EVALUASI FARMAKOLOGI BAGI EKSTRAK ORTHOSIPHON STAMINEUS DAN PEMBANGUNAN METER ANALGESIK UNTUK MODEL TIKUS ARTRITIS

oleh

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Tesis yang diserahkan untuk
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PHARMACOLOGICAL EVALUATION OF ORTHOSIPHON STAMINEUS EXTRACT AND DEVELOPMENT OF ANALGESIC METER FOR ARTHRITIC RAT MODEL

by

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This thesis is dedicated to the poor rats and mice who were made to give so much sacrifice for the sake of knowledge

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LIST OF ABBREVIATIONS

µg	microgram
µl	microliter
µM	micromolar
µm	micrometer
4-HNE	4-hydroxyalkenals
ABTS	2,2'-azino-bis(3-ethy)benz-thiazoline-6-sulfonic acid
ACN	acetonitril
ALAT	alanine aminotransferase
ALP	alkaline phosphatase
ALT	alanine transaminase
ANOVA	analysis of variance
ASAT	aspartate aminotransferase
AST	aspartate aminotransferase
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
CCD	charge-coupled device
$CCl_3^{\bullet}$	trichlormethyl free radical
CCl_4	carbon tetrachloride
CMC	carboxymethylcellulose
EDTA	ethylenediaminetetraacetic acid
EUP	eupatorin
FCA	freund complete adjuvant
$FeCl_3$	ferric chloride
GST	glutathione-*S*-transferase
Hb	haemoglobin concentration
HCl	hydrochloric acid
HDL	high-density lipoprotein
Ht	haematocrit
IC_{50}	inhibition concentration 50%
ICH	international Conference on Harmonisation
IFN-γ	Interferon gamma
IL-1	interleukin 1
iNOS	Inducible nitric oxide synthase
IPA	isopropyl alcohol
KCl	Potassium chloride
kg	kilogram
LD_{50}	lethal dose 50%

LOD	limit of detection
LOQ	limit of quantification
LPO	lipid peroxidation
m	Meter
M	molar
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
MDA	malonaldehyde
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
MRC	methylripariochromene A
NaH_2PO_4	sodium phosphate
nm	nanometer
NO	nitric oxide
NOAEL	no observable adverse effect level
NOS	Nitric oxide synthase
OGTT	oral glucose tolerance test
PBS	phosphate buffered saline
$PGF_2\alpha$	prostaglandin $F_2\alpha$
r^2	correlation coefficient
RBC	blood cell count
RP-HPLC	reversed phase high performance liquid chromatography
S.E.M.	standard error of the mean
SD rat	Sprague Dawley rat
SEN	sinensetin
SEOS	50 % methanolic extract of *O. stamineus*
SGOT	glutamic oxaloacetic transaminase
SGPT	glutamic pyruvate transaminase
STZ	streptozotocin
TAA	total antioxidant activity
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TEAC	trolox equivalent antioxidant capacity
TMF	3'-hydroxy-5,6,7,4'-tetramethoxyflavone
TNF	tumor necrosis factor
UGT	UDP-glucoronosyl transferase
UV	ultraviolet
UV-Vis	ultraviolet-visible
v/v	volume by volume

w/w	weight by weight
WBC	white blood cell count

EVALUASI FARMAKOLOGI BAGI EKSTRAK ORTHOSIPHON STAMINEUS DAN PEMBANGUNAN METER ANALGESIK UNTUK MODEL TIKUS ARTRITIS

Abstrak

Orthosiphon stamineus Benth. merupakan genus herba famili Labiatae atau Lamiaceace yang digunakan secara tradisional untuk rawatan pelbagai jenis penyakit seperti nefritis, nefrolitiasis, hidronefrosis, kalkulus vesikal, arteriosklerosis, penyakit sendi, radang, gout dan kencing manis. Walaupun terdapat ubat moden, penggunaan perubatan tradisional semakin meningkat di seluruh dunia dan ini menunjukkan keperluan untuk kajian saintifik terhadap kesan terapeutik tumbuhan ubat-ubatan dan mekanisme asasnya. Walaupun penyiasatan anti-ulser, anti-radang, anti-piretik, analgesik, kesan hepatoprotektif dan toksikologi bagi *O. stamineus* telah digunakan secara meluas, penyiasatan ini bertujuan untuk mencirikan lebih lanjut tuntutan tradisional daun *O. Stamineus*. Daun *O. Stamineus* dikeringkan, dikisar dan diekstrak dengan 50% metanol melalui kaedah meserasi. Ekstrak dikeringkan di bawah tekanan terturun dan kemudian dibeku-kering. Hasil ekstrak metanol *O. stamineus* 50% (SEOS) adalah 6%. Analisis HPLC menunjukkan bahawa SEOS mengandungi 0,46%, 1.12%, dan 0,94% 3'-hydroxy-5, 6,7,4 '-tetrametoksiflavon, sinensetin dan eupatorin, masing-masing.

Dalam kajian ketoksikan akut, kaedah atas dan bawah (had dos) telah disesuaikan. Satu dos 5000 mg/kg SEOS telah diberikan secara oral kepada 5 ekor tikus betina dan jantan dewasa normal jenis Sprague Dawley (SD). Permerhatian dilakukan selama 3 jam dan seterusnya secara berkala selama 14 hari untuk melihat tanda-tanda klinikal dan kematian. Dalam kajian

ketoksikan subkronik, ekstrak telah diberikan secara oral pada dos 1250, 2500 dan 5000 mg/kg masing-masing setiap hari selama 28 hari kepada tikus SD jantan dan betina, masing-masing. Semua haiwan tersebut dikorbankan, dan kemudian dilakukan pemeriksaan organ-organnya dan serum darah. Keputusan dalam kajian akut menunjukkan bahawa SEOS pada dos 5000 mg/kg tidak menyebabkan tanda-tanda ketoksikan yang nyata mahupun kematian. Kesemua lima tikus masih hidup lagi sehingga akhir tempoh pemerhatian. Semas kajian subkronik pemberian SEOS pada 1250, 2500, dan 5000 mg/kg selama 28 hari tidak menyebabkan kematian dan tidak terdapat perbezaan yang signifikan dalam keadaan umum, pertumbuhan, berat organ, parameter hematologi, nilai kimia klinikal dan penampilan makroskopik organ daripada kumpulan rawatan berbanding dengan kumpulan kawalan.

Perangkapan radikal DPPH, aktiviti prencatan peroksidaan lipid teraruh Fe^{3+} dan kemampuan antioksidan setrara trolox (TEAC) SEOS telah ditentukan. Keputusan menunjukkan bahawa SEOS mempunyai aktiviti perencatan peroksidaan lipid dan aktiviti perangkapan radikal bebas. Aktiviti hepatoprotektif SEOS telah dikaji dengan menggunakan ketoksikan hati teraruh CCl_4 dalam tikus. Aktiviti tersebut ditentukan melalui pemantauan ujian fungsi hati dengan kajian dan pengukuran histopatologi, alanine transaminase (ALT) dan aspartate transaminase (AST). SEOS pada dos 1000 dan 500 mg/kg merencat peningkatan serum ALT dan merencat AST denangan ketara dan mencegah nekrosis hati.

