EXTRACTION OF PHENOLIC COMPOUNDS AND FLAVONOIDS
COMPOUNDS FROM ORTHOSIPHON STAMINEUS

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UNIVERSITI MALAYSIA PAHANG
EXTRACTION OF PHENOLIC COMPOUNDS AND FLAVONOIDs
COMPOUNDS FROM ORTHOSIPHON STAMINEUS

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Thesis submitted in partial fulfillment of the requirements for the award of
the degree of Bachelor of Chemical Engineering

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SUPERVISOR’S DECLARATION

I hereby declare that I have checked this project and in my opinion, this project is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering.

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I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged. This thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

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Special dedication to my supervisor, my family members, my friends, my course mates and all faculty members for all your care, support and believe in me.
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Last but not least, I acknowledge my sincere gratitude to my family members who support me throughout my study in Universiti Malaysia Pahang (UMP).
PENGESTRAKKAN SEBATIAN FENOLIK DAN SEBATIAN FLAVONOIDS DARI ORTHOSIPHON STAMINEUS

ABSTAK

Sebatian fenolik dan flavonoid terkandung dalam Orthosiphon stamineus (OS) mempamerkan kapasiti antioksidan yang tinggi. Kajian ini telah dijalankan untuk membandingkan pengekstrakan ultrasonik (UAE) dan microwave pengekstrakan (MAE) dengan maceration dan menyiasat kesan pelarut (70% -30% v/v metanol-air, 70% -30% v/v propanol-air, 50% -50% v/v metanol-air, 50% -50% v/v propanol-air, air, metanol dan propanol), masa pengekstrakan, kuasa microwave dan suhu pengekstrakan ke atas pengestrakkan Rosmarinic asid dan Sinensetin dari serbuk OS kering. Hasil telah dianalisis dengan menggunakan Ultra Performance Liquid Chromatography (UPLC) dan telah berbanding dengan penentukuran standard dengan masa penahanan mereka. Hasil daripada Jumlah kandungan fenolik (TPC) telah digunakan untuk menyokong hasil UPLC Dengan UAE, hasil tertinggi bagi Rosmarinic asid boleh diperolehi dengan syarat berikut: 70% -30% v/v Metanol-air sebagai pelarut dengan masa pengekstrakan 90 minit pada 60 c. Hasil tertinggi untuk asid Rosmarinic yang boleh diperolehi dengan menggunakan MAE adalah dengan syarat-syarat berikut: 70% -30% v/v Metanol-air dengan masa pengekstrakan 120 s pada 450 W. Dengan UAE, hasil tertinggi untuk Sinensetin boleh diperolehi dengan syarat berikut: propanol sebagai pelarut dengan masa pengekstrakan 90 minit pada 60 C Sementara itu, hasil tertinggi bagi Rosmarinic asid yang boleh diperolehi dengan menggunakan MAE adalah dengan syarat-syarat berikut, 70% -30% v/v Metanol-air dengan pengekstrakan masa 120 s pada 450 W. UAE dan MAE boleh menggantikan maceration dalam pengekstrakan sebatian fenolik dan flavonoid sebatian kerana UAE dan MAE mempunyai hasil yang lebih tinggi dalam masa yang lebih singkat. Cadangan mencadangkan untuk mendapatkan pengoptimuman kajian ini adalah untuk mengkaji kesan yang lebih atau parameter dalam kedua-dua UAE dan MAE.
EXTRACTION OF PHENOLIC COMPOUNDS AND FLAVONOIDS
COMPOUNDS FROM ORTHOSIPHON STAMINEUS

ABSTRACT

Phenolic and flavonoids compounds present in Orthosiphon stamineus (OS) exhibit high antioxidant capacity. This research was conducted to compare ultrasonic-assisted extraction (UAE) and microwave-assisted extraction (MAE) with maceration and investigate effects of solvents (70%-30% v/v methanol-water, 70%-30% v/v propanol-water, 50%-50% v/v methanol-water, 50%-50% v/v propanol-water, water, methanol and propanol), extraction time, microwave power and extraction temperature on Rosmarinic acid and Sinensetin extraction yields from OS dried powder. The extraction yields were analyzed by using Ultra Performance Liquid Chromatography (UPLC) and were compared to standard calibration curve with their retention times. Results from Total Phenolic Content (TPC) were used to support UPLC result. By UAE, the highest yield for Rosmarinic acid could be obtained with following conditions: 70%-30% v/v Methanol-water as solvent with extraction time of 90 minutes at 60°C. The highest yield for Rosmarinic acid that could be obtained by using MAE was with following conditions: 70%-30% v/v Methanol-water with extraction time of 120 s at 450 W. By UAE, the highest yield for Sinensetin could be obtained with following conditions: propanol as solvent with extraction time of 90 minutes at 60°C. Meanwhile, the highest yield for Rosmarinic acid that could be obtained by using MAE was with following conditions, 70%-30% v/v Methanol-water with extraction time of 120 s at 450 W. UAE and MAE can replace maceration in extraction of phenolic compounds and flavonoids compounds since UAE and MAE had higher yields than maceration in shorter time. Recommendations suggested in order to obtain optimization of this research were to study more effects or parameters in both UAE and MAE.
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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Malaysia’s tropical climate contributes to tropical rainforest which supports more than 20000 plant species (Indu and Ng, 2000) and over 3000 herbs are identified as medicinal plants (Ramlan, 2009.) which are reported to have therapeutic and chemical properties. Herbal products had gained popularities and were commercialized sold in pharmaceutical dosage forms, such as capsules, tablets, pills, liquid preparations, creams, lotions, suppositories, patches (Jamal, n.d.) and also dietary supplements. One of the traditional herbs that had gained much attention was Orthosiphon stamineus or commonly known as “Misai Kucing” which was believed to have synergistic bio-enhancing ability for tamoxifen against human breast cancer antioxidant capacity according to Ahamed and Abdul in 2010. Besides, the leaves of Orthosiphon stamineus are commonly used as herbal tea or Java tea for diuresis, to treat rheumatism, diabetes, urinary lithiasis, oedema, eruptive fever, influenza, hepatitis, jaundice, biliary lithiasis, and hypertension (Hossain and Mizanur Rhaman, 2011).
In Malaysia, the plant is used for a wide range of diseases treatment such as eruptive fever, epilepsy, gallstone, hepatitis, hypertension, syphilis, and renal calculus (Akowuah et al., 2004). It had been used for kidney-related and joint ailments such as gall stones, diabetes, arthritis, rheumatism, and gout. It had been proven to remove uric acid through its diuretic activity which was the main path for its therapeutic activity (Ramlan et al., n.d.).

