OPTIMISATION OF PHENOLIC COMPOUNDS EXTRACTION FROM MISAI KUCING USING RSM

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OPTIMISATION OF PHENOLIC COMPOUNDS EXTRACTION FROM MISAI KUCING USING RSM

AININ SOFIA BINTI AZNI

Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
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JUNE 2015

SUPERVISOR'S DECLARATION

We hereby declare that we have checked this thesis and in our opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering.

Signature	:
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Date	: 16 JUNE 2015

STUDENT'S DECLARATION

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

Signature :
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Dedication

To my family especially my mom and dad for their love and support

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ABSTRACT

Misai kucing or the scientific name is Orthosiphon stamineus contains lots of phenolic compounds such as phenolic acid, flavanoids and antioxidants. These compounds have various benefits likes antifungal, antimicrobial, antitumor and antibacterial. Besides that, people also used it as remedy to treat the diseases. In order to get components from plant materials, normally they used extraction method. The yield of phenolic compounds extraction is dependent on the size of particles, solvent used, extraction method, power irradiation and condition. The method used for this work is microwave assisted extraction was studied by using response surface methodology (RSM) design for optimization. The 2 level factorial and Box behnken design have been used to determine the optimum extraction and to develop a quadratic polynomial. The highest peak for rosmarinic acid from the extraction of misai kucing is 2.203 at 2.00 to 2.30 minutes while the standard peak of rosmarinic acid is at 2.215 at 2.00 o 2.30 minutes. The highest yield of rosmarinic acid (RA) of 24.7971 mg RA/g DW was obtained at 66.6% of aqueous ethanol concentration with 112W microwave power for 3.75 minutes. The yield for total phenolic content (TPC) and total flavonoid content (TFC) are 48.51 mg GAE/g DW and 233.243 mg QE/gDW respectively. The optimum yields were obtained at 54.68% of ethanol at 170W of microwave power for 4.34 minutes. The lower total solid (TS) was produced higher rosmarinic acid per DW of material but higher TS contain more rosmarinic acid and hence it is affect by reduced its solubility in solvent and lower extraction yield. Longer extraction time increased rosmarinic acid extraction yield at lower TS (0.1 g/ml) because longer time needed for rosmarinic acid to diffuse in the solvent. Since solvent is not saturated at lower TS so the extraction process continues and become higher at longer time. The microwave assisted extraction provides rapid extraction of phenolic compounds without significantly compromising the extraction yield.

ABSTRAK

Misai kucing atau nama saintifiknya adalah Orthosiphon stamineus mengandungi banyak sebatian fenolik seperti asid fenolik, flavanoids dan antioksidan. Sebatian-sebatian ini mempunyai pelbagai manfaat suka antikulat, antimikrob, antitumor dan anti-bakteria. Selain itu, rakyat juga menggunakannya sebagai ubat untuk merawat penyakit. Dalam usaha untuk mendapatkan komponen daripada bahan-bahan tumbuhan, biasanya mereka menggunakan kaedah pengekstrakan. Hasil pengekstrakan sebatian fenolik bergantung kepada saiz zarah, pelarut yang digunakan, kaedah pengekstrakan, kuasa sinaran dan keadaan. Kaedah yang digunakan untuk kerja-kerja ini adalah gelombang mikro pengekstrakan dibantu dikaji dengan menggunakan kaedah permukaan respons (RSM) reka bentuk untuk pengoptimuman. Tahap 2 faktorial dan Box reka bentuk behnken telah digunakan untuk menentukan pengeluaran yang optimum dan untuk membangunkan polinomial kuadratik. Puncak tertinggi asid rosmarinic daripada pengekstrakan misai kucing adalah 2,203 di 2,00-2,30 minit sambil puncak taraf asid rosmarinic adalah pada 2,215 pada 2.00 o 2.30 minit. Hasil tertinggi asid rosmarinic (RA) daripada 24,7971 mg RA / g DW telah diperolehi pada 66.6% daripada kepekatan etanol akueus dengan 112W kuasa gelombang mikro untuk 3.75 minit. Hasil bagi jumlah kandungan fenolik (TPC) dan jumlah kandungan flavonoid (TFC) adalah 48,51 mg GAE / g DW masing-masing dan 233,243 mg QE / GDW. Hasil optimum diperolehi di 54,68% etanol pada 170W kuasa gelombang mikro untuk 4.34 minit. Semakin rendah jumlah pepejal (TS) telah dihasilkan asid rosmarinic lebih tinggi bagi setiap DW bahan tetapi TS tinggi mengandungi lebih banyak asid rosmarinic dan dengan itu ia memberi kesan dengan mengurangkan kelarutan dalam hasil pengekstrakan pelarut dan atas. Masa pengeluaran yang lebih panjang meningkat rosmarinic hasil pengekstrakan asid di TS lebih rendah (0.1 g / ml) kerana masa yang lebih lama diperlukan untuk asid rosmarinic untuk meresap dalam pelarut. Sejak pelarut tidak tepu di TS rendah supaya proses pengekstrakan itu berterusan dan menjadi lebih tinggi pada masa yang lebih lama. Microwave dibantu pengekstrakan menyediakan pengekstrakan pesat sebatian fenolik tanpa menjejaskan dengan ketara hasil pengekstrakan.