Suatu model lesi membran gastrik aruhan etanol absolut telah digunakan dalam kajian anti-ulser. Pemberian SEOS secara oral (125, 250, 500 dan 1000 mg/kg) mengurangkan indeks ulser secara signifikan (P<0.01, P<0.001, P<0.001, P<0.001, masing-masing). Kajian histologi suatu keratin perut tikus juga

menunjukkan pemulihan yang ketara dalam kerosakan mukosa perut pada kumpulan yang menerima SEOS. Dalam kajian selanjutnya untuk menyiasat mekanisme gastroprotektif SEOS, rembesan mukus dan tahap peroksidaan lipid telah dianggarkan secara *in vitro* dan *ex vivo*. SEOS menunjukkan perangsangan tergantung dos rembesan mukus dan perencatan peroksidaan lipid dalam homogenate gastrik mukosa perut tikus (*in vitro* dan *ex vivo*). Aktiviti antipiretik SEOS dikaji untuk kesannya terhadap suhu tubuh normal dan pireksia aruhan yis dalam tikus SD. SEOS tidak menunjukkan kesan ke atas suhu tubuh normal. Dos SEOS 500 dan 1000 mg/kg mengurangkan peningkatan suhu tubuh teraruh yis secara signifikan. Kesan ini berlanjut sehingga 4 jam berikutan pemberian ekstrak. Kesan antipiretik SEOS adalah setanding dengan parasetamol (150 mg / kg po) iatiu suatu agen antipiretik piawai.

Aktiviti anti-radang, anti-artritis dan analgesik SEOS telah dikaji dalam model haiwan. Pemberian oral SEOS pada dos 500 hingga 1000 mg/kg mengurangkan edema kaki belakang tikus secara signifikan pada 3 dan 5 jam selepas pemberian karagenan ($P<0.01$ dan $P<0.01$; $P<0.01$ dan $P<0.05$, masing-masing). SEOS (1000 mg/kg) juga mengurangkan garis pusat buku lali secara signifikan ($P<0.05$) dan meningkatkan daya pijakan tikus artritis aruhan FCA. Daya pijakan tikus diukur dengan meter analgesik yang telah dibangunkan. Meter analgesik ini dilengkapi kamera inframerah dan mampu merakam setiap gerakan tikus dan menyelaraskan gerakan itu dengan daya pijakan yang dilakukan oleh tikus. Dengan bantuan meter analgesik tersebut setiap langkah gerakan tikus dapat dibezakan dengan jelas. Disamping itu, SEOS (1000 mg/kg) juga menghasilkan yang aktiviti signifikan ($P<0.05$) dalam kedua-dua ujian geliat aruhan asid asetik dan ujian jilatan aruhan formalin (fasa lewat) dalam mencit dan tikus,

masing-masing. Walau bagaimanapun, SEOS tidak menunjukkan sebarang kesan ke atas ujian jentikan ekor dan ujian plat panas pada muncit. Keputusan kajian ini menyokong hipotesis bahawa *O. stamineus* mempunyai aktiviti anti-radang dan aktiviti analgesik bukan narkotik. Keputusan farmakologi menunjukkan bahawa *O. stamineus* mempunyai aktiviti antioksidan, hepatoprotektif, gastroprotektif, anti-artritis, aktiviti anti-radang dan analgesik bukan narkotik. Kajian toksikologi menujukkan bahawa tiada ketoksikan akut ataupun subkronik dalam tikus yang telah diberikan *O. stamineus*, ini mungkin tidak mempunyai sebrang risiko toksik. NOAEL untuk *O. stamineus* adalah 5000 mg/kg sehari untuk 28 hari.

PHARMACOLOGICAL EVALUATION OF ORTHOSIPHON STAMINEUS EXTRACT AND DEVELOPMENT OF ANALGESIC METER FOR ARTHRITIC RAT MODEL

Abstract

Orthosiphon stamineus Benth. is a genus of herb of the family *Labiatae* or *Lamiaceace* traditionally used for treatment of many diseases such nephritis, nephrolithiasis, hydronephrosis, vesical calculi, arteriosclerosis, rheumatism, inflammation, gout and diabetes. Despite the availability of modern medications, the use of traditional medicine is growing throughout the world, indicating a need for scientific investigations into the therapeutic effects of medicinal plants and their underlying mechanisms. While no previous investigation has thoroughly reported its pharmacological activities such as anti-ulcer, anti-inflammatory, anti-pyretic, analgesic, hepatoprotective and toxicological effect of such a widely used medicinal herb, this investigation set out to further characterize the traditional claims. The *O. Stamineus* leaves were dried, pulverized and successively extracted with 50% methanol using maceration method. The extract was dried under reduced pressure and freeze-dried. The yield of lyophylized 50 % methanolic extract of *O. Stamineus* (SEOS) was found to be 6%. HPLC analysis showed that SEOS contains 0.46%, 1.12%, and 0.94% of 3'-hydroxy-5,6,7,4'-tetramethoxyflavone, sinensetin and eupatorin, respectively.

In acute toxicity study, up and down method (limit dose) was adapted. A single dose of 5000 mg/kg of SEOS was given orally to 5 healthy Sprague Dawley (SD) male and female adult rats. The rats were observed for mortality and clinical signs for 3 h and then periodically for 14 days. In the subchronic toxicity study, the

extract was administered orally at doses of 1250, 2500 and 5000 mg/kg per day for 28 days to female and male SD rats, respectively. The animals were sacrificed, followed by examination of their organs and blood serum. The results in the acute study showed that SEOS at a dose of 5000 mg/kg caused neither visible signs of toxicity nor mortality. All five rats survived until the end of observation period. While in subchronic toxicity, administration of the SEOS at 1250, 2500, and 5000 mg/kg for 28 days did not produce any mortality and there were no significant differences in the general condition, growth, organ weights, hematological parameters, clinical chemistry values and macroscopic appearance of the organs from the treatment groups as compared to the control group.

DPPH radicals scavenging, Fe^{3+}-induced lipid peroxidation inhibiting activities and trolox equivalent antioxidant capacity (TEAC) of SEOS were determined. The results indicated that SEOS exhibited antioxidant, lipid peroxidation inhibition and free radical scavenging activities. The hepatoprotective activity of the SEOS was studied using CCl_4-induced liver toxicity in rats. The activity was assessed by monitoring liver function tests in histopathological study and measurement of alanine transaminase (ALT) and aspartate transaminase (AST). SEOS at the dose of 1000 and 500 mg/kg significantly inhibited the increase of serum ALT and AST activities and prevent the liver necrosis.

Absolute ethanol-induced gastric membrane lesions model was used in anti-ulcer study. Oral administration of SEOS (125, 250, 500 and 1000 mg/kg) was found to significantly decrease the ulcer index (P<0.01, P<0.001, P<0.001, and P<0.001, respectively). Histological study of a section of the rat stomach also showed a marked improvement in the gastric mucosal damage in groups receiving SEOS. In order to further investigate the gastroprotective mechanism of SEOS,

mucus secretion and lipid peroxidation level were estimated *in vitro* and *ex vivo*. SEOS exhibited dose-dependent stimulation of mucus secretion and inhibition of lipid peroxidation in rat gastric mucosal homogenates (both *in vitro* and *ex vivo*).