In the past, several techniques had been used for extraction of phenolic and flavonoids compounds from Orthosiphon stamineus such as Soxhlet extraction (Hossain and Mizanur Rahman, 2011), maceration (Mohammad et al., 2011), and reflux extraction (Matkwoski, 2008). Many types of solid-liquid extraction methods are available nowadays such as pressurized fluid extraction, microwave-assisted extraction, matrix solid phase dispersion, and supercritical fluid extraction. Extraction of flavonoids and phenolic compounds from Orthosiphon stamineus is one of the most important steps prior to their determination by Ultra Performance Liquid Chromatography (UPLC).

This research was conducted to investigate the effects of solvent, extraction temperature, ultrasonic or microwave power, and extraction time on total flavonoid content and total phenolic content of Orthosiphon stamineus leaves by three extraction methods which were maceration, ultrasonic-assisted extraction, and microwave-assisted extraction.
1.2 Problem Statement

Conventional methods that had been used to extract phenolic and flavonoids compounds from *Orthosiphon stamineus* was maceration. In recent years, new methods had been developed in extraction technologies which were pressurized fluid extraction, microwave-assisted extraction, matrix solid phase dispersion, ultrasonic-assisted extraction and supercritical fluid extraction. However, the effects of solvent types, extraction temperature, extraction time and power on extraction yields on phenolic and flavonoid compounds were not fully investigated. So, this research was conducted to investigate these parameters in ultrasonic-assisted extraction, microwave-assisted extraction and maceration.
1.3 Research Objective

This research aimed to:

- extract phenolic and flavonoids compounds from *Orthosiphon stamineus*
- compare yield of phenolic and flavonoids compounds among three extraction methods which were ultrasonic-assisted extraction, microwave-assisted extraction and maceration
- determine the best operating parameter for phenolic and flavonoids compounds for each extraction method
1.4 Scope of Study

The scope of this research was to analyze main parameters which were types of solvent, extraction temperature, ultrasonic or microwave power and extraction time. Only *Orthosiphon staminues* was used in this research. Limitations of this research were compounds extracted which are flavonoids and phenolic compounds. Only three types of extraction methods were used which are ultrasonic-assisted extraction, microwave-assisted extraction and maceration.

1.5 Significance of Study

Phenolic compounds had been found to have potential health benefits that were believed to arise mainly from their antioxidants activity. Phenolic compounds act as antioxidants by free radical-scavenging, oxygen radical absorbance, and chelating of metal ions (Halliwell *et al.*, 1995). While flavonoids possessed wide range of biological activities, such as antiallergic, anti-inflammatory, antiviral, antiproliferative and anticarcinogenic activities (Ren *et al.*, 2003). The comprehensive study highlighted that flavonoids possess various clinical and pharmalocogical properties which are antidiabetic, antiatherosclerotic, hepato- and gastro-protective, antitumour, antithrombogenic, antiosteoporotic, and antiviral effects. This study made use of *Orthosiphon stamineus* which existed abundantly and could be cultivated easily to obtain phenolic and flavonoids compounds. Once the best extraction technique was figured out, conventional technique to extract phenolic and flavonoids compounds could be replaced.
CHAPTER 2

LITERATURE REVIEW

2.1 Orthosiphon stamineus

2.1.1 Introduction

Orthosiphon stamineus or Cat’s Whiskers or “misai kucing” is one of the locally well-known herbs and normally distributed at Southeast Asia such as Malaysia, Indonesia, Thailand, Vietnam, and Myanmar. Orthosiphon stamineus is perennial herb from Lamiaceae family. Normally, its height is between 0.3 to 1 m and has 4-angled stem. Its flower is white or pale lilac coloured. And its stamens exceed more than 2 cm from the corolla-tube. The leaves have wide 2 to 4 cm, long 4 to 7 cm, egg-shaped spurs, sharp jagged edges rough and irregular, and usually arranged in opposite pairs. It is given common name as “misai kucing” or Cat’s Whiskers because of its flowers with long wispy stamens shaped like cat whiskers (Indu and Ng, 2000).
Figure 2.1  White *Orthosiphon stamineus*

Figure 2.2  Purple *Orthosiphon stamineus*
2.1.2 Medicinal Properties of *Orthosiphon stamineus*

The recent surge of interest in *Orthosiphon stamineus* has led to the isolation of more than 50 components with different biological activities (Hossain and Ismail, 2010). Twenty phenolic compounds isolated from *Orthosiphon stamineus* were nine lipophilic flavones, two flavonol glycosides, nine caffeic acid derivatives and the new compound 5, 6, 7, 8-tetrahydroxy-6-methoxy flavones (Amzad Hossain *et al.*, 2007). The major part of this plant used for medicinal purposes is its leaves. It was reported that polymethoxylated flavones, sinensetin, tetramethylscutellarein and 3’-hydroxy-5, 6, 7, 4’-tetramethoxy flavone were present in *Orthosiphon stamineus* leaves.

2.1.3 Phenolic Compounds

Phenolic compounds such as lipophilic flavones, caffeic acid derivatives (rosmarinic acid and 2, 3-dicaffeoyltartaric acid), eupatorine, cichoric acid, sinensetin and methoxy flavones were found in *Orthosiphon stamineus* (Olah *et al.*, 2003; Pietta *et al.*, 1991). Phenolic substances are the main phytochemicals with antioxidant properties found in plants. These bioactive compounds inhibit lipid autoxidation by acting as radical scavengers and act as essential antioxidants that protect the propagation of the oxidative chain. The intake of phenolic compounds is inversely correlated with the risk of coronary heart disease. These phytochemicals provide health benefits by several mechanisms: free-radical scavenging, protection and regeneration of other dietary antioxidants and chelating of pro-oxidant metal ions. The structure of phenolic compounds is a key determinant of their radical scavenging and metal-chelating activity.
A study by Huang and Zheng (2005) reported that Rosmarinic acid showed several bioactivities including anti-bacterial, anti-inflammatory and anti-carcinogenic activities.

