PHYTOCHEMICAL ISOLATION AND STANDARDIZATION OF *ORTHOSIPHON STAMINEUS* BENTH. LEAF EXTRACT FROM DIFFERENT LOCATIONS AND SELECTED ANTIOXIDANT AND TOXICITY STUDIES

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by

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PENGASINGAN FITOKIMIA DAN PEMIAWAIAN EKSTRAK DAUN *ORTHOSIPHON STAMINEUS* BENTH. DARI LOKASI YANG BERBEZA UNTUK KAJIAN ANTIOKSIDAN DAN TOKSISIITI

oleh

NUR FARAH AMALINA BINTI MUGHNI

Tesis yang diserahkan untuk
memenuhi keperluan bagi
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AAS	Atomic Absorption Spectroscopy
$AlCl_3$	Aluminium chloride
As	Arsenic
ATR-FTIR	Attenuated Total Reflection-Fourier Transform Infrared
BHA	Butylated hydroxylanisole
BHP	British Herbal Pharmacopoeia
BSA	Bovin serum albumin
BSLT	Brine shrimp lethality test
BuOH	Butanol
CAT	Catalase
Cd	Cadmium
$CHCl_3$	Chloroform
CJPK	Changkat Jering Perak
DMSO	Dimethyl sulfoxide
DSJM	Desaru Johor
DPPH	2,2-Diphenyl-1-Picrylhydrazil
EUP	Eupatorin
FCR	Folin-Ciocalteu's reagent
FTIR	Fourier transform infrared
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry

Hg	Mercury
HLSM	Hulu Langat Selangor
HMPs	Herbal Medicinal Products
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
KBPP	Kepala Batas Pulau Pinang
MLT	Microbial limit test
UV	Ultra-violet
NPCB	National Pharmaceutical Control Bureau
PPKM	Pasir Puteh Kelantan
PNSM	Pantai Negeri Sembilan
RA	Rosmarinic acid
ROS	Reactive oxygen species
SIN	Sinensetin
SKTM	Sungai Kok Terengganu
SNSM	Sendayan Negeri Sembilan
SUMM	Sungai Udang Melaka
TLC	Thin Layer Chromatography
TNC	Total nonstructural carbohydrates
TPPM	Taiping Perak
WHO	World Health Organization
WIPO	World International Property Organization

PENGASINGAN FITOKIMIA DAN PEMIAWAIAN EKSTRAK DAUN *ORTHOSIPHON STAMINEUS* BENTH. DARI LOKASI YANG BERBEZA UNTUK KAJIAN ANTIOKSIDAN DAN TOKSISITI

ABSTRAK

Kajian ini bertujuan untuk pemiawaian *Orthosiphon stamineus* daripada sepuluh lokasi berbeza di seluruh Semenanjung Malaysia termasuk ujian farmakognosi, profil kimia dan aktiviti biologi (antioksidan dan toksisiti).

Analisis gravimetrik had logam berat dan ujian had mikrob telah dilakukan ke atas serbuk daun kering untuk memeriksa kualiti dan keselamatan bahan mentah sebelum meneruskan analisis ke peringkat yang lain. Tenaga, karbohidrat, protein dan lemak dalam semua sampel telah menunjukan keputusan (per 1g bahan mentah) dalam lingkungan masing-masing 305-339 kkal, 53.1-59.9 g, 12.4-17.8 g dan 3.3-5.2 g. Pemeriksaan awal fitokimia pada bahan mentah mendedahkan kehadiran terpenoid, flavonoid, tanin dan saponin.

Profil kimia yang telah ditentukan secara kualitatif dengan menggunakan teknik spektroskopi (UV-Vis dan IR) dan kromatografi (HPLC). Keputusan menunjukkan bahawa spektrum yang sama telah diperolehi dalam *Orthosiphon stamineus* daun ekstrak metanol dari 10 lokasi yang berbeza. Asid rosmarinik, sinensetin dan eupatorin telah dipilih sebagai bahan penanda untuk dianalisis oleh HPLC. Kandungan mereka adalah dalam julat antara 2.12 $\pm$ 0.94 % - 14.61 $\pm$ 2.23 % (asid rosmarinik), 0.25 $\pm$ 2.29 %- 1.04 $\pm$ 0.78 % (sinensetin) dan 0.23 $\pm$ 2.05 % - 1.00 $\pm$ 1.12 % (eupatorin). Analisis komponen utama (PCA) telah digunakan untuk mendapatkan maklumat dengan menggunakan data

yang diperolehi daripada ujian inframerah. Perbezaan yang ditunjukkan mungkin disebabkan oleh kepelbagaian dalam faktor-faktor alam sekitar. Perbezaan suhu antara lokasi yang berbeza adalah kecil (Purata 27^0C - 29^0C) manakala perbezaan dalam lembapan adalah lebih jelas (70% - 89%). Perubahan dalam ketinggian juga boleh dilihat pada setiap lokasi yang dikaji iaitu dalam lingkungan 3.84 m-112.92 m.

Pengasingan oleh kromatografi dengan menggunakan ekstrak air daripada daun *Orthosiphon stamineus* menghasilkan pengasingan satu sebatian. Ujian analisis kimia dan analisis spektrum telah dilakukan dan memberi satu struktur yang telah dijelaskan sebagai 5-hidroksi-6,7,3',4'-tetrametoksiflavon.

Jumlah kandungan fenolik sampel termasuk dalam julat 71,63 mg asid galik/g berat kering (Kepala Batas) kepada 171 mg asid galik/g berat kering (Hulu Langat). Kandungan flavonoid jumlah adalah dalam lingkungan 105.63$\pm$0.09 mg kuersetin/g berat kering (Sungai Udang) to 245.96$\pm$0.02 mg kuersetin/g berat kering (Kepala Batas). Antara ekstrak dikaji, aktiviti antioksidan tertinggi menggunakan ujian DPPH adalah ekstrak dari Desaru dengan nilai IC_{50} 2.778 μg/ml. Dalam ujian β-karotena, aktiviti antioksidan yang tertinggi telah diperolehi dalam ekstrak daripada Hulu Langat dengan peratusan 96.46 % pada kepekatan 1000 μg/ml. Kajian ini juga membuktikan bahawa tidak wujud hubungan antara jumlah fenolik atau jumlah flavonoid dengan ujian DPPH dan β-karotena.