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LIST OF SYMBOLS

%	Percent
ºC	Degree Celcius
hr	Hour
etc	Etcetera
λ	Wavelength
ε	Dielectric constant
δ	Dissipator factor
wt	Weight
cm	Centimeter
mm	Milimeter
m	Meter
µm	Micrometer
nm	Nanometer
kg	Kilogram
g	Gram
mg	Miligram
ml	Mililiter
W	Watt
Min	Minute

LIST OF ABBREVIATIONS

BHA	Butylated hydroxyanisole
DW	Dry weight
Eup	Eupatorin
Sin	Sinensetin
RA	Rosmarinic acid
H_20	Water
HPLC	High Performance Liquid Chromatography
UPLC	Ultra Performance Liquid Chromatography
MAE	Microwave-assisted extraction
UAE	Ultrasound-assisted extraction
ME	Maceration
FDA	Food and Drug Administration
EtOH	Ethanol
Sec	Second
TPC	Total phenolic content
TFC	Total flavonoid content

CHAPTER 1

1 INTRODUCTION

1.1 Motivation and statement of problem

Misai Kucing is commonly known as cat's whiskers and the scientific name is 'Orthosiphon stamineus Benth'. In Java they called misai kucing as Java tea. Misai Kucing is belonging to the Lamiaceae family and can be found in Southeast Asian such as Malaysia, Indonesia and Thailand. Misai kucing has attracted the interest of researchers and peoples concern about the compound content and the benefits of it. In Malaysia, Misai kucing is help in treats various ailments because it is easy to access and consumed (Khamsah et al., 2006). Misai kucing also used as remedy for kidney stone and nephritis. The leaves of Misai kucing commonly used in Southeast Asia to treat diuresis, diabetes, rheumatism, oedema, hepatitis, eruptive fever, influenza and hypertension (Sumaryono et al., 1991). In Malaysia, they have formed the leaves of Misai kucing into the product in form of tablets, drinks, raw herbs, dried leaves and tea sachet herbs as a health drink (Ibrahim et al., 2010). Herbal tea of Misai kucing will help to improve health and treatment of kidney disease, bladder inflammation, gout and diabetes (Arafat et al., 2008). Misai kucing is popular as herbal tea in Southeast Asian.

In Malaysia, they use traditional method more than modern method to cure diseases. So that most of people have taken herbal drinks to take care of their health because they have lots of benefit. In 2002, there are four lead chronic illnesses that caused 29 million deaths which are cardiovascular disease, cancer, chronic lung diseases and diabetes mellitus (Organization, 2003). In 18 years period (1975 to 2005), the populations of Malaysia have been increased from 12.3 to 26.7 million peoples and have been increase about 8.3% to 28.96 million in between 2005 to 2010 (N. M. Amal et al., 2006). On 2006, the third National Health Morbidity Survey had conducted a survey about the chronic illness. Out of 57000 respondents, only 56710 (98.6%) had participated. Based on the survey, females got higher ranking having chronic illness which is 16.8% (16.3 to 17.3). The common illness was hypertension which is 7.9%

(7.6 to 8.2), followed by diabetes mellitus which is 4.0% (3.8 to 4.2) (N. M. Amal, et al., 2006). Lot of peoples suffer in handle stress. In Misai kucing leaves, the phenolic compounds to reduce the oxidative stress have been found by retard the lipid oxidation in biological systems. The extraction of Misai kucing contains many valuable bioactive compounds such as antibacterial, antifungal, antimicrobial and antitumour, and previous work have approved it (Saravanan et al., 2006).

The bioactive compounds contains in Misai kucing usually can get by performed solvent extraction method. The yields of bioactive compounds are depending on the extraction methods, type of solvent, pH, solid-to-liquid ratio and size of particles Misai kucing leaves (Wang et al., 2004), (Kosar et al., 2005) , (Durling, et al., 2007). Based on the previous work, extraction yield of flavonoid compounds is depend on the extraction method, temperature and solvent polarity (Sultana et al., 2009), (Lapornik et al., 2005). There are several types of methods that can be used to perform extraction of Misai kucing leaves. Previous studies have used the conventional methods such as soxhlet extraction and maceration extraction. These methods used high temperatures and perform for a longer time. After few years forward, there are better extraction methods such as microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE) and supercritical extraction. These methods have been developed based on the extraction time, yield and quality of extraction.

The extraction of bioactive compound from plant material is depending on the type of solvent used. Previous work have been studied the polarities of the phenolic range from polar to non-polar and also the range of solvents which are methanol, ethanol and water as their mixture (Cuvelier et al., 1996), (Kosar et al., 2005). Wang et al. (2004) have been explored the influence of the different solvents such as methanol, ethanol, water and acetonitrile on the amount of the extracted phenolic acids. The use of water as the extraction solvent gives 20% less rosmarinic acid compared to other solvents. The best extraction yields of caffeic acid and rosmarinic acid were obtained at 30 to 60% of aqueous ethanol solution. The most suitable solvents uses for extraction are water and ethanol mixture (Maja et.al, 2013).

Optimization is refer to the process of a system have improve to obtain the maximum benefit of it. In this case, to get the maximum amount of phenolic compounds

extraction in Misai kucing leaves, the optimal variables or parameters are required. In the present study, the response surface methodology (RSM) was examined for optimization variables such as extraction time, temperature, ethanol concentration and solid-to-liquid ratio to get the maximum yield of total phenolic compounds. The 2 level factorial designs with 2 replicate were used to determine the most significant effect of extraction. The RSM design is used to determine the optimum extraction condition.

1.2 Objectives

The following are the objectives of this research:

- To optimize the phenolic compounds extraction from misai kucing leaves via RSM.
- To develop a fast analysis for phenolic compounds from misai kucing
- To develop innovative extraction method which is fast efficient extraction of phenolic compounds from misai kucing

1.3 Scope of this research

The following are the scope of this research:

i. Extraction of the phenolic compounds from misai kucing.

The work will focus on the method use to extract the phenolic compounds from misai kucing. There are many types of extraction method such as microwave assisted, ultrasound assisted, maceration, soxhlet, supercritical and so on. Therefore, microwave assisted has been chosen to extract the compounds.

ii. Quantification or analysis of total phenolic content (TPC), total flavonoid content (TFC) and ultra-performance liquid chromatography (UPLC) of the plant leaves extract.

The work will focus on the method use to determine the total phenolic content, total flavonoid content and UPLC analysis from the plant extract.

iii. Optimization study via RSM

The RSM have been used to optimize the extraction. 2 level factorial designs with 2 replicate have been used to determine the most significant effect of the extraction and Box-behnken design model have been used to develop the quadratic polynomial. The methods help in produce the higher yield of extraction.

1.4 Main contribution of this work

Firstly, this work aim to optimize the phenolic compounds from Misai kucing leaves by using extraction methods such as microwave-assisted extraction, ultrasound-assisted extraction and maceration method. Response surface methodology (RSM) was used to examine the optimization variables or parameters to produce higher yield of extraction.