The anti-pyretic activity of SEOS was investigated for its effect on normal body temperature and yeast-induced pyrexia in SD rats. The SEOS showed no effect on normal body temperature. Doses of 500 and 1000 mg/kg body weight of SEOS significantly reduced the yeast-induced elevation in body temperature. This effect persisted up to 4 hours following the administration of the extract. The anti-pyretic effect of SEOS was comparable with that of paracetamol (150 mg/kg p.o.), a standard anti-pyretic agent. Anti-inflammatory, anti-arthritic and analgesic activities of SEOS were evaluated in animal models. Oral administration of SEOS at doses of 500 and 1000 mg/kg significantly reduced the hind paw edema in rats at 3 and 5 hours after carrageenan administration ($P<0.01$ and $P<0.01$; $P<0.01$ and $P<0.05$, respectively). SEOS (1000 mg/kg) also significantly ($P<0.05$) reduced the ankle diameter and increased the stepping force of Freund complex adjuvant (FCA)-induced arthritic rat. The stepping force was measured using a novel developed analgesic meter. The apparatus was fabricated with a built-in infrared CCD camera integrated within the analgesic meter. This camera captures the locomotion of the rats and synchronizes the stepping force concurrently. Using this feature, the steps produced by the rat can be correctly identified and the stepping force caused by the front paw can be differentiated from that of the hind paw. Moreover, SEOS (1000 mg/kg) also produced significant ($P<0.05$) activity in both the acetic acid-induced writhing test and the formalin-induced licking test (late phase) in mice and rats, respectively. However, SEOS showed no effect on the tail flick and hot plate tests in mice.

The results of the present study support the hypotheses that *O. stamineus* has anti-inflammatory and non-narcotic analgesic activities. The pharmacological results suggest that *O. stamineus* has antioxidant, hepatoprotective, gastroprotective, anti-arthritic, anti-inflammatory and non-narcotic analgesic activities. Toxicology study revealed that no acute or subchronic toxicity in *O. stamineus* treated rat was observed and this plant could be devoid of any toxic risk. The NOAEL for the *O. stamineus* is 5000 mg/kg per day for 28 days.

CHAPTER 1

LITERATURE REVIEW

1.1 Botanical aspect of *Othrosiphon stamineus*

Orthosiphon stamineus Benth. is a genus of herb of the family *Labiatae* or *Lamiaceace*. Generally, it is found in Africa and from South Eastern Asia to the Pacific. [syn: *Orthosiphon aris-tatus* (B1) Miq., *Orthosiphon grandiflorus* Bold., *Orthosiphon spicatus* (Thumb) Bak.] (Burkill, 1966, Awale et al., 2002b, Perry, 1980).

Originally, the genus name *Orthosiphon* was basically coined from two Latin words, *lorthos* and *siphon*. The word *lorthos* referred to straight while *siphon* meant tube-like or cylindrical. These two words jointly referred to the straight tube-like flowers produced by the *Orthosiphon* spp., one of the main characteristic features of the *Labiatae* or *Lamiaceae* family (Burkill, 1966). Originally, the genus name *Orthosiphon* was basically coined from two Latin words, *lorthos* and *siphon*. The word *lorthos* referred to straight while *siphon* meant tube-like or cylindrical. These two words jointly referred to the straight tube-like flowers produced by the *Orthosiphon* spp., one of the main characteristic features of the *Labiatae* or *Lamiaceae* family (Wiart, 2000). The herb grows well on wet soil and can be found in both temperate and tropical gardens (Hsuan, 1986). It is generally propagated vegetatively by cuttings of the mature stem. Therefore, the plant is considerably distributed in countries with those aforementioned climates conditions.

It is found from India and China to tropical Australia and the Pacific; in the Malaysia peninsula it occurs wild in the North and in gardens elsewhere (Burkill, 1966). This herb is known by its vernacular or traditional local names based on its anecdotal heritage in that particular region or country. For instance Java tea (UK), Rĕmuk jung (Java), moustaches de chat (Jaganth and Ng, 2000) or thé de Java (Akanae et al., 2010) (France), or Neko no hige (Awale et al., 2002b) (Japan), ruku hutan (woodland patchouli), balbas-pusa and kabling-gubat (Philippines), kapen prey (Cambodia), hnwàd méew (Laos), yaa nuat maeo (Thailand), r[aa]u m[ef]o in Vietnam (Burkill, 1966, Akanae et al., 2010), misam kucing in Malaysia and Singapore (Burkill, 1966, Lee and Chan, 2004a, Lee and Chan, 2004b, Akanae et al., 2010).

The Malaysian cat's whiskers are believed to consist of two varieties based on floral and calyx colors and leaf characteristics (Lee, 2004) which are not very distinct if not carefully observed. One of the varieties produces white flowers (Figure 1A) while the other one gives corolla with light purplish tint at the edges of the petal lobes (Figure 1B), hence named as the white and purple varieties, respectively. The purple variety was reported to possess higher bio-active compounds than the white variety (Lee, 2004) which are not very distinct if not carefully observed.

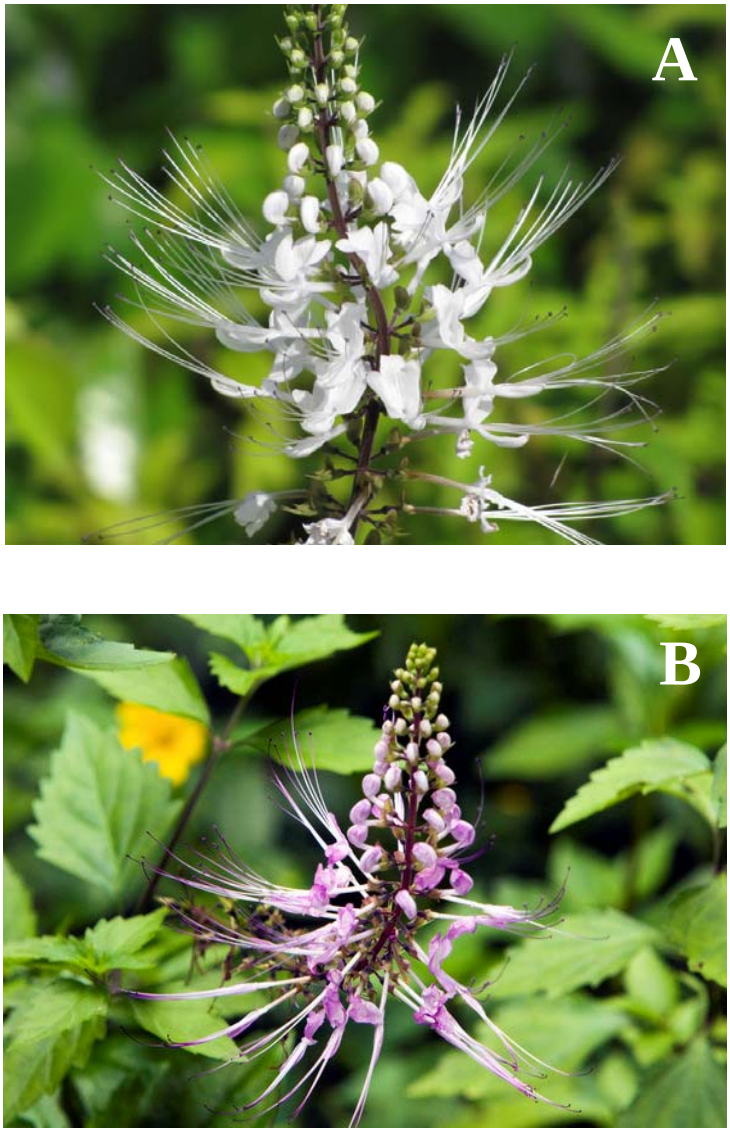

Figure 1.1. *Orthosiphon stamineus* varieties. (A) *O. stamineus* white variety and (B) *O. stamineus* purple variety.