Kajian toksisiti daripada ekstrak dikaji dengan menggunakan ujian anak udang. Kematian anak udang dengan 50% kepekatan (LC_{50}) adalah dalam lingkungan 106.09-234.7 μg/ml.

Kesimpulannya, kajian yang telah dilakukan menunjukkan bukti kualiti dan keselamatan, aktiviti antioksidan dan toksisiti ekstrak *Orthosiphon stamineus* adalah seragam dari 10 lokasi yang berbeza.

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ABSTRACT

This study aimed to standardize *Orthosiphon stamineus* extract from ten different locations throughout Peninsular Malaysia that includes pharmacognosical tests, chemical profile and biological activity (antioxidant and toxicity using a brine shrimp bioassay).

Gravimetric analysis, heavy metal limit and microbial limit test were done on the dried leaf powder to determine the quality and safety of the raw material. Energy, carbohydrate, protein and fat content were estimated in all samples and the results (per 1g of raw material) were in the range of 305-339 kcal, 53.1-59.9 g, 12.4-17.8 g and 3.3-5.2 g respectively. Preliminary phytochemical screening revealed the presence of terpenoids, flavonoids, tannins and saponins.

The chemical profile was determined qualitatively using spectroscopic (UV-Vis and IR) and chromatographic techniques (HPLC). The results showed that similar spectra were obtained in the methanolic leaf extracts from all locations. Rosmarinic acid (RA), sinensetin (SIN) and eupatorin (EUP) marker compounds were analyzed by HPLC. Their content was in the range of 2.12$\pm$0.94 % - 14.61$\pm$2.23 % (RA), 0.25$\pm$2.29% - 1.04$\pm$0.78 % (SEN) and 0.23$\pm$2.05 % - 1.00$\pm$1.12 % (EUP). The similarity and difference between spectra (which has been done using FT-IR) were analyzed using Principle Component Analysis (PCA) as tools for extracting relevant chemical information from obtaining infrared data. These differences may be explained

due to the variations in the environmental factors. The difference in temperature between the different locations was marginal (average 27^0C – 29^0C) whereas the difference in relative humidity was more obvious (70 % - 89 %). Changes in altitude can also be seen amongst the studied locations, the altitude values were in the range of 3.84m-112.92m.

Separation by column chromatography from the *Orthosiphon stamineus* leaf water extract resulted in the isolation of one compound. On the basis of chemical and spectral data, the structures was elucidated as 5-hydroxy-6,7,3',4'-tetramethoxyflavone.

The total phenolic content of the samples from ten locations are within the range of 71.63±0.02 mg Gallic acid/g dry weight (Kepala Batas) to 171±0.02 mg Gallic acid/ g dry weight (Hulu Langat). The total flavonoid content was in the range of 105.63±0.09 mg quercetin/g dry weight (Sungai Udang) to 245.96±0.02 mg quercetin/g dry weight (Kepala Batas). Methanolic leaf extracts of the *Orthosiphon stamineus* were then screened for antioxidant activity. Amongst the extracts studied, the highest antioxidant activity in DPPH scavenging assays was obtained in the extract prepared from Desaru with IC_{50} values 277.8±0.10 µg/ml. In the *β*-carotene assay, the highest antioxidant activity was obtained in the extract prepared from Hulu Langat, with the percentage inhibition of 96.46±0.02 % at 1000 µg/ml. Statistical analysis was done and resulted no correlation exist between total phenolic or total flavonoid with DPPH assay and *β*-carotene assay.

Toxicity of the extracts was studied using the brine shrimp lethality assay. The median lethal concentration (LC_{50}) was in the range of 106.09±1.45 – 323.47±4.43 µg/ml.

In conclusion, the current study provides evidence on quality and safety, antioxidant activity and toxicity assay using a brine shrimp assay of the standardized *Orthosiphon stamineus* extract from 10 different locations.

CHAPTER 1

INTRODUCTION

1.1 General introduction

Ever since decades ago, herbs have been proven to have potentials to cure and heal diseases and illnesses. Besides decorative plants and flavor enhancing, herbs are widely used as healing sources for diseases and illness prior to the discovery of modern medicine. Throughout the centuries, herbs are found to be able to treat various illnesses involving digestive problems, toothaches, open wounds, etc. Extensive researches have then been carried out to further develop and explore the potentials of these herbs.

Based on the statistics carried out by the World Health Organization (WHO), 80% of the world population prefers to choose herbs as medicine to treat ailments. WHO states that about 119 medicinal substances are extracted from various plants and 74% of them are still used without any modification. Up to date, the extracted substances from herbs have become the basis of manufactured medication for diseases involving cardiovascular problems, asthmas and hypertensions (WHO, 1998).

Originally, the word herb is derived from the word “Herba”, a Latin word referred to grasses, green crops and other leafy plants. Nowadays, herbs extend to plants, trees or shrubs possessing culinary, medicinal and aesthetic properties. Generally, a plant can be used as a whole, by part or even with combination with other plants with the purpose of the treatments. The World Health Organization (WHO) stated that herbal

medicines (plants or parts of plants) contain an incipient as an active ingredient, whether it is in the crude or processed state (WHO, 1998).

Traditional medicine is commonly practiced as it is a natural and safe medical approach since centuries ago. Nowadays, herbal products play important roles due to their compatibility with the human body, besides being able to prevent diseases from occurring rather than to cure a post-occurred disease. The chemical constituents present in herbs are part of the physiological function (Kamboj, 2000).

Although traditional medicines involving herbal remedies are trusted and oftenly safe, negative side effects might arise if these remedies are consumed together with prescriptive drugs (Benzie and Watchel-Galor, 2011). Several case reports of the inverted effect after consuming herbal products such as allergic reactions and direct toxification. Fairbairn (1980) suggests that standardization of herbal remedies should be prioritized in producing herbal products to prevent unnecessary side effects.