UPLC method has been used in this work to separate and identify the major active components such as rosmarinin acid, sinensetin and eupatorin. UPLC method is the best analytical method because it is take a shorter time to detect the peak or the particles. It is produce accurate qualification and quantification analysis of the Misai kucing extract.

Lastly, the total phenolic content (TPC) and total flavonoids content (TFC) are determined by using their method. TPC was determined using Follin-Ciocalteu reagent (Trabelsi, et al., 2010). The absorbance was measured at λ = 760 nm using a calibrated ultraviolet-visible spectrometer (Hitachi U-1800, Japan). TFC was determined by aluminium chloride colometric assay (Abouzid & Elsherbeiny, 2008) was measured at λ = 414 nm using a calibrated ultraviolet-visible spectrometer (Hitachi U-1800, Japan).

1.5 Organisation of this thesis

The structure of the reminder of the thesis is outlined as follow:

Chapter 2 is review the previous work by the researchers on Misai kucing leaves from characteristics, extraction, analytical method and compound contains in the Misai kucing leaves.

Chapter 3 presents the experimental method to extract the phenolic compounds. The methods to extract the active compounds have been discussed in this part such as microwave assisted extraction, ultrasound assisted extraction and maceration method. Qualification and quantification analysis (determination of total phenolic and total flavonoid) and ultra-performance liquid chromatography was presented in this chapter.

Chapter 4 shows the results and discussion on the yield of extraction from misai kucing leaves. The optimum parameters to get better extraction yield also have been discussed in this chapter. The influences of various percentages of solvents, time and power of extractor on the phenolic compounds are presented in detail.

Chapter 5 is a conclusion and summary of the thesis and outlines of future work which might be derived from the technique developed for this work.

CHAPTER 2

2 LITERATURE REVIEW

2.1 Overview

This chapter have reviews the previous work on Misai kucing. The famous active compounds in Misai kucing and their benefits had been discussed. Based on the previous journal, they are various methods to extract the active compounds such as microwave-assisted extraction, ultrasound-assisted extraction and maceration method. The literatures present the method of extraction that can be used to optimize the phenolic compounds from Misai kucing using response surface methodology (RSM). The best method will help to get the best result of extraction yield.

2.2 Introduction

The extraction of Misai kucing leaves have a lot of useful bioactive compounds such as terpenoids, phenolic and sterol that expose diuretic (Arafat et al., 2008), antidiabetic (Mohamed et al., 2013), antiangiogenic and antiproliferative properties (Doleckova et al., 2012). There are twenty phenolic compounds found from misai kucing. There are nine lipophilic flavones, two flavonol glycoside and nine caffeic acid derivatives such as rosmarinic acid, sinensetin, eupatorin and 2,3,-dicaffeoyltartaric acid (Akowuah et al., 2004). In aqueous methanol extracts, caffeic acid derivatives have become the most abundant polyphenol. They also will be appearing in the polymethoxylated flavones predominate. The special part found in Misai kucing is polymethoxylated flavones, because of the rare structural features which is the methoxy group at C-5. The other groups of chemically active constituents also are found in Misai kucing likes terpenoids such as diterpenes and triterpenes and sterols.

Misai kucing contain phenolic compounds that have great interest due to their health-benefit antioxidant properties. The examples of antioxidants are butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Spigno & de Faveri,

2007). The presences of phenolic compounds which are flavonoids have made the Misai Kucing popular in search engine. The researchers believe that the leaves of misai kucing have antiallergenic, antihypertensive and anti-inflammatory properties. Phytochemicals are popular to have several health-benefit properties, reduce the risks of cancer, cardiovascular, heart and neurodegenerative diseases (Dahmoune et al., 2015).

2.2.1 Misai kucing plant characteristic

Table 2. 1: The characteristics of Misai kucing plant

CHARACTERISTICS	DESCRIPTION
Height	• It can grow up to 1.5 metres.
Stem	• Rectangular and it easy to break
Leaves	• Opposite, oval-shaped, acuminate and grossly dentate, elongated and pointed.
Flowers	• Lax and grow in terminal pseudo spikes. • Pale violet to white colour with remarkably long stamens extending far beyond the flower.

Based on Table 2.1, the characteristic of the plant have shown about its height which can grow up to 1.5 metres. The stem of the Misai kucing plant is in rectangular shape and it is easy to break. There are two types of Misai kucing plant which are Orthosiphon stamineus (white flower) and Orthosiphon aristatus (purple flower).

2.3 Previous work on Misai kucing

Table 2. 2: Previous study on Misai kucing

Author	**Study**	**Remarks**
Arafat et al. (2008)	Biological activity	Analyze the diuretic impact of a methanol extract of O. stamineus in regular rat.
Akowuah et al. (2004)	HPLC method development and biological activity	Separation was developed from HPLC method to determine the methoxylate flavones (sinensetin, eupatorin, 3'-hydroxy-5,6,7,4'-tetramethoxyflavone and rosmarinic acid) and estimate the antioxidative properties.
Anna et al. (2008)	Physiological and biological activity	Carry out the physiological studies by using gamma irradiation and evaluate the concentration of rosmarinic acid.
Olah et al. (2003)	Biological activity and HPLC method development	Higher diuretic and uricosuric action have been found in 50% ethanol extract compared to 70% ethanol extract. HPLC method was used for quantification analysis.
Maheswari et al. (2008)	Biological activity	O. stamineus extract has decreased the level of lipid peroxidation and extraction of O. stamineus leaves contain hepatoprotective activity.
Adam et al. (2009)	Biological activity	O. stamineus have shown contain diuretic activity but it was less effective than hydrochlorothiazide and furosemide.
Yasuhiro et al. (2000)	Biological activity	Examine the diterpene compound from methanol extraction.
Amzad et al. (2008)	Biological activity	O. stamineus extract possess the anti-fungal properties of the essential oil and crude extraction.