1.2 Traditional uses

O. stamineus is one of the popular traditional folk medicines extensively used in Southeast Asia for the treatment of a wide range of diseases:

From Taiwan south to Pulau, an infusion or tea of the leaves from wild or cultivated plants of *O. stamineus* is used as diuretic. Van der Sleesen stated that its use is almost universal in Indonesia but less so in the Philippines (Perry, 1980). Moreover, in Indonesia, it is locally known as one of *jamu* ingredients, a traditional functional beverage (Mardisiswojo and Rajakmangunsudarso, 1975), used for rheumatism, diabetes, hypertension, tonsillitis, epilepsy, menstrual disorder, gonorrhoea, syphilis, renal calculus and gallstone (Bwin and Gwan, 1967, Awale et al., 2003a, Awale et al., 2003b).

In Java, it is not used alone but with other plant ingredients which stimulate the kidneys. It contains of glucoside, orthosiphonin and high percentage of potassium salts which themselves act on the kidneys (Burkill, 1966).

In Vietnam, it is used for urinary lithiasis, edema, eruptive fever, influenza, hepatitis, jaundice and biliary lithiasis and in Myanmar it is commonly employed to alleviate diabetes, urinary tract and renal diseases (Tran, 1970). In Malay Peninsula, it is used as a remedy for catarrh of the bladder, the leaves act as diuretic and does not cause injury to the kidneys and was admitted into the fourth Dutch Pharmacopoeia (Burkill, 1966). It is used as a folk medicine for various disorders such as nephritis, nephrolethiasis, hydronephrosis, vesical calculi, arteriosclerosis, gout and rheumatism. The latter three ills are mentioned by Van

Steenins-Kruseman; the latter two as well as gallstones and diabetes by Kloppenburg-Versteegh (Perry, 1980).

The leaves have been introduced to Europe and Japan as a health tea (Lee and Chan, 2004a). As it has been the subject of experiments in Germany and was found to deserve its reputation, for its diuretic effect surpasses that of ordinary diuretics (Englert and Harnischfeger, 1992, Matsubara et al., 1999)

Additionally, owing to its beneficial pharmaceutical utility, it is under systematic cultivation in Okinawa Prefecture, Japan and consumed as a healthy Java tea to facilitate body detoxification (Awale et al., 2002b). A wealth of information has been generated in the literature regarding this species. The crude herb is said to cause vomiting. Early reported constituents are a glucoside, orthosiphonin; leaves and stems have a high potassium content, urea and urides (Perry, 1980) while recent literatures have reported the presence of many classes of natural compounds mainly polymethoxylated flavonoids, terpenoids and caffeic acid derivatives. To the best of our knowledge, many of these natural constituents are not novel compounds and have been reported elsewhere in other plants. However, some have been isolated for the first time.

1.3 Phytochemical studies

Recent investigations on *O. stamineus* chemical profiling have revealed that the major constituents present in various extracts of this plant can broadly be categorized into three components, namely polymethoxylated flavonoids (Lyckander and Malterud, 1996, Hollman and Katan, 1999) caffeic acid derivatives

(polyphenols) (Olah et al., 2003, Loon et al., 2005) and terpenoids (mainly diterpenes and triterpenes) (Masuda et al., 1992a, Tezuka et al., 2000, Awale et al., 2001, Awale et al., 2002c).

The most prominent flavonoids that have been isolated and/or identified from the hydro-alcoholic extract of *O. stamineus* leaves include sinensetin, eupatorin, 3'-hydroxy-5,6,7,4'-tetramethoxyflavones (Pietta et al., 1991, Yam et al., 2009b, Mohamed et al., 2011a) tetramethylscutellarein (Pietta et al., 1991) salvegenin, ladanein, vomifoliol, 7,3',4'-tri-O-methylluteolin, and scutellarein tetramethylether (Takeda et al., 1993, Lyckander and Malterud, 1996, Malterud and Rydland, 2000, Tezuka et al., 2000).

Among the famous constituents of *O. stamineus* is a group of organic acids known as caffeic acid derivatives. Major derivatives of caffeic acid are predominantly present in the aqueous extract of *O. stamineus* and these involve caffeic acid, rosmarinic acid (Sumaryono et al., 1991, Olah et al., 2003), cichoric acid (Olah et al., 2003), 2,3-dicaffeoyltartaric acid (Sumaryono et al., 1991).

On the other hand, a large number of terpenoid constituents have been characterized in this plant by chemical and spectroscopic methods. For instance, orthosiphols A-H (Awale et al., 2002b, Awale et al., 2003b, Awale et al., 2003a, Awale et al., 2001, Masuda et al., 1992a, Stampoulis et al., 1999a, Awale et al., 2002c, Awale et al., 2002a, Nguyen et al., 2004), staminols A–D (Stampoulis et al., 1999b, Tezuka et al., 2000, Awale et al., 2003a, Nguyen et al., 2004), staminolactones A and B (Stampoulis et al., 1999a, Ohashi et al., 2000b, Ohashi et al., 2000a), norstaminols A–C (Awale et al., 2002b, Awale et al., 2003a, Tezuka et

al., 2000, Stampoulis et al., 1999b), siphonols A–E (Awale et al., 2003a, Awale et al., 2003d) and many other diterpenes have been characterized from this plant (Figure 1.2).

Recently, seven triterpenes namely ursolic acid, oleanolic acid, betulinic acid, hydroxybetulinic acid, maslinic acid, α-amyrin and β-amyrin have been isolated from the leaves of the Malaysian *O. stamineus* with α-amyrin isolated from this plant for the first time (Hossain and Ismail, in press). Apart from diterpenoids and triterpenes, oils from *O. stamineus* contain a complex mixture consisting of mainly oxygenated monoterpene and sesquiterpene hydrocarbons. For example, β-caryophyllene, α-humulene, β-elemene, 1-octen-3-ol, β-bourbonene, β-pinene, caryophyllene oxide, camphene and limonene were identified as the major compounds obtained from the hydrodistilled essential oils of leaves and stems of the Malaysian *O. stamineus* (Hossain et al., 2008). In contrast, α-Pinene, 1,8-cineol, borneol, linalool, camphor, eugenol, *p*-cymene, carvone, bornyl acetate and δ-cadinene were reported as minor components of *O. stamineus* leaves and stem oils (Hossain et al., 2008) (Figure 1.2).

Further investigations have reported the presence of other classes of naturally-occurring constituents in *O. stamineus* such as saponins, hexoses, chromene and myo-inositol (Malterud et al., 1989, Tezuka et al., 2000, Olah et al., 2003) and sterols like β-sitosterol (Tezuka et al., 2000). Kannappan *et al.* (2010) reported the presence of flavonoids, phenols, carbohydrates, steroids, tannins, glycosides, terpens and saponins but the absence of alkaloids, gums and mucilage in the methanolic extract of Indian *O. stamineus* (Kannappan et al., 2010).