![Structure of Rosmarinic Acid](image)

**Figure 2.3** Structure of Rosmarinic Acid

### 2.1.4 Flavonoids Compounds

Flavonoids are large group of secondary metabolites in plants that do not have direct involvement with growth of plants. Flavonoids are biological pigments that provide colours from red to blue in flowers, fruit and leaves. Flavonoids compounds exhibit wide range of biological activities and clinical properties including antimicrobial, antibacterial, anti-inflammatory, anti-allergic and antithrombotic actions (Yao *et al.*, 2004). According to the unsaturation and oxidation degrees of the three-carbon segment, several sub-classes of flavonoids can be distinguished which are flavonols, flavanones, flavones, flavan-3-ols, anthocyanidins and isoflavones (Biesaga, 2011). Flavonoids compounds that can be isolated from *Orthosiphon stamineus* are eupatorin, sinensetin, 5-hydroxy-6,7,3’ 4’-tetramethoxyflavone, salvigenin, 6-hydroxy-5, 7, 4’-trimethoxyflavone and 5, 6, 7, 3’-tetramethoxyflavone and 5, 6, 7, 3’-tetramethoxy-4’-hydroxy-8-C-prenylflavones (Hossain and Rahman, 2011). Factors such as place of
plant growth, the genus, extraction conditions and technology contribute to the content of flavonoids compounds (Mao et al., 2008). In a study (Arai et al., 2000), the total intake of quercetin was inversely related to total cholesterol and low-density lipoprotein (LDL) plasma levels. Sinensetin helps to relax the muscles of the walls of the internal vessels thus facilitating easier flow of urine and even the small particles that become stones. Also, Sinensetin was reported to have high chemo synthesizing effect which was used for the synthesis of the multi-drug resistance cell for anti-cancer drugs (Ahmad et al., 2008).

![Figure 2.4 Structure of Sinensetin](image_url)
2.2 Extraction

2.2.1 Introduction

Extraction is a separation process where the distribution of the phenolic compound and flavonoids between two immiscible phases is made in order to achieve the appropriate distribution coefficient. The extraction procedure is sequential and systematically carried out using an aqueous organic solvent to extract phenolic compounds and flavonoids in fruit and vegetables samples. Stability of flavonoids and phenolic compounds will be affected by extraction method.
2.2.2 Ultrasonic-Assisted Extraction (UAE)

2.2.2.1 Introduction

The use of ultrasound to enhance the extraction yield is a technique that started in the 1950s with laboratory scale experiments. The extraction of bioactive compounds under ultrasound irradiation about 20 to 100 KHz offers high reproducibility in shorter times, simplified manipulation, reduced solvent consumption and temperature and lower energy input (Khan et al., 2010). Usually, UAE performed in experimental work is indirect sonication by using a small cleaning bath. Additional agitation or shaking is attached to avoid standing waves or the formation of solid free regions for the preferential passage of the ultrasonic waves. Very few plant materials require more than two hours of sonication. Interestingly, when a process was scaled up in a large ultrasonic cleaning bath, for example 10 litres solvent and over 1000g of plant material, the ultrasonic procedure seems to be a significant improvement when extraction time is taken into account. It is noted that when high frequency ultrasound is employed, the extraction yield did not increase significantly however the degradation of the herb constituents was diminished (Vinatoru et al., 1997).

Since vegetal tissue consists of cells surrounded by walls, UAE mechanism involves two types of physical phenomena which are diffusion through the cell walls and rinsing the cell contents once the walls are broken. Both phenomena are significantly affected by ultrasonic irradiation. Some cells exist in the form of internal and external glands that are filled with essential oil. For internal glands, the milling degree of the vegetal material plays important role. Reducing the size of the vegetal
material particles will increase the number of cells directly exposed to ultrasonically induced cavitation. External glands have thin skin and can be easily destroyed by sonication (Vinatoru, 2001). During sonication, cavitation process causes swelling of cells or breakdown of cell walls allows high diffusion rate across cell wall (Vinatoru, 2001).

2.2.2.2 Ultrasonic Cavitation

Ultrasonic cavitation is the momentary creation of “bubbles” in the fluid which immediately and violently implode to produce millions of microscopic jets of liquid which gently scrubs the parts which are submerged in the tank. These cavities are created tens of thousands of times each second to gently remove contaminants without damage, as long as the ultrasonic frequency selected is correct. Ultrasonic cavitation is capable of selectively disrupting the subcutaneous cells through thousands of microscopic implosions impacting the cell membranes. When high power ultrasonic is doing in liquid material media, tens of thousands times ultrasonic vibration will be produced per second continuously, to prompt liquid molecular fast movement.
2.2.3 Advantages

Ultrasonic extraction is a more complete extraction process since the surface area between the solid and liquid phase is significantly larger due to the cell disruption and particle dispersion. By the use of sonication also the operating temperature can be reduced, allowing the extraction of temperature-sensitive components.

A substantial advantage of ultrasonic is the influence on the most important processing parameters: amplitude, time, temperature, pressure, and viscosity. Thereby, the extraction process can be optimized to ensure that the structure of the extracts does not become damaged.

The main advantages of UAE are reproducibility of the technique, applicability of the method to a range of sample sizes, reduction in time needed to perform highly efficient extraction and efficient extraction of polar organic compounds (Lee et al., 2001).