Table 2. 3: Size of sieve tray based on previous study

Author	Title	Plants	Size of sieve tray
Chung et al. (2013)	Modelling and prediction of extraction profile for microwave assisted extraction based on absorbed microwave energy.	Theobroma cacao L. leaves (cocoa)	0.25 – 0.60 mm
Dahmoune et al. (2015)	Optimization of microwave-assisted extraction of polyphenols from Myrtus communis L. leaves.	Myrtus communis L. leaves	125 µm
Zhizhe et al. (2014)	Comparison of four kinds of extraction techniques and kinetics of microwaves-assisted extraction of vanilla planifolia Andrews.	Vanilla planifolia Andrews	0.630 mm
Ma et al. (2013)	Microwave-assisted aqueous two-phase extraction of isoflavonoids from Dalbergia odorifera T. Chen leaves.	Dalbergia odorifera leaves	30-80 mesh
Ghasemzadeh et al. (2014)	Optimization of ultrasound-assisted extraction of flavonoid compounds and their pharmaceutic activity from curry leaf (Murraya koenigii L.) using RSM.	Murraya koenigii L. (curry leaf)	80 mesh
Majid et al. (2014)	Optimization of ultrasonic-assisted extraction of phenolic compounds from bovine pennyroyal (Phlomidoschema parviflorum) leaves using RSM.	Phlomidoschema parviflorum leaves	149 µm
Vetal et al. (2014)	Microwave-assisted extraction of urolic acid and oleanolic acid from ocimum sanctum.	Ocimum sanctum leaves	0.50-1.0 mm
Zhang et al. (2014)	Ultrasound-assisted extraction of bergenin from Astilbe chinensis.	Astilbe chinensis leaves	250 µm

2.4 Analysis methods for bioactive compounds from Misai kucing leaves

The leaves of Misai kucing have lots of benefits to human to treat various diseases such as diuresis, diabetes, rheumatism, oedema, hepatitis, eruptive fever, influenza and hypertension. There are lots of compound that will be good to human like antioxidant itself can help in treat various ailment. The one of most important step is extraction of compound from plant material. Extraction is a mass transfer process. The previous studies have used conventional extraction methods to extract phenolic compounds from Misai kucing leaves because they have found out that active compounds are more able to extract by using lower polarity solvent. The old version of solvent extraction such as heating, boiling or refluxing are associated with longer extraction times and lower production yield. It also used large quantity of organic solvents and bad extraction efficiency (Shirsath et al., 2012), (Zhang et al., 2011).

The extraction of phenolic compound from misai kucing leaves usually used conventional extraction method because it is provide more area for improvement such as to reduce the extraction time without affect the quality of extraction. Maceration is one of the conventional extraction method had been found by researcher Akowuah et al. (2004) that sinensetin and eupatorin are more able to extract by using lower polarity solvent. Maceration method also able to extract active component of O. stamineus such as toxicity and biological activity test but Olah et al. (2003) and Mohamed et al. (2011) have approve that maceration method take a longer time to extract the active compound. Olah et al. (2003) able to get the extract of active component after 5 days by using 50% and 70% ethanol while Mohamed et al. (2011) have used 50% ethanol and able to extract the toxicity in 24 hours.

Many researchers have studies how to improve the extraction method of phenolic compound due to lack of previous method. Recently, they have been develop better extraction methods of phenolic compound such as microwave-assisted extraction (MAE), ultrasonic-assisted extraction (UAE), accelerated solvent extraction (ASE) and supercritical extraction (Zhang et al., 2011). Microwave-assisted extraction (MAE) and ultrasonic-assisted extraction (UAE) are accepted because the function of this method can cut down the working time, increase yield and able to keep the quality of extract.

2.4.1 Microwave assisted extraction method

Microwave extraction method is the process of heating solvents in contact with sample with microwave energy to partition compound. Microwave is an electromagnetic radiation that can transmit as a wave. The two principle of heating using microwave energy are ionic conduction and dipole rotation. Heat and mass gradient must work in the same way to produce higher extraction yield (Chemat et al., 2009). Heat is dissipated volumetrically inside the irradiated medium in MAE. The most important factor is solvent to select microwave physical constants. It is important to select a solvent that have high extracting power and strong interaction. As a new method extraction, MAE is known as a more environmental-friendly process with economic advantages than the traditional extraction methods. Microwave-assisted extraction is the simplest and the inexpensive technique for the extraction of nutraceuticals (Hemwimon et al., 2007). Recently, this technique has been commonly used for sample preparation (Chen et al., 2008). In the present study, the extraction of polyphenols was under microwave dry process without adding any organic solvent or water, which is different from conventional solvent extraction techniques. The benefits of MAE are the extensive reduction in time and solvent consumption with better extraction yield (Eskilsson & Bjorklund., 2000) (Wang & Weller, 2006). Besides, it is also produce higher extraction rate and keep the quality of the extraction.