Standardization is defined as the establishment of pharmaceutical quality that is repeated through comparison with reference materials and to determine the amount of established qualitative or quantitative for one or more compounds or groups of compounds. In phytomedicine field, standardization is used in extraction (Gurib-Fakim *et al.*, 2005). Mosihuzzaman and Choudhary (2008) defined standardization as the control on quality of herbal medicinal products by means of modifying the herbal medicine planning for describing content constituent or group of substances with known therapeutic activity. Standardization is the most important step which allows the identification of known active constituents. However, there are still unknown active

constituents in the herbs. For these cases, herbal products need to be standardized using certain marker compounds. Based on Ong (2004), chemical standardization usually contains chemical identifications (using spectroscopic or chromatographic fingerprint) and chemical assays (active constituents or marker compounds).

It is very important to maintain the quality of herbal products to ensure the safety and effectiveness of the products (Busse, 2000). There are some variables that give effect to the safety, efficiency and quality of the herbal medicines such as the freshness of the plants, temperature, water availability, light exposure, nutrients and the time of collection (Calixto, 2000).

In traditional mediciation, old folks believe that *Orthosiphon stamineus* possesses diuretic properties, antiallergic, antiinflammatory, antihypertensive and antitumor. It is also used to treat diabetes, rheumatism and gout (Burkhill, 1966).

In this study, the leaves of *Orthosiphon stamineus* Benth plant from different locations were studied. This plant is locally known as "Misai kucing" or literally cat's whiskers due to its unique flower which resembles a cat's whisker. The entire plant possesses therapeutic properties; such as the leaves of the plant are believed to contain antioxidant property (Akowuah *et al.*,2005 (b) ; Khamsah *et al.*,2006; Yam *et al.*,2007), antiinflammatory property (Masuda *et al.*,1992; Yam *et al.*,2008) and diuretic property (Arafat *et al*., 2008).

1.2 Justification of study

Orthosiphon stamineus Benth is the source of many secondary metabolites compounds. Secondary metabolites perform a significant part in the adoption of plants to the surroundings besides being able to overcome stress conditions. The concentration of secondary metabolites in *Orthosiphon stamineus* plants are regulated by physiological, genetic, environmental and chemical factors such as light, rainfall, temperature, chemicals, soil and plant growth regulators (Ibrahim and Jaafar, 2013).

Various products derived from *Orthosiphon stamineus* were registered under National Pharmaceutical Control Bureau (NPCB) (Table 1.1). According to the World International Property Organization (WIPO), there are several patents available for *Orthosiphon stamineus* (Table 1.2).

Table 1.1: Products *Orthosiphon stamineus* registered under National Pharmaceutical Control Bureau (NPCB)

Product Name	**MAL No**	**Company Name**
Misai kucing	MAL20012878T	Herbal Science Sdn. Bhd.
Misai kucing plus	MAL20040150T	Herbal Science Sdn. Bhd.
Golden leaf cat's whisker tea plus	MAL05101755T	Herbal Land Manufacturing Sdn. Bhd.
Yang yen bao capsule	MAL07101275TC	Kangyew Healthcare
Forest'secret precious misai kucing herbal extract	MAL09111786TC	Forest Secret Sdn. Bhd.
Orthosiphon stamineus plus	MAL09021103T	Duha Sdn. Bhd.
Misai kuching herbal tea 3 gm	MAL07061365T	Natural Wellness Industries Sdn. Bhd.
Naturax misai kucing 2g tea	MAL09071080T	Biotropics Malaysia Berhad

http://www.portal .bpfk.gov.my/product (27/3/2013)

Table 1.2: Patents published base of the World International Property Organization (WIPO)

Title	**Applicant**
WO/2011/078652 Orthosiphon stamineus extracts with beneficial use as cognition enhancer	Biotropics Malaysia Berhad
Wo/2011/129680 A use of an effective amount of a composition comprising o. Stamineus leaf extract	Universiti Sains Malaysia Usm
2010143862 Composition containing plant extract	Sunstar Inc.
2005139136 Insulin secretagogue	National Institute Of Advanced Industrial & Technology
02180232 Agent eliciting antitumor activity	Gorshkov Anatolij Nikolaevich
2001224330 Food comprising lactobacillus symbiotic culture product and medicinal plant and method for producing the same	Uechi Hideko

http://www.patentscope.wipo.int/search (3/4/2013)

This study aimed to:-

1. Provide a comparative study of *Orthosiphon stamineus* leaf selected from Peninsular Malaysia based on a humidity, temperature, altitude, collection season and soil condition.
2. Provide information regarding free radical scavenging and brine shrimp lethality bioassay of *Orthosiphon stamineus* leaf extract.
3. Prove the potential relationship between the complex chemical constituent and geographical origin of the samples.

1.3 Objectives of study

1. To analyse the quality (microscopic, macroscopic, colour and gravimetric), safety (microbial and heavy metal) and nutritive value of *Orthosiphon stamineus* from 10 different locations based on the Malaysian Herbal Monograph Volume 2.
2. To profile and standardize methanol extracts of *Othosiphon stamineus* Benth. leaf by using spectroscopic and chromatographic methods of selected marker compounds from all locations.
3. To isolate and identify selected compound from the water extract of *Orthosiphon stamineus* leaf.
4. To evaluate the selected antioxidant and toxicity (brine shrimp lethality assay) properties of the methanolic extracts.

CHAPTER 2

LITERATURE REVIEW

2.1 *Orthosiphon stamineus*

Orthosiphon stamineus Benth, also known as "Misai Kucing" is a genus in the family of Lamiaceae. "Misai Kucing" plant is an herbal species originated from the South-East Asia region. In Malaysia, the plant's leaves are used as a treatment to diuretic, diabetes and hypertension. In Vietnam, the arterial part of the plant is used to cure urinary lithiasis, eruptive fever, oedema, influenza, rheumatism, hepatitis, jaundice and biliary lithiasis. Since 1930s, phytochemical and pharmacological studies of this plant have been studied (Tezuka et al., 2000).