2.2.4 Applications

UAE has been recognized and widely applied in the phyto-pharmaceutical industry for a wide range of herbal extracts (Vilkhu, 2008). Besides, it also had been applied in oil, protein and bioactives from plant materials.
2.2.3 Microwave-Assisted Extraction (MAE)

2.2.3.1 Introduction

The first reported analytical use for microwave ovens was almost 35 years ago for the digestion of samples for metal analysis, with the first use of microwaves for organic compound extraction ten years later. Refer to Mandal, Mohan and Hemalatha in 2007, microwaves are non-ionizing electromagnetic waves which has frequency between 300MHz to 300GHz positioned between x-ray and infrared rays in electromagnetic spectrum. Microwaves are made up of two oscillating perpendicular fields which are electric field which is in-charge of heating and magnetic field. In MAE, heating occurs in targeted manner with no heat lost to the environment as heating occurs in a closed system and helped to reduce the extraction time which usually within 30 minutes.

2.2.3.2 Types of Solvent

The solvent used for MAE must be able to absorb microwave radiation and thereby becomes hot. Two parameters define the dielectric properties of materials which are $\varepsilon'$, the dielectric constant and $\varepsilon''$, dielectric loss factor. The former describes the polarizability of the molecule in electric field while the latter measures the efficiency with which into the absorbed microwave energy can be converted into heat. The ratio of these two terms is called the dissipation factor, $\delta$ shown in Eq. (2.1). To get maximum heated distributed through the matrix, it is vital to choose a solvent that has a high
dielectric constant as well as high dissipation factor. A range of solvents and their respective physical constants is shown in Table 2.1.

\[
\delta = \frac{\varepsilon''}{\varepsilon}
\]  

(2.1)

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Dielectric constant((\varepsilon'), \text{ F/m})</th>
<th>Dielectric loss factor((\varepsilon''), \text{ F/m})</th>
<th>Dissipation factor (\delta \times 10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>80</td>
<td>12</td>
<td>1500</td>
</tr>
<tr>
<td>Acetone</td>
<td>20.7</td>
<td>11.5</td>
<td>5555</td>
</tr>
<tr>
<td>Methanol</td>
<td>23.9</td>
<td>15.2</td>
<td>6400</td>
</tr>
<tr>
<td>Ethanol</td>
<td>7</td>
<td>1.6</td>
<td>2286</td>
</tr>
<tr>
<td>Hexane</td>
<td>1.88</td>
<td>0.00019</td>
<td>0.10</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>6.02</td>
<td>3.2</td>
<td>5316</td>
</tr>
</tbody>
</table>
2.2.3.3 Microwave Power

Generally, extraction yield increases proportionally with increasing microwave power up to a limit before the increase becomes insignificant or decline. High microwave power might cause poor extraction yield due to degradation of thermal sensible compounds. Microwave power provides localized heating in the sample and acts as a driving force for MAE to destroy the plant matrix so that analyte can diffuse out and dissolve in the solvent. Increasing the power will improve the extraction yield and result in shorter extraction time. Temperature and microwave power are interrelated. Increasing temperature causes the solvent power to increase due to a drop in viscosity and surface tension (Chan et al., 2011). It has been reported by Xiao in 2008 that extraction yield decreases if the extraction temperature is higher than 110 °C due to instability of the flavonoids at that particular temperature.

2.2.3.4 Advantages

Microwave irradiation reduces overheating problems which minimizes degradation of analytes (Biesaga, 2011). Microwave extraction results in shorter time at the same temperature using less solvent. Microwave heating allows all sample fluid to be heated and extraction solution to reach desired temperature more rapidly and also avoids thermal gradient.
2.2.3.5 Applications

MAE has been applied to a diverse range of sample types such as soils, sediments, sewage sludge, plants and marine samples for the determination of organic compounds. Other applications include the extraction of high-value compounds from natural sources such as phytonutrients, nutraceutical and functional ingredients and pharma actives from biomass (Jain et al., 2009).
Table 2.2  Extraction Methods Used to Extract Compounds from Plants

<table>
<thead>
<tr>
<th>Author</th>
<th>Compounds extracted</th>
<th>Plant</th>
<th>Extraction method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ma et al. (2005)</td>
<td>Flavonoids</td>
<td><em>Glycyrrhiza uralensis</em></td>
<td>Soxhlet extraction: 90% aqueous ethanol; Liquid-liquid extraction: ether and ethyl acetate</td>
</tr>
<tr>
<td>Zhou et al. (2005)</td>
<td>Flavonoid glycosides</td>
<td><em>Trollius ladebouri</em></td>
<td>Soxhlet extraction: 60% or 70% aqueous ethanol</td>
</tr>
<tr>
<td>Biesaga (2011)</td>
<td>Flavonoids</td>
<td>Maize corn</td>
<td>Heated reflux extraction, Microwave-assisted extraction, Ultrasonic extraction, Maceration extraction</td>
</tr>
<tr>
<td>Xiao et al. (2008)</td>
<td>Flavonoids</td>
<td><em>Radix Astragali</em></td>
<td>Microwave-assisted extraction</td>
</tr>
</tbody>
</table>

Table 2.3  Extraction Methods Used to Extract Compounds from *Orthosiphon stamineus*

<table>
<thead>
<tr>
<th>Author</th>
<th>Compounds extracted</th>
<th>Extraction methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maheswari <em>et al.</em> (2008)</td>
<td>Phenolic compound &amp; flavonoids</td>
<td>Soxhlet extraction: 1kg powdered leaves with methanol for 36 hours</td>
</tr>
<tr>
<td>Chun <em>et al.</em> (2010)</td>
<td>Rosmarinic acid</td>
<td>Maceration: 50g powder macerated with 500ml methanol of different concentrations (25%, 50%, 75%, 100%) in water bath at 40 °C for 8 hours</td>
</tr>
<tr>
<td>Hossain and Rahman (2011)</td>
<td>Flavonoids</td>
<td>Soxhlet extraction: 1Kg dried leaves extracted with 10L for 36 hours</td>
</tr>
<tr>
<td>Akowuah <em>et al.</em> (2004)</td>
<td>Sinensetin, Eupatorin, Rosmarinic acid, 3'-hydroxy-5, 6, 7,4’ tetramethoxyflavone</td>
<td>Continuous stirring: 1 g of samples extracted with 100mL methanol at 40 °C for 4 hours</td>
</tr>
</tbody>
</table>
2.3 Analysis