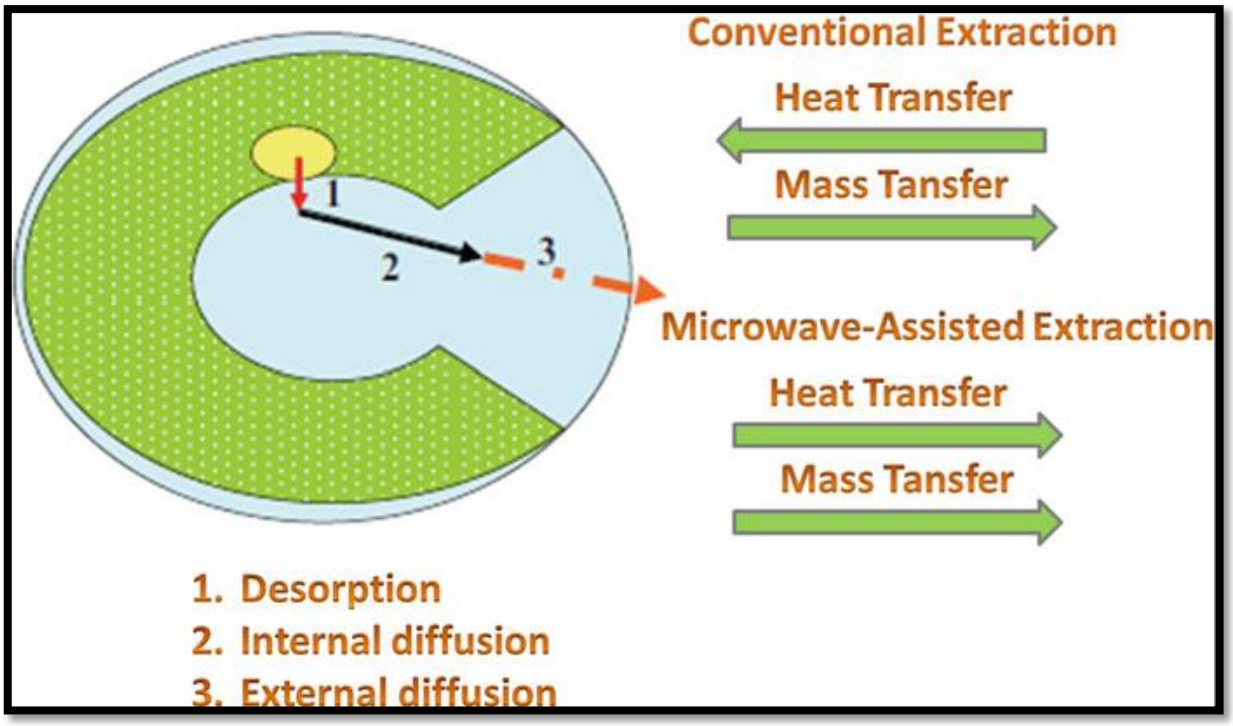

Figure 2. 1: Basic heat and mass transfer mechanisms in microwave and conventional extraction (Perino-Issartier et al., 2011)

2.4.2 Ultrasound-assisted extraction

Ultrasound-assisted extraction was sequentially used in extraction which is can accelerate heat and mass transfers (Chemat et al., 2004). In the modern industry, eco-friendly techniques such as ultrasound-assisted extraction (UAE) have gained popularity, as ultrasound irradiation (20–100 kHz) is able to offer high ability to produce, shorter extraction times, reduced solvent consumption, lower temperature and lower energy input compared with other extraction methods (Bimakr et al., 2012). During sonication, ultrasound produces cavitations bubbles from ultrasonic waves that permit greater penetration of the extraction solvent into the plant cell walls compared to conventional extraction methods, effectively releasing the intracellular products of the plant (Lee & Lin, 2007).

Physical and chemical properties of the plant material will be changing when the interaction happen between ultrasound waves and the plant material itself (Chemat & Khan, 2011). Ultrasounds also apply a mechanical effect which is allow better penetration of solvent into the sample and it is increase the contact surface area between solid and liquid phase (Jing et al., 2008), (Rostagno et al., 2003). An ultrasound probe was chosen for this study instead of the more commonly used ultrasonic bath because the bath lacks uniformity in the distribution of the ultrasound energy, and the power of the bath declines with time. By contrast, an ultrasound probe is able to focus on a localized sample zone, which guarantees a higher efficiency extraction (Priego-Capote & Luque de Castro, 2004).

2.4.3 Maceration extraction

Maceration is one of the conventional extraction method had been found by researcher Akowuah et al. (2004) that sinensetin and eupatorin are more able to extract by using lower polarity solvent. Maceration method also able to extract active component of O. stamineus such as toxicity and biological activity test but Olah et al. (2003) and Mohamed et al. (2011) have approve that maceration method take a longer time to extract the active compound. Olah et al. (2003) able to get the extract of active

component after 5 days by using 50% and 70% ethanol while Mohamed et al. (2011) have used 50% ethanol and able to extract the toxicity in 24 hours. Besides that, maceration extraction methods have to use higher temperatures for a long period but, gallic acid was simply ruined when exposed to high temperatures for a longer time (Amirah et al., 2012).

2.5 Bioactive compounds from misai kucing leaves

Misai kucing have lot of useful active compound and it has been used as remedy to treat various illnesses. It is widely used for treatment of many diseases such as diabetes mellitus, hypertension, rheumatisms and menstrual disorder. The diuretic effect of Misai kucing leaves is used to treat various kidney diseases from infection to renal calculi. The benefits of Misai kucing have been supported by alienation of several active chemical constituents from Misai kucing plant such as flavonoids (Malterud et al., 1989), (Sumaryono et al., 1991), terpenoids (Matsuda et al., 1992), (Tezuka et al., 2000), organic acid, caffeic acid derivatives and others. The main components in Misai kucing leaves are phenolic compounds which are polymethoxylate flavonoids such as sinensetin and eupatorin; caffeic acid derivatives such as rosmarinic acid, cichoric acid and caffeic acid. Figure 2.2 have shown the structural of active components in Misai kucing leaves (Olah et al., 2003). The selected components in this study are rosmarinic acid, sinensetin and eupatorin and the structure of these three components are shown in Figure 2.2.

Misai kucing contain phenolic compounds that have great interest due to their health-benefit antioxidant properties. The examples of antioxidants are butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Spigno & de Faveri, 2007). The bioactive components that extract from Misai kucing leaves have a lot of useful properties such as antisalmonella, antimicrobial, antipyretic, diuretic effect, anti-inflammatory, antihypertensive, anti-diabetic, hepatoprotective, anti-ploriferative and toxicity. Yam et al. have studies the anti-inflammatory and analgesic properties by standardized extract the Misai kucing leaves and it was able to avoid the inflammatory process by induce Carageenan. An analgesic effect is presence when the extract was

able to inhibit acetic acid induced. The extraction of Misai kucing leaves by using solvent methanol/water (50/50) have showed an ability to reduce fever better than Paracetamol which is the effect last until 4 hours. It was induced by yeast. An analytical method, HPLC have shown that the extract contains of rosmarinic acid, sinensetin, eupatorin and tetramethoxyflavone (Yam et al., 2009). The diuretic properties have been widely used to treat kidney diseases in traditional medicine. Many researchers have been studied about diuretic properties. In recent work, diuretic activity was shown that has dose dependent effects on the urine output in Sprague-Dawley rat (Adam et al., 2009). The anti-diabetic properties are one of the useful bioactive components that contain in Misai kucing leaves. It have been approved that extract was able to decrease plasma glucose level in dose dependent manner in both normal and diabetic rats (Sriplang et al., 2007). The previous works have been studied about the toxicity of the Misai kucing extraction. Acute toxicity of standardized extraction has been reported that at single dose of 5000 mg/kg body weight, there are no observe during the 4 days period (Abdullah & Ismail., 2009).

