenolics in extracts. Therefore, presence of other secondary metabolites and primary metabolites which may act as hydrogen donors in the plant materials might be responsible for this activity. Following years, Khamsah et al. (2006) reported the antioxidant activity of *O. stamineus* extracts might be due to presence of staminane-type diterpenes and triterpenes. They were reported that, there is no simple relationship between the antioxidant activity of *O. stamineus* extracts with the level of total phenolics and flavonoids component presence in the extracts. In another study by Yam et al., (2016) reported the evaluation of the antioxidant activity of methanolic extract (50%) of *O. stamineus* leaves. Superoxide radical scavenging, hydroxyl radical scavenging, and ferrous ion chelating methods were used to evaluate the antioxidant properties of the extract. The results indicated that the respective extract showed good superoxide radical scavenging, hydroxyl radical scavenging, and ferrous ion chelating

On 2010, Siddique et al., was reported the evaluation of primary metabolite contents (total proteins, total polysaccharides and glycosaponins), cytotoxicity and antiangiogenic activity of freeze dried and spray dried methanolic (50%) extract. The result showed freeze dried extract contain high content of proteins and glycosaponins while contents of polysaccharides were high in spray dried extracts. Concurrently, the