2.3.1 UPLC Analysis

Ultra-Performance Liquid Chromatography (UPLC) is the newest technology in liquid chromatography. UPLC involves the liquid chromatography at high pressures, providing chromatography that outperforms traditional High-Performance Liquid Chromatography (HPLC) in peak resolution. Additionally, UPLC delivers separations in 3 to 10 minutes, as compared to 15 to 20 minute separations in traditional HPLC. UPLC offers a lot of advantages such as high resolution, high speed, high sensitivity, quick and ease of instrument handling. Even neon particles can be separated easily. It is up to 9 times faster, has up to twice the resolution and three times the sensitivity than that of HPLC. By using UPLC, separation and identification of amphetamine, methamphetamine, ephedrine, pseudoephedrine, phentermine, MDA (3, 4-methylenedioxyamphetamine), MDMA (3, 4-methylenedioxy-\textit{N}-methylamphetamine), MDEA (3.4-methylenedioxy-\textit{N}-ethylamphetamine) and ketamine can be done in less than 3 minutes.
2.3.2 UV-Vis Analysis

Ultraviolet-Visible (UV-Vis) is an electromagnetic radiation between visible light and X-rays. The wavelength range is from 1nm to 400nm. UV-Vis is a type of vibrational spectroscopy, similar to infra-red. A sample in a cuvette is exposed to light energy between 190nm (in the UV) and 1000nm (in the Vis). Certain parts of an organic molecule will absorb some of this energy to create peaks on a spectrum for quantitative (primarily) and qualitative analysis. A cuvette is a standard cell holder. It is made of Quartz for UV range while glass and plastic for Vis range. It has 1 to 100mm path lengths. UV-Vis analysis is primarily used for quantitative analysis of known compounds. UV-Vis is one of the most popular techniques the pharmaceutical, foods and paints industries, as well as water laboratories.
CHAPTER 3

METHODOLOGY

3.1 Introduction

Optimizations of extraction in this experiment were achieved based on one-factor-one time approach. One-factor-one time approach or single factor experiment meant only one factor was the variable at one time while others were kept constant. This approach was used in previous study to optimize phenolic extraction from OS (Chew et al., 2011). This approach was used in this experiment to determine the range of factor that had significant effect on recovery of phenolic compounds and flavonoids compounds.
3.2 Materials

3.2.1 Plant Material

*Orthosiphon stamineus* (OS) was collected from land near Gambang. The leaves part of the OS was used. Leaves were collected and washed thoroughly. After drying in oven with 34°C, the dried leaves were tested its moisture content in moisture analyzer model MS-70 and grounded to a homogenous powder in a blender branded Pensonic.

3.2.2 Equipment/ Apparatus

Ultrasonic bath (CREST, US), Domestic microwave oven (Samsung, MW71E), Oven (MEMMERT, Germany), Blender (Pensonic), Analytical Balance, Micropipette, Universal Bottle, Nylon membrane filter (Dioflow, 0.45um), Syringe, Vacuum pump, Filtration set.

3.2.3 Chemicals

Dimethylsulfoxide (DMSO), Folin-Ciocalteu reagent and two standards: rosmarinic acid and sinensetin used for the UPLC analysis were obtained from Sigma-Aldrich. Methanol (99.8% purity) and propanol (99.8% purity) used were obtained from Merck (Darmstadt, Germany). Gallic acid, and sodium carbonate were purchased from Sigma chemical Co. (St. Louis, MO). All the chemical reagents used were analytical
grade and the deionized water used throughout experiment was obtained from Milli-Q water purification system (Millipore Corporation, USA).

3.3 Method

3.3.1 Ultrasonic-assisted Extraction (UAE)

1.0 g of OS dry powder was accurately weighed into 500 mL conical flask and mixed with 100 mL of solvents. The conical flask contained powder and solvent was put in ultrasonic bath. The flask was wrapped with aluminum foil to prevent light exposure. After extraction, the samples were filtered through 0.45 µm nylon membrane filter by vacuum pump filter and kept at – 80°C for UPLC analysis later.

3.3.1.1 Types of Solvent

By fixing extraction time and extraction temperature at 90 minutes and 50°C respectively, samples were extracted using different types of solvent: pure water, pure methanol, pure propanol, 70%-30% v/v methanol-water and 70%-30% v/v propanol-water.
3.3.1.2 Extraction Temperature

By using the best solvent determined in step above which was 70%-30% v/v propanol-water and extraction time of 90 minutes, samples were extracted at different temperatures: 40, 50, 60 and 70°C.

3.3.1.3 Extraction Time

Samples were extracted in 70%-30% v/v propanol-water for 30, 60, 90, 120 and 180 minutes by fixing the extraction temperature constant at 50°C.
3.3.2 Microwave-Assisted extraction (MAE)

0.25 g of OS dry powder was accurately weighed into 50 mL PTFE Teflon vessel and mixed with 25 mL of solvent. Then, the vessel filled with powder and solvent was treated under microwave irradiation. After extraction, the samples were filtered through 0.45 µm nylon membrane filter by vacuum pump filter and kept at –80°C for UPLC analysis later.

3.3.2.1 Types of solvent

By fixing extraction time and microwave power at 2 minutes and 200W respectively, samples were extracted using different types of solvent: pure water, pure methanol, pure propanol, 70%-30% v/v methanol-water and 70%-30% v/v propanol-water.
3.3.2.2 Microwave Power

OS powder was mixed with 70%-30% v/v propanol-water, the best solvent determined in step above. Different microwave powers were tested: 100, 200, 300 and 450W. Extraction time was set at 2 minutes.

3.3.2.3 Time

OS powder was mixed with 70%-30% v/v propanol-water, the best solvent determined in step previously. Microwave power was set at 300 W. Extraction times were tested: 30s, 40s, 2 minutes and 5 minutes.