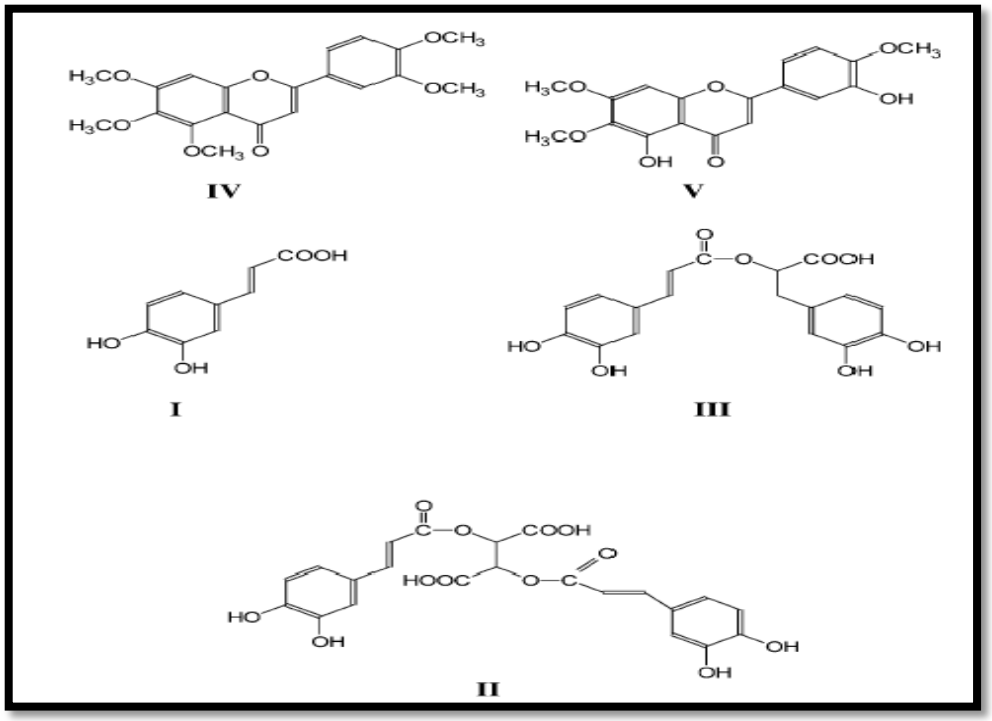

Figure 2. 2: Active component in Misai kucing extract (IV) sinensetin, (V) eupatorin, (I) caffeic acid, (III) rosmarinic acid and (II) cichoric acid (Olah et al., 2003)

2.6 Optimization study via RSM

Optimization is refer to the process of a system improve to obtain the maximum benefit of it. It is applied to the experiment to produce the best results. Response surface methodology (RSM) is a design or model of experiment to optimize the response (output variable) which will influence the independent variables (input variable). The RSM method is used for the extraction of phenolic compounds only. The parameters are used in the experiment to obtain the optimal extraction. The variables use in this extraction methods are extraction times (0.5 – 6 min), solid to solvent ratio is from (0.1 – 10 v/w), the ethanol proportion is from 40 to 100% and the microwave irradiation power (25 – 250 W). Table 2.4 have shown the example of 2 level factorial designs. The design is used to determine the most significant effect of the extraction. Table 2.5 have shown the Box-behnken model design. It is used to develop the quadratic polynomial.

Table 2. 4: Example of 2 level factorials designs

Run	TS (g/100ml)	Power (W)	Time (min)	% Etoh	Response			
					RA	Eup	Sin	Actual Solid
1	1	25	0.5	40				
2	10	25	0.5	40				
3	1	250	0.5	40				
4	10	250	0.5	40				
5	1	25	6	40				
6	10	25	6	40				
7	1	250	6	40				
8	10	250	6	40				
9	1	25	0.5	100				
10	10	25	0.5	100				
11	1	250	0.5	100				
12	10	250	0.5	100				
13	1	25	6	100				
14	10	25	6	100				
15	1	250	6	100				
16	10	250	6	100				

Table 2. 5: Example of Box Behnken design

Run	TS (g/100ml)	Power (W)	Time (min)	% Etoh	Respone			
					RA	Eup	Sin	Actual solid
1	0.1	137	6	40				
2	0.1	137	6	100				
3	0.1	250	6	70				
4	0.1	250	3.25	100				
5	0.1	25	6	70				
6	0.1	137	3.25	70				
7	0.1	25	3.25	40				
8	0.1	25	3.25	100				
9	0.1	137	0.5	40				
10	0.1	137	0.5	100				
11	0.1	137	3.25	70				
12	0.1	137	3.25	70				
13	0.1	25	0.5	70				
14	0.1	137	3.25	70				
15	0.1	250	3.25	40				
16	0.1	250	0.5	70				
17	0.1	137	3.25	70				

2.7 Ultra-performance liquid chromatography (UPLC)

Quantification and qualification of the bioactive compounds need a good analytical method to get the better results. The previous studies have analysed the bioactive compounds by using HPLC and most of them use this analytical method. High-performance liquid chromatography (HPLC) is an analytical method use to separate the component in the mixture, to identify the components and to quantify the components. Previous work have approved by using reverse phase column, rosmarinic acid produced was low retention and not separate well (Mohamed et al., 2011), (Yam et al., 2008). The problem of this matter has been improved by using a column with a smaller particle size. Liquid chromatography (LC) is the best method to analyse the bioactive component. HPLC method needs longer times to detect the component. Recently, a new analytical method has introducing which is ultra-performances liquid chromatography (UPLC) to overcome the weakness of HPLC method. UPLC as shown in Figure 2.3 is column chromatography used to separate, identify and quantify the compounds. It can separate small particles quickly and effectively. LC is the process of passing a mixture of particles through the column to separate. UPLC usually runs at higher pressure to increase speed, efficiency and resolution and the standard column usually packed with small particle size which is 1.7µm.