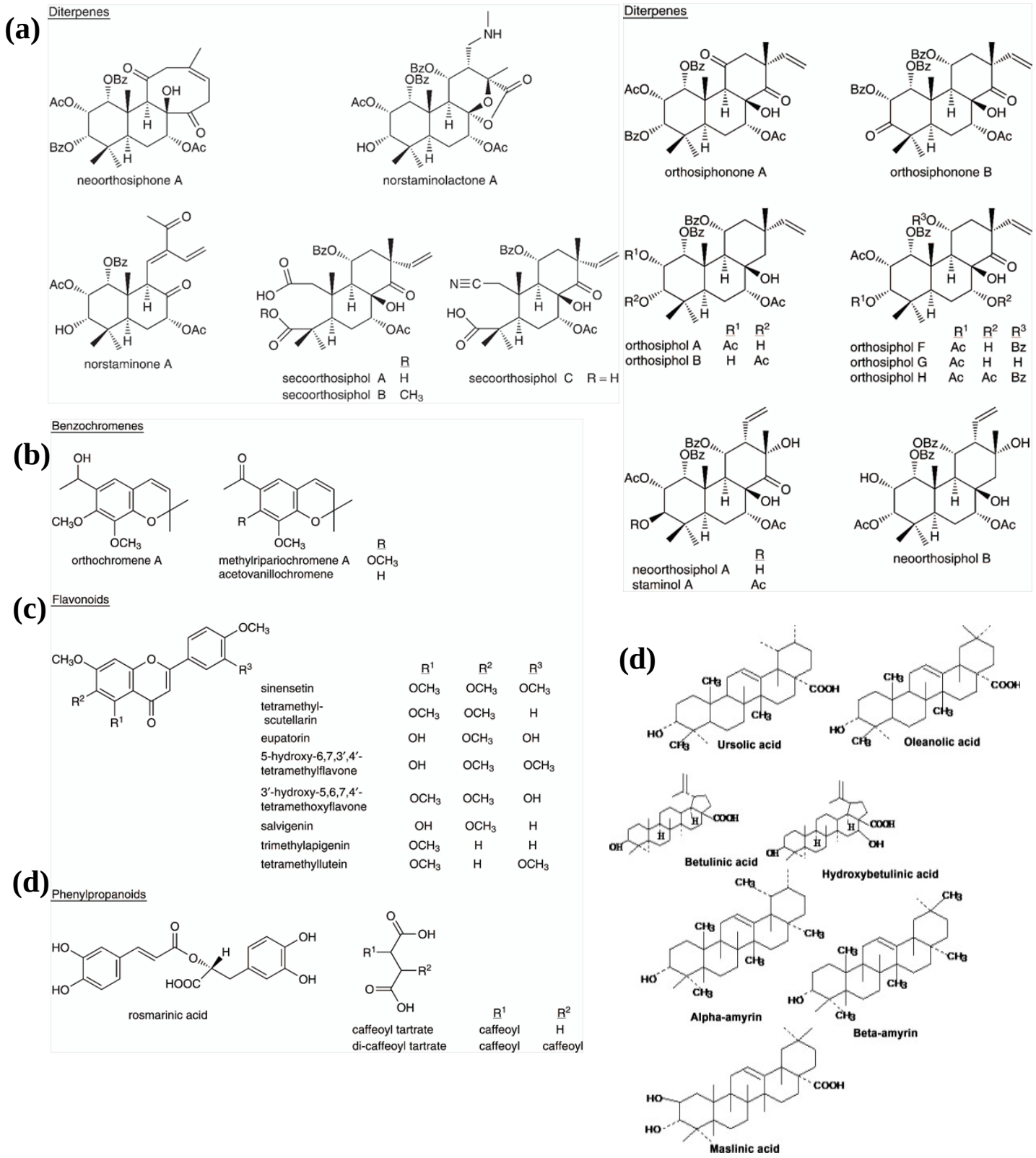

Figure 1.2. Chemical structure of isolated compounds from *O. stamineus* (a) diterpenes; (b) benzochromenes; (c) flavonoids; (d) phenylpropanoids; (e) other.

1.4 Pharmacological studies

Many investigations on *O. stamineus* have been conducted and are currently extrapolated around the world to justify its huge traditional and folk uses. This medicinal herb has been a crucial source of many novel components that have evidently been proven to alleviate modern diseases.

1.4.1 Diuretic, hypouricemic and antistone activities

A study on the diuretic, saluretic and uricosuric actions of 50% and 70% ethanol extracts of *O. stamineus* from Germany on white Wistar Bratislava male rats (Olah et al., 2003) revealed some interesting results. It was found that the 50% ethanolic extract of the herb has a better diuretic action than the 70% ethanolic extract. Moreover, the former extract eliminated better the sodium than the later or furosemide (a high ceiling loop diuretic drug used as a control), and it preserved the potassium for body better than furosemide or the 70% ethanolic extract. Furthermore, the experiments on the same extract (50% ethanol) indicated a very good elimination of uric acid. Olah *et al.* (2003) concluded from this study that the more polar extract (50% ethanolic extract) has better diuretic and uricosuric actions compared to the less polar one (70% ethanolic extract). They ascribed the effect of the 50% ethanolic extract to its higher contents of caffeic acid derivatives (except for rosmarinic acid), polymethoxylated flavonoids from 70% ethanolic extract. Thus, on the basis of these results, the high polyphenolic contents of *O. stamineus* may contributes to its diuretic and beneficial effects in gout treatment.

Another group of researchers used a modified Schneider's gel slide method, image analysis method and multivariate techniques of principle component analysis and self-organizing map to monitor the inhibitory effect of a 50% methanol extract of Malaysian *O. stamineus* on the growth of calcium oxalate crystals, major contributors to urinary stones (Dharmaraj et al., 2006). They concluded that compounds in the hydroalcoholic extract of *O. stamineus* possessed a prominent inhibitory effect on growth and morphology of calcium oxalate stones. Arafat *et al.* (2008) reported that the hydroalcoholic extract of Malaysian *O. stamineus* produced marked diuretic, natriuretic, kaliuretic and hypouricemic effects when administrated orally to Sprague–Dawley (SD) rats using acute and chronic regimens (Arafat et al., 2008).

Diuretic action is a key factor in kidney stone treatment since an increase in the volume of fluid flowing through the kidney helps to dissolve the stones, assist their passing to avoid further retention, and flush out the deposits (Modlinger and Welch, 2003a). (Modlinger and Welch, 2003b). A number of researchers have studied the adenosine A1 receptor binding effect of *O. stamineus* from the Netherland as A1 receptor antagonists which are able to enhance renal water and sodium excretion (Khatib et al., 2009, Yuliana et al., 2009). In these investigations, they related the diuretic action of *O. stamineus* to the presence of several methoxyflavonoids, fatty acids and/or terpenoids obtained through a combination of thin layer chromatography of different extracts prepared by extraction with diverse solvents (*n*-hexane, chloroform, *n*-butanol and water), and multivariate data analysis based on orthogonal partial least squares. They concluded that a possible

pathway by which *O. stamineus* constituents could have induced diuresis and natriuresis was by adenosine A1 receptor antagonistic activity.

It has further been reported that a water extract of *O. stamineus* from Malaysia, administered orally to SD rats, exhibits a dose-dependent diuretic activity that is associated with minor increases in sodium and chloride excretions, yet markedly elevated urinary excretion of potassium (Adam et al., 2009). Water extract of *O. stamineus* has recently been found to cause a slight increase in the serum BUN, creatinine and blood glucose levels. However these levels were still within the normal range of the measured parameters (Adam et al., 2009). It is noteworthy to mention that in this study the authors have purposely used the water extract of *O. stamineus* rather than alcoholic extract since the former is the most likely used extract by people seeking therapeutic benefits. In addition, they have addressed the issue that the water extract is still less potent compared to furosemide and hydrochlorthiazide and acts via different mechanisms to bring about diuresis. Lacking of robust tools to elucidate the exact mode of action, Adam *et al.* attributed the diuretic action of this water extract to its rich electrolyte content, or the presence of group of active compounds that might act individually or synergistically to promote vasodilation, or might be to unknown secondary active metabolite that can cause diuresis. Despite those discrepancies, they have proved that the activity of the most likely used *O. stamineus* water extract comes with the agreement of the traditional uses of this plant in dysuria treatment.