3.3.3 Maceration

1.0 g of OS powder was mixed with 100mL 70%-30% v/v propanol-water at 60°C for 2 hours and 4 hours maceration with the help of magnetic stirrer in a closed conical flask by referring procedures done by Akowuah et al. in 2004. The extract later was filtered through 0.45 µm membrane filter and stored at -80°C until UPLC analysis later.
3.4 Analysis

3.4.1 UPLC Analysis

The analysis was done by using Ultra Performance Liquid Chromatography (UPLC). Before the analysis was done, the mobile phases were prepared. UPLC analysis was performed by using Water Acquity UPLC® system equipped with photodiode array detector and connected to a computer running Waters Empower 2® software. Acquity UPLC HSS T3 C18 Column (2.1 x 75 mm, 1.8 µm inner diameters) was used. The mobile phases consisted of acetonitrile: trifluoroacetic acid (20: 0.001); ultrapure water: trifluoroacetic acid (20:0.001). The temperature was maintained at 30°C, with injection volume of 2 µl and flow rate of 0.170 ml/min. The peaks were detected at 340 nm and identified by standard substances. The reference compounds used as markers were Rosmarinic acid and Sinensetin. The external standard method was used for the UPLC quantification. The results were reported as percent of dry powder weight.

3.4.2 Total Phenolic Content (TPC)

The effects of types of solvent, microwave power, extraction time and extraction temperature were studied on the recovery of phenolic compounds. The phenolic compounds were analyzed by total phenolic content (TPC) according to study done by Trabelsi (2010). Gallic acid was used as standard for calibration curve for TPC.
The standard gallic acid was accurately weighed and then dissolved in appropriate volume of methanol to produce corresponding stock standard solutions. The stock standard solution was diluted with methanol to different concentrations for the calibration curve. TPC test used was suggested by Trabelsi in 2010. 0.125 mL of diluted sample extract was added to 0.5 mL of distilled water and 0.125 mL of Folin-Ciocalteu reagent. After 3 minutes, 1.25 mL of sodium carbonate solution (7g/100mL) were added and the final volume was made up to 3 mL with distilled water. Blank was prepared by replacing extract with deionized water. The absorbance was measured at 760 nm using UV-Vis spectrophotometer after incubation for 90 min at 23 °C in dark. Quantification was performed on the basis of linear calibration plots of the UV absorption peak area against concentrations.
3.5 Standard Curves

3.5.1 Standard Curve for Rosmarinic Acid

The standard Rosmarinic acid was accurately weighed and then dissolved in appropriate volume of methanol to produce corresponding stock standard solutions. The stock standard solution later was diluted with methanol to different concentrations and analyzed by UPLC to plot standard calibration curve. The range of the concentrations was 0.00225 till 5.0 mg /mL. The graph of area versus varies concentration was plotted and showed in Figure 3.1. The calibration equation for Rosmarinic acid was \( y = 2.25 \times 10^7 x - 2.33 \times 10^4 \). The linear regression coefficient was 0.9997. Figure 3.4 showed peak of standard Rosmarinic acid detected at its retention time. The peaks of Rosmarinic acid were confirmed by comparison of its retention times with reference standards showed in Figure 3.6.

![Graph of area versus concentration](image)

**Figure 3.1** Standard Curve of Rosmarinic Acid
3.5.2 **Standard Curve for Sinensetin**

The standard Sinensetin was accurately weighed and then dissolved in appropriate volume of DMSO to produce corresponding stock standard solutions. The stock standard solution later was diluted to different concentrations with DMSO and analyzed by UPLC to plot standard calibration curve. The graph of area versus varies concentration was plotted and showed in Figure 3.2. The range of concentration was 0.00008 till 0.01 mg/mL. The calibration equation for Sinensetin was \( y = 2.26 \times 10^8 x + 1.31 \times 10^4 \). The linear regression coefficient was 0.9974. Figure 3.5 showed peak of standard Sinensetin detected at its retention time. The peaks of Sinensetin were confirmed by comparison of its retention times with reference standards showed in Figure 3.6.

![Standard Curve of Sinensetin](image)

**Figure 3.2** Standard Curve of Sinensetin
3.5.3 Standard Curve for Gallic Acid

The standard Gallic acid was accurately weighed and then dissolved in appropriate volume of methanol to produce corresponding stock standard solutions. The stock standard solution later was diluted with methanol to different concentrations and analyzed by UV-Vis Spectrophotometer to plot standard calibration curve. The range of the concentrations was 0.01 till 0.5 mg /mL. The graph of absorbance versus varies concentration was plotted and showed in Figure 3.3. The calibration equation for Gallic acid was \( y = 4.6625x + 0.0045 \). The linear regression coefficient was 0.9982. the absorbance was detected at wavelength of 760nm.

![Figure 3.3 Standard Curve for Gallic Acid](image.png)
Figure 3.4  Rosmarinic Acid Peak Shown

Figure 3.5  Sinensetin Peak Shown
Figure 3.6 Identification of Rosmarinic Acid and Sinensetin Peaks
CHAPTER 4

RESULT AND DISCUSSIONS

4.1 Introduction

This research was able to extract phenolic and flavonoids compounds from *Orthosiphon stamineus* via microwave-assisted extraction (MAE) and ultrasonic-assisted extraction (UAE). Extraction yields of both methods were compared with maceration. Results obtained from UPLC were discussed. Results from Total Phenolic Content (TPC) were used to support UPLC results.
4.2  UPLC Result

4.2.1  UAE

4.2.1.1 Types of Solvent

![Bar Chart](image)

**Figure 4.1**  Effects of Solvents on Rosmarinic Acid Yield (Time: 90 Minutes; Temperature: 50°C)

Based on the Figure 4.1 showed above, 70%-30% v/v Methanol-water was the best solvent for Rosmarinic acid extraction with yield of 4.01 mg RA/ g DW, followed by 70%-30% v/v Propanol-water (3.88 mg RA/ g DW), 50%-50% v/v Methanol-water (3.62 mg RA/ g DW), 50%-50% v/v Propanol-water (3.62 mg RA/ g DW), pure methanol (3.63 mg RA/ g DW). Pure propanol and water were poor solvent with yield of 29.60 µg RA/ g DW and 0 respectively. Binary-solvent system was more favorable
compared to mono-solvent system (Chew et al., 2011). Water in methanol and propanol was easy to penetrate into OS powder and enhanced the absorbing capacity of ultrasonic energy. Besides, the extraction yield of Rosmarinic acid increased with increasing percentage of methanol and propanol. This supported the findings of Akuwoah et al. (2005) of an increasing amount of Rosmarinic acid extracted from OS leaves using different solvent system (50% methanol > 100 % methanol).