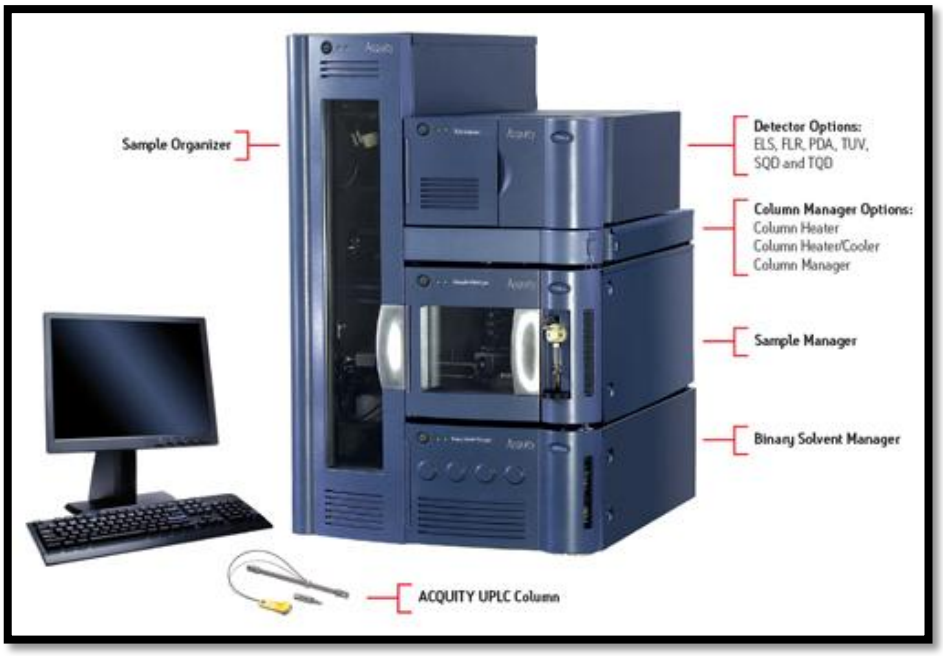

Figure 2. 3: The Acquity UPLC system

2.8 Summary

Misai kucing contain lots of useful bioactive components such as rosmarinin acid, eupatorin and sinensetin. Based on the previous work, MAE and UAE had many advantages over conventional method. So, the result is MAE was chosen as extraction method to extract the phenolic compound. The best analytical method for liquid chromatography is a must to obtain the better results of qualification and quantification of these bioactive components.

CHAPTER 3

3 MATERIALS AND METHODS

3.1 Overview

This chapter presents the materials and chemical used for the experimental study. The details methods study for the extraction, proximate analysis (total phenolic content (TPC) and total flavonoids content (TFC)) and ultra-performance liquid chromatography (UPLC) were presented in this chapter.

3.2 Chemicals

Ethanol (99%), HPLC grade acetonitrile, trifluoroacetic acid (99%), sodium hydroxide (98%), sodium nitrite and aluminum hexachloride (99.9%) were purchased from Sigma Aldrich (St. Louis, MO). Propanol was obtained from Fisher Scientific (Pittsburgh, PA). HPLC grade methanol was obtained from Fluka (USA) and Folin-Ciocalteu reagent was obtained from Merck (Darmstadt, Germany). The chemical of ethanol, propanol, methanol, HPLC grade acetonitrile, HPLC grade methanol, trifluoroacetic acid and Folin-Ciocalteu reagent were in liquid phase. Sodium hydroxide, sodium nitrite and aluminum hexachloride were in solid phase.

3.3 Plant Materials

White flowered of Orthosiphon stamineus leaves samples is collected in Gambang, Pahang, Malaysia. Then, the leaves were washed and dried in an oven at 35 °C for a few days. After that, the dried leaves were grounded to powder form and kept in the tight plastic bag at room temperature to avoid the moisture absorption.

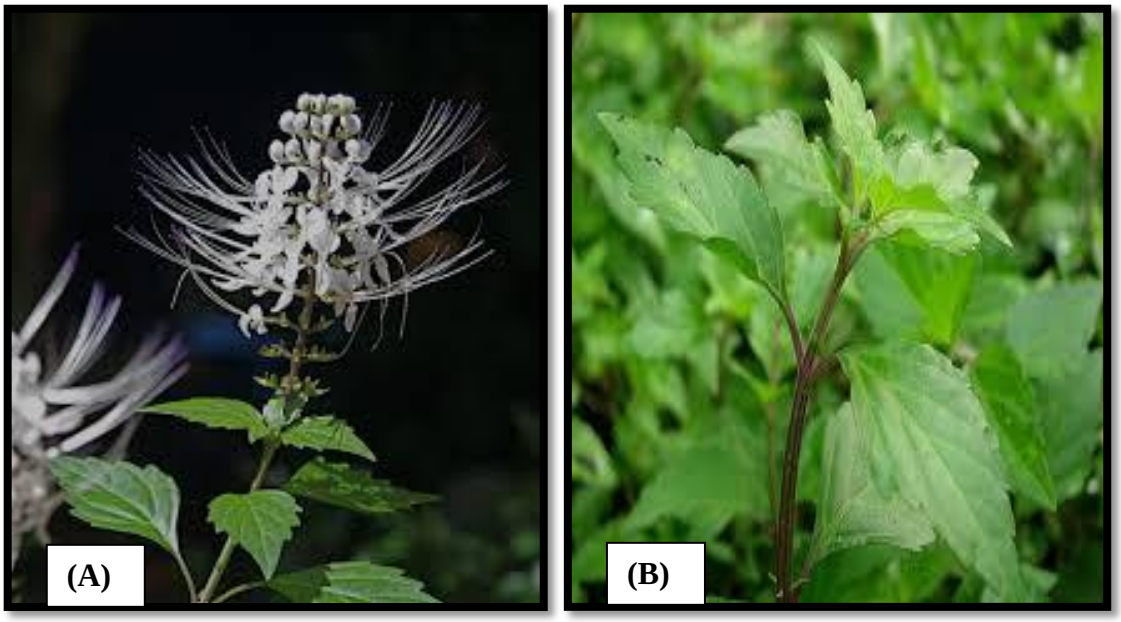

Figure 3. 1: (A) Orthosiphon stamineus (white) flower, (B) Orthosiphon stamineus leaves