2.1.1 Classification and description

Taxonomically, this plant is categorized into the following scheme:

Family	: Lamiaceae
Genus	: *Orthosiphon*
Species	: *Stamineus*
Scientific name	: *Orthosiphon stamineus* (Benth)
Local name	: Misai Kucing

According to Anon (2001), *Orthosiphon aristatus*, *Orthosiphon grandiflorum* and *Orthosiphon spicatus* are synonyms to the scientific term, *Orthosiphon stamineus*. Every country has its own vernacular name for this plant such as Thé de Java (France), kumis kucing (Indonesia), kumis kucing (Sudan), kumis kucing or misai kucing (Malaysia), balbas-pusa and kabling-gubat (The Phillippines), kapen prey (Cambodia), hnwàd méew (Laos), yaa nuat maeo (Thailand) and r[aa]u m[ef]o (Vietnam).

2.1.2 Botanical description

Orthosiphon stamineus is a quadrangular perennial herb, with a height of 25-200 cm, inadequately ramified and ascending stems. The plant is an herbaceous shrub, grows to a stature of 1.5 m and can be found in tropical and subtropical regions. The stem is intensely arranged in a quadrangular manner, reddish in colour, erect and branches profusely. As shown in Figure 2.1, the leaves are organized in inverse sets, straightforward, sleek, hued green, glabrous with a lanceolate leaf cutting edge and serrate edge. The leaf apices are sharpened with an intense leaf base. The petiole is moderately short, measuring around 0.3 cm in the ballpark and ruddy purple in the shade.

Figure 2.1: Picture of *Orthosiphon stamineus* leaf

In Figure 2.2, it is observed that the flowers are borne on the verticals and length of about 16 cm. The terminal inflorescence is borne on a maroon pubescent. The flowers are campanulas, white or blue in colour, with long filament over mid-green foliage, making the flowers resembles cat's whiskers. The calyx is gamosepalous with two lips (bilabiate), which is greenish red in colour and measuring about 6 mm in length. One of the calyx lips has a toothed margin while the other lip is enclosed with minute white hairs. The corolla is also partially gamapetalous with two lips (bilabiate) covered with minute hairs. The corolla is pale violet in colour with lips shorter than the corolla tube. The labellum is pale violet in colour, hairy and pinkish on the under surface. Four stamens are inserted near the base of the corolla tube. The stamens are not the same in length, measuring from 4.7 to 5.2 cm. There is a single, central terete style with a clavate stigma (Muhamad and Mustafa, 1994).

Figure 2.2: Flower of *Orthosiphon stamineus*

2.1.3 Plant habitat and cultivation

Jaganath and Ng (2000) explained that *Orthosiphon stamineus* has been cultured for quite a while. Wild *Orthosiphon stamineus* grow along roadsides, forest sides and on wasteland. This plant is easily propagated through three or four nodded stem cuttings. These stem cuttings are obtained from the mother plant of more than five months age. The middle portion of the stem is then chosen to obtain a higher rate of success in propagation. Before transferring to the fields, the stem cuttings are propagated for about a month. The leaves and branches can be harvested after 3 to 4 months after the field transfer. *Orthosiphon stamineus* thrives in well-drained soils in full sunlight. These plant branches more profusely and generally do better with regular applications of organic fertilizers such as chicken dung. *Orthosiphon stamineus* is not significantly susceptible to disease, but it's prone to insect attack (Jaganath and Ng, 2000).

2.1.4 Chemical constituent

Orthosiphon stamineus plants have been reported to contain several chemical compounds. *Orthosiphon stamineus* has been investigated phytochemically and pharmacologically since 1989, when oil namely methylripariochromene A was isolated from this plant (Guerin *et al.*, 1989). Phytochemically investigation of the dry leaves of *Orthosiphon stamineus* has led to the isolation of highly-oxygenated isopimarane-type diterpenes, orthosiphol A-E (Masuda *et al.*, 1992; Sumaryono *et al.*,1991; Takeda *et al*., 1993), and also reported on monoterpenes, triterpenes, saponins, flavonoids, hexoses, organic acids, rosmarinic acid, chromene and myo-inositol. Other bioactive compounds

have also been isolated such as sterols organic acid and caffeic acid derivatives (Olah *et al.,* 2003). Olah *et al*. (2003) stated that the most important compound present in the *Orthosiphon stamineus* leaves is the polyphenols which are polymethoxylated flavonoids: sinensetin, eupatorin, etc. and caffeic acid derivatives: rosmarinic acid, cichoric acid, caffeic acid, etc. The detail of chemical constituents identified in *Orthosiphon stamineus* is listed in Table 2.1 while the structures of some compounds are shown in Fig. 2.3 to Fig. 2.7.

Table 2.1: Chemical constituents of *Orthosiphon stamineus*

Class of compounds	Chemical constituents		Reference
Diterpenes	• Orthosiphols A [1] • Orthosiphols B [2] • Orthosiphols C-J [3-7] • Orthosiphols K-N • Orthosiphols R-T • Orthosiphols S [8] • Secoorthosiphol A-C	• Staminols A [9] • Staminols B [10] • Staminolactones A [11] • Staminolactones B [12] • Norstaminol A [24] • Orthosiphonone A [25] • Orthosiphonone B [26]	• Tezuka *et al* (2000) • Awale *et al*. (2001) • Masuda *et al*. (1992)
Triterpenes	• Oleanolic acid [27] • Ursolic acid [28] • Orthosiphonic acid	• Betulinic acid [29] • B-sitosterol [13] • Maslinic acid	• Tezuka *et al*. (2000) • Hossain and Ismail (2003)
Flavones	• Eupatorin [15] • Sinensetin [16] • Rutin • Salvigenin [18] • Ladanein • Tetramethylscutallarein [19] • 3'-hydroxy-5,6,7,4'-tetramethoxyflavone [17] • 7,3', 4'-tri-O-methylluteolin [14] • 6-hydroxy-5,7,4'-trimethoxyflavone [20] • Kaempferol-3-O-β-glycoside [21] • Quercetin-3-O-β-glucoside [22]		• Tezuka *et al*. (2000) • Sumaryono *et al*. (1991) • Hossain and Ismail (2003)
Phenolic Acid	• Caffeoyl tartrate [23] • Rosmarinic acid [30] • Vomifoliol [32] • Aurantiamide acetate [31] • 2,3-dicaffeoyl tartrate		• Sumaryono *et al.* (1991)
Benzochromene	• Methylripariochromene A [35] • Acetovanillochromene [36] • Orthochromene A [34]		• Shibuya *et al.* (1999)