Doan *et al.* assessed the diuretic effect of four traditional Vietnamese herbal remedies including *O. stamineus* on 40 healthy volunteers aged 18 to 27 years, on

the basis of a claimed increase of diuresis through daily triple oral doses of *O. stamineus* water extract for 2 weeks period. No influence was recorded for the 12- and 24-hr urine output or on the sodium excretion for the extract when tested under standardized conditions in a placebo controlled double-blind crossover model (Doan et al., 1992). However, the authors indicated that there was an impact of the external factor of temperature logged during the trial with *O. stamineus*. Additionally, it could be attributed to the use of a water extract of *O. stamineus* rather than a hydroalcoholic one.

1.4.2. Anti-inflammatory

Natural compounds isolated from *O. stamineus* cultivated in different parts of Asia have been found to possess an inhibitory action on nitric oxide (NO). NO is an important signaling molecule that acts in many tissues to regulate a diverse range of physiological processes. When certain cells are activated by specific proinflammatory agents such as endotoxin, tumor necrosis factor (TNF), interferon-gamma (IFN-γ), and interleukin-1 (IL-1), NO is produced by inducible nitric oxide synthase (iNOS) and acts as a host defense by damaging pathogenic DNA and as a regulatory molecule with homeostatic activities (Kuo et al., 2008). However, excessive production has detrimental effects on many organ systems of the body leading to tissue damage, even leading to a fatal development such as septic shock (Vincent et al., 2000). Therefore, effective inhibition of NO accumulation by inflammatory stimuli presents a beneficial therapeutic strategy.

The ability of compounds isolated from *O. stamineus* to block NO synthesis has been verified using a variety of non-selective NOS inhibitors. For instance, Awale *et al.* compared the NO inhibitory action of some *O. stamineus* constituents to a number of positive controls such as N^G-monomethyl-l-arginine (L-NMMA), polymixin B and dexamethasone using lipopolysaccharide (LPS)-activated macrophage-like J774.1 cells. Interestingly, orthosiphols A, B, D, X (Awale et al., 2003c), H, K, M and N, 7-*O*-deacetylorthosiphol B, 6-hydroxyorthosiphol B, 3-*O*-deacetylorthosiphol I, 2-*O*-deacetylorthosiphol J, neoorthosiphols A and B, norstaminol A (Awale et al., 2003a), siphonols A, B, C, D and E(Awale et al., 2003a, Awale et al., 2003d) staminols A, B (Awale et al., 2003a), C and D, orthosiphonone C and D, 14-deoxo-14-*O*-acetylorthosiphol Y, 2-*O*-deacetylorthosiphonone A (Nguyen et al., 2004) and neoorthosiphonone A (Awale et al., 2004) evidently inhibited NO production. Moreover, the NO inhibitory activity in endotoxin-activated macrophages by the diterpenes further verifies the antiinflammatory activity of *O. stamineus* (Masuda et al., 1992b).

1.4.3. Antioxidant, hepatoprotective and nephroprotective activities

Akowuah *et al.* demonstrated the antioxidative potency of *O. stamineus* methanol extract from different parts of Malaysia. It was found that these methanolic extracts were comparable to that of the pure quercetin and synthetic antioxidant butylated hydroxylanisole (BHA) in their antioxidant capacity. Furthermore, they attributed this activity to the high phenolic contents of *O. stamineus* (Akowuah et al., 2004).

In another comparable investigation, Akowuah *et al.* screened various extracts of *O. stamineus* for free radical-scavenging potential using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) *in vitro* model. The extracts exhibited significant radical-scavenging activity and the acetone extract showed the highest activity amongst water, methanol and chloroform extracts of *O. stamineus* (Akowuah et al., 2005).

Interactions between modern drugs and *O. stamineus* have not been largely explored. The liver is considered as a major organ for metabolism. Thus, Han *et al.* (2009) examined the effect of a 14-day oral administration of methanol leaf extract of Malaysian *O. stamineus* on hepatic phase I and phase II drug metabolising enzymes using streptozotocin-(STZ)-induced diabetic female SD rats. Aminopyrine, p-nitrophenol (pNP) and 1-chloro-2,4-dinitrobenzene (CDNB) were used as a substrate to monitor cytochrome P450-mediated N-demethylase, UDP-glucoronosyl transferase (UGT) and glutathione-*S*-transferase (GST) activity respectively in rat liver. It was observed that methanol leaf extracts of *O. stamineus* was able to increase both UGT and GST activity in diabetic rat liver (Han et al., 2009).

Apart from the antioxidant and hepatoprotective effects of *O. stamineus*, a group of investigators studied the nephroprotective activity of Indian *O. stamineus* in a rat animal model (Kannappan et al., 2010). The nephroprotective effect of *O. stamineus* methanol extract was challenged against gentamycin-induced nephrotoxicity. Renal functional parameters (serum creatinine, blood urea and urinary protein) and the extent of renal damage manifested by the histopathological sections were markedly alleviated in the extract-treated renal failure rats. Based on

these observations, Kannappan *et al.* (2010) concluded the presence of renoprotective elements in *O. stamineus* methanol extract (Kannappan et al., 2010).

1.4.4 Hypoglycemic, hypolipidimic and antihypertensive activities

Preliminary investigations were carried out by Mariam *et al.* (1996) to evaluate the effect of an aqueous extract of Malaysian *O. stamineus* on blood glucose levels in both normal and diabetic rats. Interestingly, *O. stamineus* aqueous extract was found to possess hypoglycemic and antihyperglycemic properties when orally administered to normal and STZ-induced diabetic rats, respectively (Mariam et al., 1996).

Sriplang *et al.* (2007) studied the effects of a 14-day oral treatment with an aqueous extract of *O. stamineus* from Thailand on plasma glucose concentration and lipid profile in normal and STZ-induced diabetic male Wistar rats. They found that the oral glucose tolerance test (OGTT) of the aqueous extract of *O. stamineus* dose-dependently reduced plasma glucose concentration in euglyceamic and hyperglycaemic animals. Moreover, the extract plasma glucose lowering effect was close to that of glibenclamide, a standard sulfonylurea antidiabetic drug. However, one drawback on this study was the use of a positive control for the treatment of type 2 diabetes in rat model of type 1 diabetes. Sriplang *et al.* (2007) further demonstrated a significant plasma triglyceride lowering effect in the extract-treated diabetic rats with no evident change in the cholesterol levels. By contrast, plasma HDL-cholesterol concentration was significantly higher in diabetic rats treated with

the extract. In perfused rat pancreas, the extract did not increase insulin secretion in the presence of glucose but rather potentiated glucose-induced insulin secretion. Collectively, findings of Sriplang *et al.* (2007) have clearly suggested that *O. stamineus* aqueous extract is effective for alleviating the signs and symptoms of hyperglycemia and improving lipid profile in diabetic rats (Sriplang et al., 2007).