![Figure 4.2](image.png)

**Figure 4.2** Effects of Solvents on Sinensetin Yield (Time: 90 minutes; Temperature: 50°C)

Based on Figure 4.2, propanol was the best solvent for Sinensetin extraction with yield of 24.82 µg Sin / g DW, followed by pure methanol (23.96 µg Sin/ g DW), 70%-30% v/v Propanol-water (23.82 µg Sin/ g DW), 50%-50% v/v Propanol-water (18.56 µg Sin/ g DW) and 70%-30% v/v Methanol-water (15.98 µg Sin/ g DW). The extraction yield of Sinensetin increased with increasing percentage of methanol and propanol. This was probably due to the relative polarity, increase in effective swelling the surface area.
for solute-solvent contact and higher solubility of flavonoids in alcohol than in water (Biesaga, 2011).

4.2.1.2 Temperature

The trend shown in Figure 4.3 indicated that temperature of 60°C was the most suitable temperature for Rosmarinic acid and Sinensetin extraction as it showed highest yield. Extraction yield of Rosmarinic acid reached peak at 60°C (7.63 mg RA/ g DW). The Sinensetin yield reached a peak, 61.99 µg Sin/ g DW at 60°C, but at 70°C there was a sharp drop to 59.41 µg Sin/ g DW. It was noticed that the graph showed increasing trend from 40°C to 60°C. Increasing temperature increased Sinensetin extraction yield by increasing diffusion rate. Sinensetin yield showed dramatic fall at 70°C. This was
probably because thermal degradation of Sinensetin compound occurred because of increased temperature which was beyond certain limit.

4.2.1.3 Time

Figure 4.4 Effect of Time on Rosmarinic Acid and Sinensetin Yields (Solvent: 70%-30% v/v Propanol-water; Temperature: 50°C)

Figure 4.4 showed that extraction yield of Rosmarinic acid increased tremendously from 30 to 90 minutes, then, the extraction yield decreased at 120 minutes. Peak was observed at 120 minutes with Rosmarinic acid yield value of 4.09 mg RA/ g DW. At 120 minutes, Rosmarinic acid yield showed value of 3.86 mg RA / g DW. Over exposure of OS powder on solvent caused degradation. 90 minutes was the most suitable time for Rosmarinic acid extraction. Figure 4.4 showed increasing trend of Sinensetin yield. The trend showed linear relationship between Sinensetin yield and time. The value
of Sinensetin yield increased sharply from 30 to 90 minutes and decrease at 120 minutes with value of 25.04 µg Sin /g DW. This was probably due to the high concentration gradient between OS powder and solvent at the beginning of extraction, the concentration gradient reduced with time, so, only a small increment as time continued.
4.2.2 MAE

4.2.2.1 Types of Solvent

There was a suggestion by Chew in 2011 that phenolic compounds presented in OS had moderately polar characteristic. Rosmarinic acid yield of pure propanol and 70%-30% v/v Propanol-water were compared. Pure propanol showed higher extraction yield, 7.83 mg RA/ g DW than 70%-30% v/v Propanol-water (7.47 mg RA/ g DW). Excessive solvent caused poor microwave heating as the microwave irradiation would be absorbed by the solvent and additional power was required. Methanol had high dielectric constant as well as high dissipation factor, so methanol was able to absorb microwave radiation and thereby becomes hot. Rosmarinic acid yield of pure methanol
and 70%-30% v/v Methanol-water were 5.50 and 8.03 mg RA/ g DW respectively. 70-
%-30% v/v Methanol-water showed the highest Rosmarinic acid yield.

Figure 4.6  Effects of Solvents on Sinensetin Yield (Microwave Power: 200 W; Time:
2 Minutes)

Figure 4.6 showed that 70%-30% v/v Methanol-water and 70%-30% v/v
Propanol-water showed high Sinensetin yield with values of 51.72 and 51.49 µg Sin/ g
DW respectively. Meanwhile, Sinesetin yields of 50%-50% v/v methanol-water, 50%-50%
v/v Propanol-water, pure methanol and pure propanol were 47.37, 50.28, 28.07 and
18.72 µg Sin/ g DW respectively. Methanol and propanol were water miscible. Water in
methanol and propanol solutions was easy to penetrate into the OS and enhanced the
absorbing capacity of microwave energy. The mass transfer of Sinensetin from the
sample to the solution was enhanced. It was indicated that the solvent with higher

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concentration of methanol and propanol in water resulted in higher extraction yield of Sinensetin from OS. This result was supported the findings done by Wang et al. (2011).

4.2.2.2 Microwave Power

![Graph showing effect of microwave power on Rosmarinic Acid and Sinensetin yields](image)

**Figure 4.7** Effect on Microwave Power on Rosmarinic Acid and Sinensetin Yields  
(Solvent: 70%-30% v/v Propanol-water; Time: 2 Minutes)

From 100 W to 450 W, there was a rise in Rosmarinic acid yield by using MAE. The highest extraction yield, 3.93 mg RA / g DW was observed at 450 W. Microwave power provided localized heating in the sample and it acted as driving force to destroy the OS matrix. Hence, Rosmarinic acid diffused out and dissolved in the solvent.

It was obvious that Sinensetin yield fluctuated from 100 W (7.51 μg Sin/ g DW) to 300 W (22.21 μg Sin/ g DW), then, the yield fell at 450 W (21.66 μg Sin/ g DW). The
fluctuation of yield value was due to higher microwave power in closed vessels caused the extraction process to be performed at evaluated temperature. When the microwave power increased from 100 to 300 W, more electromagnetic energy was transferred to the extraction system and improved the extraction efficiency. Similar explanation was also given to support the effect of microwave power on the MAE of flavonoids from *Radix astragali* (Xiao *et al.*, 2008) and silybinin from *Silybum marianum* (Dhobi *et al.*, 2009). Higher microwave power which was 450 W caused poor extraction yield due to degradation of Sinensetin which was thermal sensible compound.