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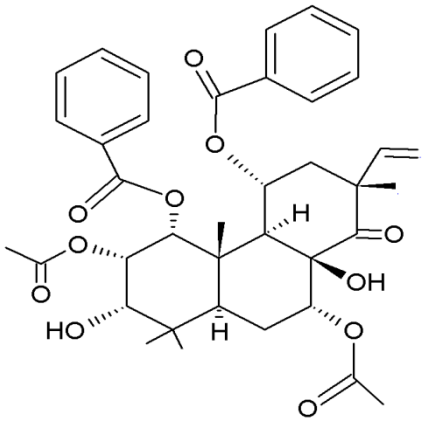
<td> 
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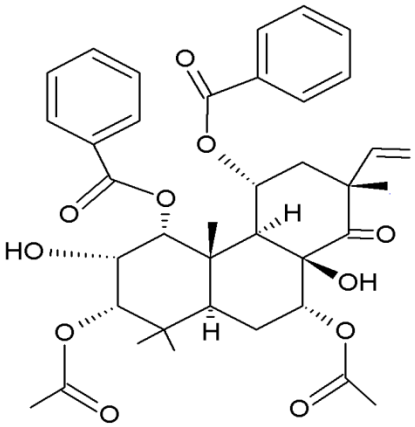
<td> 
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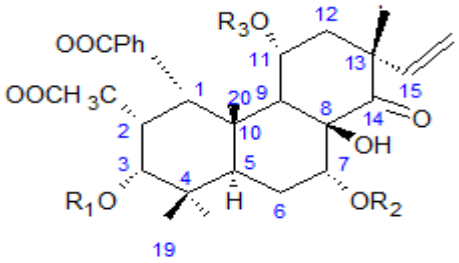
<td> 
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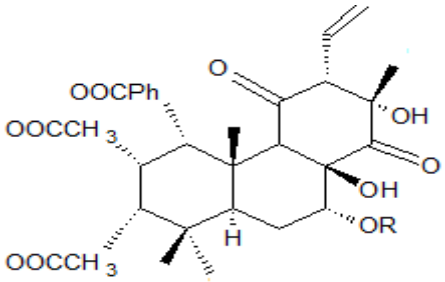
<td> 
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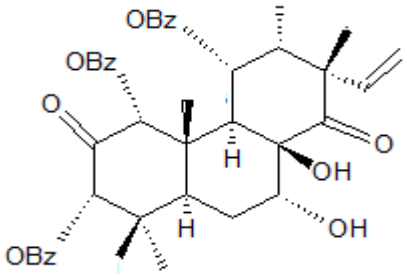
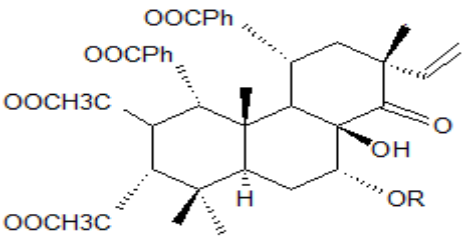
<td> 
Orthosiphol S [8]</td>
<td> 
R= $COCH_3$, Staminol A [9]
R= H , Staminol B [10]</td>
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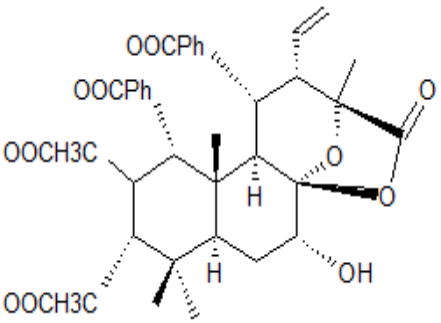
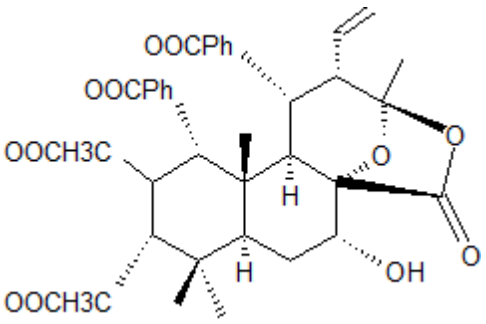
<td> 
Staminolactone A [11]</td>
<td> 
Staminolactone B [12]</td>
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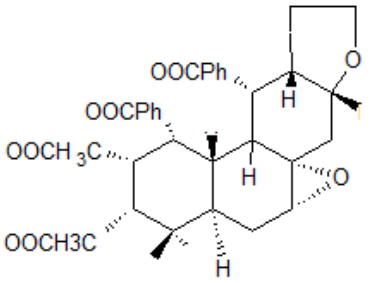
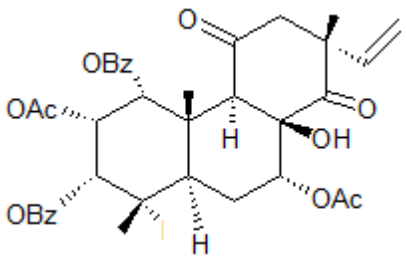
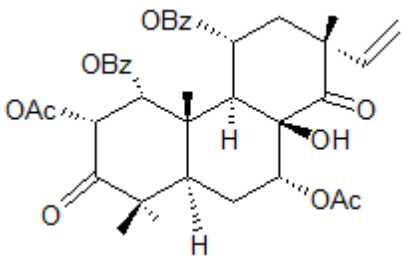
Figure 2.3: Chemical structures of *Orthosiphon stamineus* (diterpenes)



Norstaminol A [24]

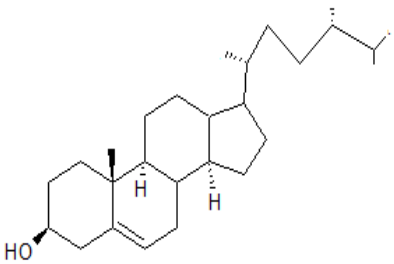


Orthosiphonone A [25]

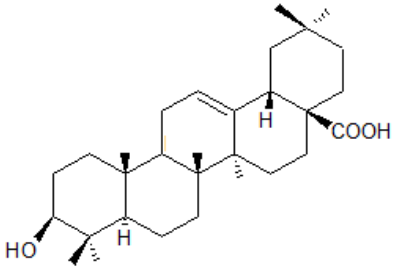


Orthosiphonone B [26]