In addition to the hypoglycemic and hypolipidemic actions of *O. stamineus*, evidence for its antihypertensive potentials was also described in the literature. For instance, methylripariochromene A (MRC) was isolated from the leaves of Indonesian *O. stamineus* and subjected to the examination of several pharmacological actions related to its antihypertensive activity (Matsubara et al., 1999). Four significant and worth mentioning findings were revealed from this investigation: firstly, MRC caused a continuous decrease in systolic blood pressure and a decrease in the heart rate (bradycardia) after subcutaneous administration to conscious male spontaneously hypertensive rats. Secondly, MRC exhibited a concentration-dependent suppression of contractions induced by high potassium, phenylephrine, a selective α_1 adrenoceptor agonist, or prostaglandin $F_2\alpha$ ($PGF_2\alpha$) in endothelium-denuded rat thoracic aorta. Thirdly, MRC showed a marked suppression of contractile force (negative inotropic effect) without a significant reduction in the beating rate in isolated bilateral guinea pig atria, and lastly, MRC increased urinary volume and excretion of sodium, potassium and chloride for 3 hour after its oral administration to saline-preloaded fasted rats. These findings ultimately indicated that MRC of *O. stamineus* possesses some actions related to a decrease in blood pressure, i.e., a decrease in cardiac output, vasodilatory and

diuretic actions. Hence, Matsubara *et al.* presumed that the traditional use of *O. stamineus* as a therapy for hypertension may, at least partly, be ascribed to its MRC contents.

Shibuya *et al.* (1999) tested the vascular effects of two diterpenes isolated from a water decoction of Javanese *O. stamineus* namely, neoorthosiphols A and B using an endothelium-denuded rat thoracic aorta. They demonstrated a concentration-dependent suppression of contractions induced by high potassium and phenylephrine in rat aorta. Together, these observations, to a degree, have justified the folk use of *O. stamineus* in the treatment of hypertension, perhaps due to the presence of vasodilator diterpenes (Shibuya et al., 1999).

Water decoction of Javanese *O. stamineus* leaves was also explored for its antihypertensive activity by Ohashi *et al.* (Ohashi et al., 2000b). The extract was partitioned against chloroform and the activities of the resulting fractions were tested in rat thoracic aorta. The experiments showed that the chloroform-soluble portion had a marked inhibitory effect on the contractile responses of potassium chloride-precontracted aortic smooth muscle; yet, the water fraction, in contrast, showed no significant effect. Phytochemical investigations revealed the presence of several classes of terpenoids in the chloroform-soluble portion. MRC was the major isolated constituent in the water decoction of *O. stamineus* leaves which exhibited a continuous decrease in systolic blood pressure after subcutaneous administration in conscious stroke-prone spontaneously hypertensive rats (Ohashi et al., 2000b), findings which were in agreement with those of Matsubara *et al.* (Matsubara et al., 1999).

1.4.5 Antiproliferative, cytotoxic and antiangiogenic activities

In the search of cancer antiproliferative agents, many investigators have isolated and/or extracted various components from *O. stamineus* and tested them through different experimentations. Stampoulis and colleagues (1999) found that the methanol extract of the aerial parts of Vietnamese *O. stamineus* exhibits a cytotoxic activity against highly liver-metastatic colon 26-L5 carcinoma cells. Upon fractionation, the chloroform-soluble fraction of the extract showed the strongest activity against colon 26-L5 cells (Stampoulis et al., 1999a). Separation by silica gel column chromatography followed by preparative TLC procedures revealed five diterpenes, namely staminol A and orthosiphols F–I, which possibly contribute to the cytotoxic activity of the methanol extract of *O. stamineus*. In another study by the same research group three highly oxygenated staminane-type diterpenes from Vietnamese *O. stamineus* namely, staminolactones A, B and norstaminol A were isolated. However, they showed mild cytotoxic activities against highly liver-metastatic colon 26-L5 carcinoma cells (Stampoulis et al., 1999b). Additionally, Tezuka *et al.* (2000) also demonstrated that orthosiphols F, G, H and J, staminols A and B, staminolactones A and B, norstaminol A, staminolactones A and B, norstaminol A, sinensetin , 5-hydroxy-6,7,3',4'-tetramethoxyflavone, salvigenin, tetramethylscutellarein, vomifoliol, aurantiamide acetate, rosmarinic acid, caffeic acid, oleanolic acid, ursolic acid, betulinic acid and β-sitosterol isolated from Vietnamese *O. stamineus* proved experimentally to have substantial cytotoxic potentials against highly liver metastatic murine colon 26-L5 carcinoma cells. By contrast, orthosiphols A, B, D, E and K–Q, norstaminone A, neoorthosiphol A,

nororthosiphonolide A and orthosiphonone A isolated from Myanmar *O. stamineus* showed mild to weak antiproliferative activities toward highly liver metastatic colon 26-L5 carcinoma and human HT-1080 fibrosarcoma cell lines (Awale et al., 2001, Awale et al., 2002a).

Awale *et al.* further studied the possible cytotoxic activity of compounds isolated from Japanese *O. stamineus* towards highly liver metastatic murine colon 26-L5 carcinoma (Ohnishi et al., 1997) and human HT-1080 fibrosarcoma cell lines. Norstaminolactone A, norstaminols B and C, secoorthosiphols A–C and orthosiphols R–T showed selective dose-dependent activity towards murine colon 26-L5 carcinoma cell line but with relatively different order of potency. Among these compounds, norstaminolactone A showed the most potent antiproliferative activity (Awale et al., 2002c).

Of particular interest among the various mechanisms of action of anticancer drugs are those which counteract the process of angiogenesis. Angiogenesis is the process of new blood vessel formation from pre-existing one regulated by a variety of endogenous cytokines (Auerbach et al., 2003). This process plays a pivotal role in the growth and metastasis of tumours and several chronic inflammatory diseases, including rheumatoid arthritis and proliferative diabetic retinopathy (Folkman, 1995, Beaux et al., 1999). Antiangiogenic therapies aimed at halting new blood vessel growth are being developed to treat these conditions. Recently, the concept of using antiangiogenic agents with conventional chemotherapy has been materialised in clinical setting with the approval of the drug bevacizumab (Avastin®) for the treatment of metastatic colon cancer (Tezuka et al., 2000). Such

approach in treatment strategy is considered ideal with natural products that exhibit antiangiogenic activity using similar treatment regime.

Regarding antiangiogensis in general and *O. stamineus* in particular are in concern, Sahib and co-workers (2009) exclusively investigated the possible antiangiogenic activity of different extracts obtained from Malaysian *O. stamineus*. Experimentally, it was proven that the methanolic extract of *O. stamineus* possessed the highest antiangiogenic activity in rat aortic assay followed by the chloroform, petroleum ether and water extracts in descending order. The significant antioxidant properties of *O. stamineus* were suggested as a possible element in the inhibition of new blood vessel development (Sahib et al., 2009a , Sahib et al., 2009b). One possible explanation for this proposition is that the decrease in free radicals turnover is known to activate the hypoxia responsive element gene. This, in turn, acts as a trigger for vascular endothelial growth factor (VEGF), a key cytokine in angiogenesis activation (Goodwin, 2007). Apart from VEGF, transforming growth factor α. (TGFα) is widely acknowledged as one of the potent angiogenic agents and antioxidants to possess a remarkable aptitude to inhibit the expression of this factor, hence resulting in a decline in the process of angiogenesis (Shklar, 1998). The presence of significantly high phenolic contents in *O. stamineus* perhaps plays a major role in the herb's antiangiogenic potentials (Sahib et al., 2009a).