**4.2.2.3 Time**

![Graph](image)

**Figure 4.8** Effect of Time on Rosmarinic Acid and Sinensetin Yields (Solvent: 70%-30% v/v Propanol-water; Microwave Power: 300W)

Figure 4.8 showed the effect of microwave irradiation time on the Rosmarinic acid and Sinensetin yields, with increased yields with the increase of the microwave
irradiation time and reached the highest at 120 s. The yields of both Rosmarinic acid and Sinensetin were 3.83 mg RA/ g DW and 22.60 µg Sin/ g DW respectively. These findings were supported by the study conducted by Wang et al in 2011. Longer exposure of extract in solvent caused higher values of Rosmarinic anid and Sinensetin yields. But, the prolonged exposures caused degradation by heating. These findings were supported by study conducted by Rafiee et al. in 2011. So, 120s was the optimal irradiation time.
4.2.3 Maceration

![Graph showing the effect of time on Rosmarinic Acid yield.]

**Figure 4.9** Effect of Time on Rosmarinic Acid Yield (Solvent: 70%-30% v/v Propanol-water; Temperature: 60°C)

Rosmarinic acid yield was increasing with time. Rosmarinic acid yield at 4 hours (3.91 mg RA/ g DW) was higher than 2 hours (3.83 mg RA/ g DW). It can be observed that only little increment which was 0.08 mg RA/ g DW within 2 hours.
**Figure 4.10** Effect of Time on Sinensetin Yield (Solvent: 70%-30% v/v Propanol-water; Temperature: 60°C)

Sinensetin yield was increasing with time. Sinensetin yield at 4 hours (25.46 µg Sin/ g DW) was higher than 2 hours (24.96 µg Sin/ g DW). It can be observed that only little increment which was 0.50 µg Sin/ g DW within 2 hours.
4.3.1 UAE

4.3.1.1 Types of Solvent

Figure 4.11 Effects of Solvents on TPC

Binary-solvent system was more favorable in the extraction of phenolic compounds from plant as compared to mono-solvent system. Solvents would only extract those compounds which have similar polarity with the solvents. In 2011, Chew et al. suggested that most of the phenolic compounds presented in Orthosiphon stamineus has moderately polar characteristic. 70% Methanol-water showed highest Gallic acid yield with value of 9.63 mg GA/ g DW.
4.3.1.2 Temperature

Figure 4.12  Effects of Temperatures on TPC

Figure above showed that extraction of TPC from OS extract increased with increasing temperature up to 60°C (9.91 mg GA/g DW) and followed by a slight decrease (9.80 mg GA/g DW) as a result of thermal degradation of polyphenols. Similar findings were reported by Tabaraki and Nateghi, which reported that TPC of rice bran extracts increased with increasing temperature up to 54°C and followed by slight decrease.
4.3.1.3 Time

Peak was observed at 120 minutes with highest TPC value which was 9.42 mg GA/ g DW followed by dramatic fall at 180 minutes with Gallic acid yield of 9.17 mg GA/ g DW. TPC was decreased when the extraction time was longer than 120 minutes. Prolonged extraction would lead to decrease in the phenolic content of extract as oxidation of phenolic compounds occurred by extending the exposure to light and oxygen.
4.3.2 MAE

4.3.2.1 Types of Solvent

Based on the result shown above, it showed that phenolic compounds presented in OS had moderately polar characteristic. 50% Methanol-water was the most suitable solvent for TPC with highest Gallic acid yield (19.56 mg GA/ g DW).

Figure 4.14  Effects of Solvents on TPC
4.3.2.2 Microwave Power

Figure 4.15 Effect of Microwave Power on TPC

Figure 4.15 showed increasing trend with increasing microwave power applied. Highest value obtained was at 450 W with value of 10.08 mg GA/ g DW. As microwave power increases, more electromagnetic energy was transferred to the extraction system.
4.3.2.3 Time

Extraction yield was rapidly increased in the beginning of extraction and reached 9.42 mg GA/ g DW at 120s. Further irradiation was only slightly increased extraction yield to 9.69 mg GA/ g DW.

Figure 4.16  Effect of Time on TPC
4.3.3 Maceration

![Graph showing the effect of time on TPC yield.](image)

**Figure 4.17** Effect of Time on TPC

TPC yield was increasing with time. TPC yield at 4 hours (9.68 mg GA/ g DW) was higher than 2 hours (9.22 mg GA/ g DW). It can be observed that only little increment which was 0.46 mg GA/ g DW within 2 hours.
CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

5.1.1 Rosmarinic Acid

The highest yield for Rosmarinic acid that could be obtained by using UAE was with following conditions, 70%-30% v/v Methanol-water as solvent with extraction time of 90 minutes at 60°C. The highest yield for Rosmarinic acid that could be obtained by using MAE was with following conditions, 70%-30% v/v Methanol-water with extraction time of 120 s at 450 W.
5.1.2 Sinensetin

The highest yield for Sinensetin that could be obtained by using UAE was with following conditions: propanol as solvent with extraction time of 90 minutes at 60°C. Meanwhile, the highest yield for Rosmarinic acid that could be obtained by using MAE was with following conditions, 70%-30% v/v Methanol-water with extraction time of 120 s at 450 W.

5.2 Comparison

Both UAE and MAE can replace maceration in extraction of phenolic compounds and flavonoids compounds since UAE and MAE have higher yields than maceration method in shorter time. MAE can obtain higher yield than UAE since MAE can have extremely high yield within few minutes.

5.3 Recommendations

A few recommendations were suggested in order to obtain optimization of this research. It was suggested to study more effects or parameters in both UAE and MAE. Parameters that can be added in UAE including ultrasonic power, frequency and solvent to sample ratio. Meanwhile, parameters that can be added in MAE including extraction cycle, temperature and solvent composition.
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