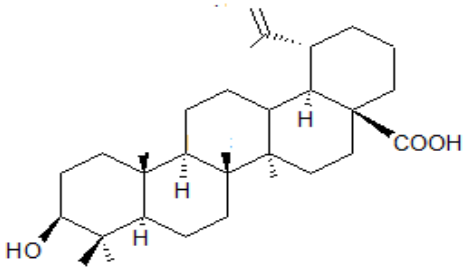
Figure 2.3 (Continued): Chemical structures of *Orthosiphon stamineus* (diterpenes)



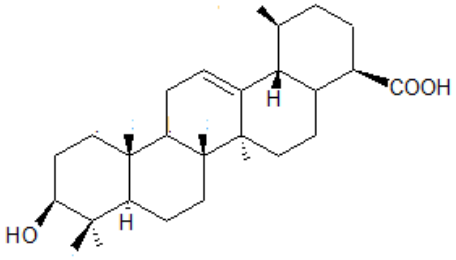
B- sitosterol [13]



Oleanolic acid [27]

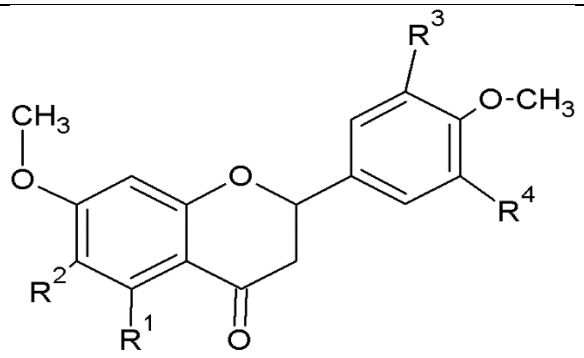


Ursolic acid [28]

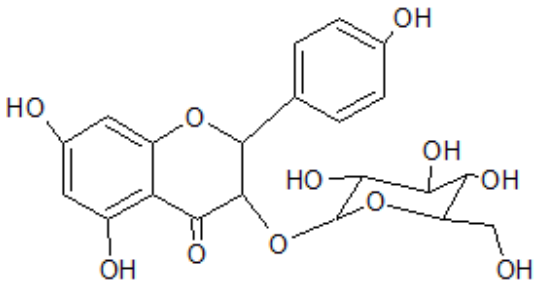


Betulinic acid [29]

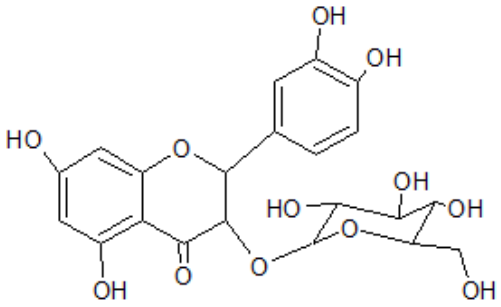
Figure 2.4: Chemical structures of *Orthosiphon stamineus* (triterpenes)



R_1=R_2=H, R_4=OCH_3, 7,3',4' –Tri-O-methylluteolin [14]
R_1=R_3=OH, R_2=OCH_3, R_4=H , Eupatorin [15]
R_1=R_2=R_3=OCH_3, R_4= H, Sinensetin [16]
R_1=OH, R_2=R_3=OCH_3, R_4=H, 3-hydroxy-5,6,7,4-tetramethoxyflavone [17]
R_1=R_4=H, R_2= OCH_3 , Salvigenin [18]
R_1=CH_3, R_2 =OCH_3, R_4= H, Tetramethylscuteliarein [19]
R_1=CH_3, R_2 =OH, R_4= H, 6-Hydroxy-5,7,4-trimethoxyflavone [20]



Kaempferol-3-O-β-glucoside [21]



Quercetin-3-O-β-glucoside [22]

Figure 2.5: Chemical structures of *Orthosiphon stamineus* (flavones)

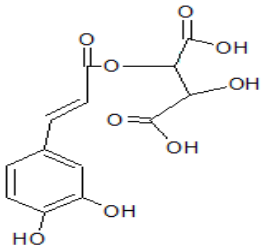
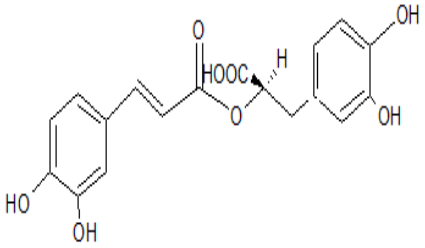
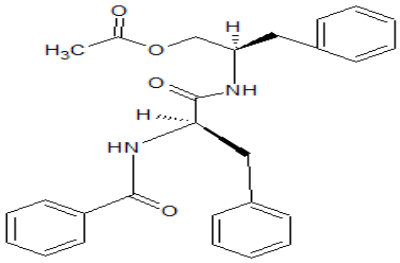
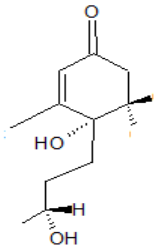
 Caffoeul tartrate [23]	 Rosmarinic acid [30]
 Aurantiamide acetate [31]	 Vomifoliol [32]

Figure 2.6: Chemical structures of *Orthosiphon stamineus* (phenolic acid)

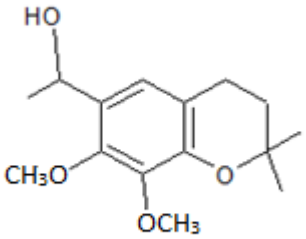
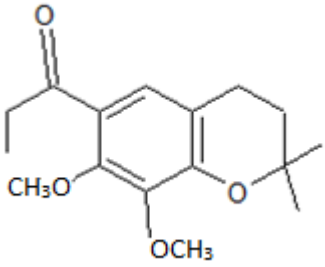
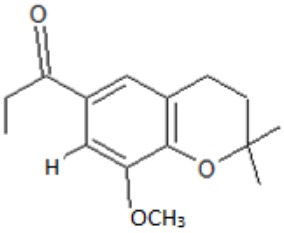
 Orthochromene A [34]	 Methylripariochromene A [35]	 Acetovanillochromene [36]