A methanolic extract from *O. stamineus* has been found to enhance the anticancer efficacy of the estrogen receptor antagonist, tamoxifen; yet by itself it exerts no appreciable cytotoxic effect (Sahib et al., 2009b). In experimental

settings, a combination of *O. stamineus* methanol extract and tamoxifen increases the antiproliferative activity of the latter by five folds towards MCF-7 hormone sensitive breast cancer cell line relative to the administration of tamoxifen alone (Sahib et al., 2009b). In other words, *O. stamineus* synergistically enhances the activity of tamoxifen against hormone-responsive breast cancer cells *in vitro* and may, therefore, prove to be useful adjuvant for the treatment of metastatic breast cancer.

1.4.6 Anti-sebum activity

It has been found that *O. stamineus* possesses remarkable capabilities to reduce the oily appearance of skin owing to its ability to decrease 5-α reductase type 1 (an enzyme which plays a major role in the control of sebum secretion) expression in normal human epidermal keratinocytes *in vitro* (Vogelgesang et al., 2011). *Ex vivo* studies have further shown that an extract from *O. stamineus* leaves is able to reduce 5-α reductase activity as well as the production of squalene, one of the main components of sebum (Vogelgesang et al., 2011). Using instrumental techniques as well as clinical and self evaluations, Vogelgesang *et al.* (2010) observed that an oil/water cosmetic formula containing 2% of *O. stamineus* leaf extract could visibly reduce the oily appearance of skin as well as the size of pores, thus leading to a significant improvement of complexion, evenness and radiance.

1.4.7 Antibacterial and antifungal activities

Interest in using natural antibacterial compounds, such as extracts of spices and herbs for preserving food, has become increasingly popular as consumers today ask for products free of synthetic additives (Suhaj, 2006). Plant extracts, especially herbs and spices, are rich in phenolic secondary metabolites, and some have antimicrobial activity (Lin et al., 2005). Therefore, extracts of *O. stamineus* from Malaysia were tested for antimicrobial and antioxidant activities against selected food-borne bacteria *in vitro* (Ho et al., 2010). Using disc diffusion assay, *O. stamineus* 50% methanol extract demonstrated variable antibacterial action against *Bacillus subtilis, Bacillus cereus*, *Staphylococcus aureus, Listeria monocytogenes, Escherichia coli, Vibrio parahaemolyticus, Salmonella enteritidis*, *Salmonella Typhimurium and Klebsiella pneumoniae,* with the highest growth inhibitory action against *Vibrio parahaemolyticus*, a bacterium that causes mild gastroenteritis in humans upon consumption of infected sea food. The effective inhibition of *Vibrio parahaemolyticus* growth by *O. stamineus* methanol extract and its most potent fraction was further found promising when tested for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) and revealed comparable susceptibility to the inhibition seen with the use of 5% lactic acid, a natural food preservative. This is likely due to the high concentration of rosmarinic acid found in the *O. stamineus* extracts whereby the highest concentration of rosmarinic acid seemed to have the best antibacterial and free radical scavenging activities (Ho et al., 2010). This possibly suggests that rosmarinic acid content is

closely associated with antibacterial and free radical scavenging activities of *O. stamineus* extracts.

O. stamineus extracts have been utilized as a source of compounds with antifungal activity to especially combat those fungi which are causal agents of plant diseases. For instance, the essential oils and the methanol extract of *O. stamineus* as well as the derived fractions were tested for antifungal activity by Hossain *et al.* (2008). Using disc diffusion and MIC determination methods, oils, extracts and fractions derived form *O. stamineus* displayed prominent antifungal activity as mycelial growth inhibitors against a set of tested phytopathogenic fungi, such as *Botrytis cinerea*, *Rhizoctonia solani*, *Fusarium solani*, *Colletotricum capsici* and *Phytophthora capsici* (Hossain et al., 2008).

1.5 Toxicological studies

It is particularly important for herbalists or herbal products manufacturers to understand the correlation between pharmacological activities of herbal active compounds and the possibility of these compounds to harmfully interact in the body leading to undesirable toxic manifestations upon administration.

Based on *O. stamineus* popularity and demonstrated effectiveness, it has vigorously been investigated through countless phytochemical and pharmacological studies since 1930 (Beaux et al., 1999). However, toxicological investigations have not been thoroughly addressed in the literature.

Chin *et al.* (2008) investigated the possible toxic effects of orally-administered *O. stamineus* methanol extract in female SD rats. The extract was

given over a period of 14 days and toxicity was practically evaluated by the incidence of lethality, side-cage observation and the analysis of some biochemical parameters. During the experimental period, no lethality, obvious adverse manifestations or delayed toxic effect and lethality were seen at a dose of as high as 5 g/kg. Surprisingly, liver hypertrophy with peculiar significant drop in AST and ALT levels were observed at the end of the study protocol. Despite that Chin *et al.* (2008) suggested that *O. stamineus* methanol extract is practically non-toxic and the 14-day 5g/kg regimen in female SD rats is regarded as the no observable adverse effect level (NOAEL), the increase in liver size, by itself, in the absence of any significant changes in the liver enzymes' levels remains questionable and warrants further investigation (Chin et al., 2008). Likewise, a comparable dose regimen was employed by Abdullah *et al.* (2009) to explore the acute toxicological effects of *O. stamineus* using a standardized extract of *O. stamineus* and male SD rat model. Their results reported no deaths or signs of toxicity during the experimental period. They further estimated the LD_{50} to be >5g/kg (Abdullah et al., 2009). Curiously, contrary to the study by Chin *et al.* (2008), liver size and biomarkers remained unaffected and comparable to the control.

Recently, the genotoxicity of *O. stamineus* has been evaluated by Muhammad *et al.* (2011). In this study the genotoxic potential of *O. stamineus* aqueous extract was investigated by the Salmonella/microsome mutation assay and the mouse bone marrow micronucleus test. Basically, the Salmonella/microsome assay (TA97a, TA98, TA100 and TA1535; plate incorporation method) was performed in the presence or in the absence of extrinsic

metabolic activation (S9 mixture). In the mouse micronucleus assay, *O. stamineus* water extract was administered by gavage (0, 500, 2000 and 4000 mg/kg body weight/day for 3 days) to male and female Swiss Webster mice (N= 6 per dose per sex) and bone marrow cells were harvested 24 h after the last dose. Ethoxy-resorufin-O-dealkylase and benzyloxy-resorufin-O-dealkylase activities were determined in mouse liver microsomes. Results have showed that tested at doses up to 5000 µg/plate, the aqueous extract of *O. stamineus* was not toxic to Salmonella tester strains and did not increase the number of revertant colonies over the background incidence. Interestingly, in the mouse bone marrow assay, the extract did not alter the polychromatic: normochromatic erythrocytes ratio, nor did it increase the incidence of micronucleated polychromatic erythrocytes. No overt toxicity and no change of CYP1A and 2B9/10 activities were noted. Therefore, Muhammad et al., 2011 concluded that the use of *O. stamineus* in the traditional medicine poses no genotoxic risk (Muhammad et al., 2011).

1.6 *In vitro* herb-drug interaction studies

Research on *O. stamineus* was not confined to its pharmacological uses and traditional applications but rather extended beyond these limits. Herein, we address some of these studies.

The topic drug-herbal extract interactions have been of a potential interest in many occasions where the consumption of mixture of pure drugs with medicinal extractives is useful. On the other hand, herbal products and drugs are reported to have potential in herb-drug interaction if they are administered simultaneously