Figure 2.7: Chemical structures of *Orthosiphon stamineus* (benzochromene)

2.1.5 Traditional uses of *Orthosiphon stamineus*

Orthosiphon stamienus is a useful traditional medicine herbs as it can be used to treat many illnesses which involve diuretic, antiinflammatory, anticholestatic, urolithiatic, antirheumatic, and antidiabetic activities (Wiart, 2002). *Orthosiphon stamineus* have been used differently in different countries. Decoction of *Orthosiphon stamineus* leaf in certain places especially in Malaysia and Indonesia are used to treat arteriosclerosis, alleviate bladder and kidney discomfort, gout and rheumatism (Awale *et al*., 2001; Tezuka *et al*., 2000; Wiart, 2002). The Vietnamese uses this plant to treat urination, reduce eruptive fever urination, rheumatism, influenza, hepatitis, biliary lithiasis and jaundice. In India, *Orthosiphon stamineus* issued to treat diabetes and this plant is mixed with *Andrographis paniculata* (Wiart, 2002).

2.1.6 Review of biological activity of *Orthosiphon stamineus*

Aqueous extract from the leaves of *Orthosiphon stamineus* in Germany exhibited diuretic effect (Englert and Harnischfeger, 1992). In Vietnam, urine output or on the sodium excretion shows no influence after 24 hours of treatment under consistent conditions (Doan *et al.*, 1992). After oral administration of aqueous extracts of the plant in different doses (400 mL/day of 3.75% extract, 400 ml/day of 15% of extract, and 500 mL/day of 3.3% extract) in volunteers, increased the diuresis (Schuman, 1927). In another study, 14 patients were treated (dose 9500 ml/day of 12% infusion of leaves for 10 days) and increase in diuresis and excretion of chloride and urea was observed. In more recent research conducted on 40 volunteers for diuretic effects showed no influence on urine output or sodium excretion recorded in a placebo-controlled double

blinded crossover study at a dose of 0.6 liter/day of an infusion equivalent to 10 g of the dried leaves (Doan *et al*., 1992). Increased acidity in the urine has been reported in a study which was carried out on 6 healthy male volunteers who consumed 250 ml of *Orthosiphon stamineus* tea every 6-hour for one day (Nirdnoy and Muangman, 1991).

In Thailand, research conducted has shown that the leaves of *Orthosiphon stamineus* have both favorable and non favorable effects on stone prevention. The study showed that uric acid containing stones may be prevented after drinking the tea but higher risk of stone formation may occur due to the excretion of oxalate in the urine. However, since the research was done on a healthy patient, where all the parameters were normal and may not be applicable to kidney stone patients (Nirdnoy and Muangman, 1991). In another research conducted in Russia, it was shown that the administration of dried extract in experimental and therapeutic doses to white rats having post-ischemic acute renal insufficiency was observed to significantly decrease the concentration of lipid peroxidation in the kidneys. The preparation of the extract inhibits hemolysis induced by the Fenton reagent and photoactive chlorpromazine. The inhibition may be due to scavenging of free radicals by phenolic compounds of the extract contained in the preparations (Shantova, 1998).

Orthosiphon stamineus Benth is believed to also contain antihypertensive properties (Hossain *et al.,* 2008; Sriplang *et al.,* 2007; Yam *et al.,* 2009). Shibuya *et al*. (1999) studied the water decoction of the air-dried *Orthosiphon Arristatus* leaf in the mixture of water and chloroform and resulted in an inhibitory effect which is caused by the major constituent of the leaf (methylripariochromene A) on the contractive responses in the rat thoracic aorta smooth muscle stimulated with KCI beforehand (Shibuya *et al.,*

1999). Azizan *et al*. (2012) studied the systolic blood pressure of spontaneously hypertensive rats (SHR) using *Orthosiphon stamineus* leaf. This study showed result in a significant mean reduction of systolic blood pressure with SHR and its efficacy is comparable to a modern recent antihypertensive agent (Azizan *et al.,* 2012).

HPLC method has been designed for separation of three methoxylated flavones (eupatorin, sinensetin and 3'-hydroxy-5,6,7,4'-tetramethoxyflavone) and rosmirinic acid derivatives using a methanol extract of *Orthosiphon stamineus* from locations in Malaysia. It was observed that there was a difference in antioxidant activities (ranging from 55.5% to 84.2%) and variation of total phenolics (ranging from 6.7 to 10.1 mg caffeic acid/g dry weight) (Akowuah *et al*., 2004). In further study, *Orthosiphon stamineus* methanol extract was used to determine the antioxidant activity by measuring the scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and on superoxide anion which resulted in variation of free-radical (ranging from 62.82% to 92.34%) and superoxide-anion scavenging activities (ranging from 53.29% to 75.88%). This study concluded that methanol extract was active. Its antioxidative efficiency was comparable to that of pure synthetic antioxidant butylated hydroxylanisole (BHA) and quercetin (Akowuah *et al.,* 2005). Khamsah *et al.* (2006) stated that the antioxidant activity of *Orthosiphon stamineus* extract was not only due to the phenolic compounds but also staminane-types diterpenes, triterpenes and some other components present in the plant's leaf.

Orthosiphon stamineus plays the role in bacteriostatic activity and antibiotic activity due to the presence of the saponins and ceffeic acid derivatives, respectively (Chen, 1989). The oral administration of Orthosiphon stamineus extract has been

performed on patients and it was reported to increase choleresis and cholekinesis, together with an antibacterial action in cholecystitis (Chen, 1989). 3-hydroxy-5,6,7,4 tetramethoxyflavone and flavones sinensetin did not confirm these findings, which administered intravenously at a dose of 10 mg/kg body weight (Schut and Zwavig, 1993).

The antiinflammatory activity was verified using *Orthosiphon stamineus* leaf chloroform extract by using carrageenan-induced hind paw edema method. HPLC method was done to identify the active compounds contributing to its antiinflammatory activity and the result showed that the flavonoids rich chloroform extract fraction (CF2) which contained eupatorin, sinensetin and 3'-hydroxy-5,6,7,4'-tetramethoxyflavone may affect this activity because of the presence of flavonoid compounds fit for influencing the nitric oxide pathway (Yam *et al.*, 2010).

Sripalang *et al*. (2007) suggested that *Orthosiphon stamineus* (aqueous extract) is advantageous for enhancing the profile in diabetic rats. The study carried out showed the effects of *Orthosiphon stamineus* aqueous extract on plasma glucose concentration, lipid profile in normal and stretozotocin-induced diabetic rats. In the oral glucose tolerance tests which were dose-dependent in both normal and diabetic rats, the extract (0.2-1.0 g/kg) significantly decreased plasma glucose concentration. The most effective decrease in plasma glucose concentration occurred when the extract at 1 g/kg and it was compared with the result of glibenclamide (5 mg/kg). The extract had significantly reduced plasma glucose concentration. The plasma glucose concentration was found to be lower in the extract-treated diabetic rats (at day 7 and 14) that after rehashed every day oral administration of the extract (0.5 g/kg) for 14 days. Towards the conclusion of

the study, the plasma triglyceride concentration was lower in the extract- treated diabetic rats and plasma HDL-cholesterol concentration was significantly increased in diabetic rats which were treated with extract (Sriplang *et al*., 2007).

Isolation of bioassay-guided on *Orthosiphon stamineus* aqueous extract was used to carry out antiangiogenic studies and showed that hexane fraction gave three componds which were oleanolic acid, betulinic acid and ursolic acid. The extract exhibited 20% antiangiogenic activity while the hexane fraction exhibited 80% antiangiogenic activity. The three isolated compounds gave 100% antiangiogenic activity. It can be concluded that the isolated compounds and the hexane fraction had promising antiangiogenic activity (Hussain *et al*., 2012).

The methanol extract of the plant (dried aerial parts) was studied for cytotoxicity. The fractions of the extract, hexane, chloroform, etanoic acid, butanol and water showed that the cytotoxicity at IC_{50} was less than 100 µg/mL. Five new isopimarane-type diterpenes were given in chloroform and etanoic acid fractions using chromatography. It showed mild to weak antiproliferative activities toward highly liver metastatic colon 26-L5 carcinoma and human HT-1080 fibrosarcoma cell lines (Tezuka *et al*., 2000).

Table 2.2: Summary of scientific studies on the *Orthosiphon stamineus* from the reviewed literature

Biological activity	Methodology	Compound present (positive results)	References
Antioxidant	In vitro assay : B-carotene linoleic acid system	• Sinensetin (SEN) • Eupatorin (EUP) • 3'-hydroxy-5,6,7,4'-tetramethoxyflavone (TMF) • Rosmirinic Acid (RA)	Akowuah *et al*., 2004
	In Vitro assay: Free radical scavenging using DPPH	• Sinensetin (SEN) • Eupatorin (EUP) • 3'-hydroxy-5,6,7,4'-tetramethoxyflavone (TMF) • Rosmirinic Acid (RA)	Akowuah *et al*., 2005
	In vitro assay: Free radical scavenging using DPPH, β-carotene linoleic acid system	• Diterpenes • Triterpenes	Khamsah *et al*., 2006
	In vitro assay: Free radical scavenging using DPPH, Fe^{3+}-induced lipid peroxidation inhibiting activities, Trolox equivalent antioxidant capacity (TEAC)	• Sinensetin (SEN) • Eupatorin (EUP) • 3'-hydroxy-5,6,7,4'-tetramethoxyflavone (TMF)	Yam *et al*., 2007
	In vitro assay: Free radical scavenging using DPPH	• Polyphenols	Zakaria *et al*., 2008
Antiinflammatory	In vivo assay: The inflammation induced by tumor promoters, 12-O-tetradecanoylphorbol-13-acetae, on mouse ears	• Orthosiphol A • Orthosiphol B	Masuda *et al*., 1992

	In vitro assays: Lipopolysaccharide (LPS) -stimulates nitric oxide (NO), prostaglandin E2 (PGE2) and intracellular reactive oxygen species (ROS) production RAW 264.7 cells.	• Oleanolic acid • Ursolic acid	Hsu *et al.*,2010
	In vivo assay: Carrageenan-induced hind paw edema method	• Flavonoids • Phenolic compounds	Yam *et al*., 2008
	In vivo assay: Carrageenan-induced hind paw edema method In vitro assay: Nitric oxide (NO) inhibition	• Sinensetin • Eupatorin (EUP) • 3'-hydroxy-5,6,7,4'-tetramethoxyflavone (TMF)	Yam *et al*., 2010
Diuretic	In vivo assay: Placebo controlled double-blind crossover model	• Flavonoids	Doan *et al*., 1992
	In vivo assay: Enhances ion excretion in rat to a level comparable to that obtained with furosemide	• Flavonoids • Triterpenes	Englert and Harnischfeger, 1992
	In vivo assay: Diuretic induced by hydrochlorothiazide and hypouricemic endorsed by allopurinol	• Flavonoids • Triterpenes • Caffeic acid derivatives	Arafat *et al*., 2008
	In vivo assay: Induced by hydrochlorothiazide	• Diterpene • Triterpene • Glycosides • Phenolic compounds (flavonoids, tannins and coumarins)	Beaux *et al.*, 1999

Antibacterial	In vitro assay : Antibacterial activity against food-borne bacteria such as: B. subtilis, B. cereus, S. Areas, Listeria monocytogenes, Klebsiella pneumonia, E. coli, V. parahaemolyticus, Slamonella enteritidis and Salmonella typhimurium, compared to inhibition of 5% lactic acid (the natural food preservative)	• Rosmarinic acid	Ho *et al.*, 2010
Antifungal	In vitro assay: Disc diffusion and minimum inhibitory concentration (MIC) determination method	• β-caryophyllene • caryophyllene oxide • α-humulene • β-pinene • Limonene • B-elemene • 1-octan-3-ol	Hossain *et al.*, 2008
Antipyretic	In vivo assay: The yeast-induced pyrexia model and compared with paracetamol (acetaminophen in U.S)	• Rosmarinic acid	Yam *et al.*, 2009
AntiAngiogenic	Ex vivo assay: Rat aorta assay	• Rosmarinic acid • Sinensetin • Other flavonoids • Diterpenes • Triterpenes • Phenolic compounds	Siddiqui *et al*., 2009 Aisha *et al*., 2009