3.4 Extraction methods

3.4.1 Microwave-assisted extraction

Misai kucing extracts were prepared by using Microwave Organic Synthesis Reactor (M.A.D Technology, 2012) at 230°C as control temperature and the range of power within 25W to 250W for 0.5min to 6min. Hence, before the extraction part, the powder has been sieves and 125µm were chosen to use and the powder was moisture content by using a moisture analyzer (AND MS-70) at temperature 105°C. The dried plant powder were weighted based on solid to liquid ratio (0.1 to 10%) and mixed with

5mL of various concentration of ethanol (40 to 100%) into vial. The parameter is manipulated by using RSM in order to get better extraction yield. Use the different concentration of ethanol in water for extraction of phenolic compound will be safe and efficient (Li et al., 2012).

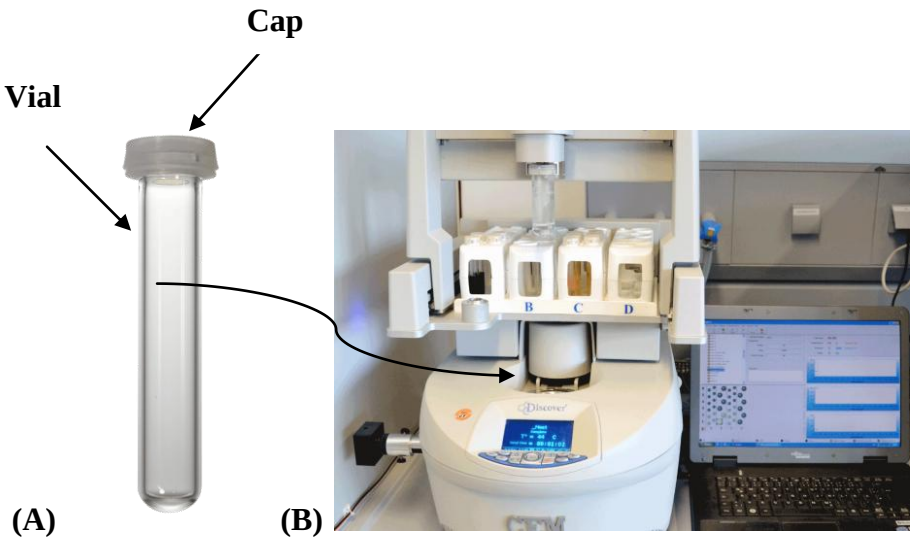

Figure 3. 2: (A) Vial with cap, (B) Microwave Organic Synthesis Reactor

3.4.2 Experimental procedure

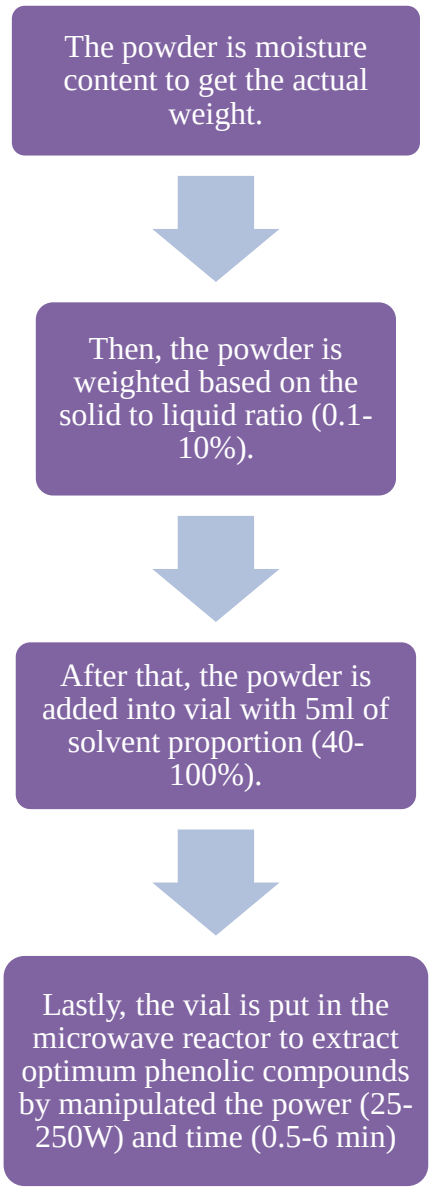

Figure 3. 3: Experimental procedure of extraction

3.4.3 Total Phenolic Content

Total phenolic content (TPC) was determined spectrophotometrically using the Folin–Ciocalteu reagent, according to Singleton's method as described by Trabelsi et al. (2010). Diluted crude extracts (0.125 ml) or standard solution of gallic acid (0.005 – 0.5mg/ml) was added to a centrifuge tube containing 0.5 ml of ultrapure water and 0.125 ml of the Folin–Ciocalteu reagent. After 3 minutes, 1.25 ml of 7% Na_2CO_3 solution were added and the final volume was made up to 3 ml with ultrapure water.

The solution was mixed well and incubated for 90 minutes at 23 °C in the dark. The absorbance was measured against prepared blank reagent at λ = 760 nm using a calibrated ultraviolet–visible spectrometer (Hitachi U-1800, Japan). Total phenolic content of the leaves was expressed as mg gallic acid equivalents per gram dry weight (mg GAE/g DW) by comparing with the calibration curve for gallic acid (Figure 3.4) using Eq. 3.1 (Pan et al., 2012).

$$\text{Total phenolic content (mg/g)} = \frac{Y \times N \times V}{W} \qquad \text{Eq. (3.1)}$$

where Y – the sample fluid concentration of total phenolic calculated by regression equation, mg/ml; N-dilution; V – extract volume, mL; W – quantity of *O. stamineus* dry powder, g.

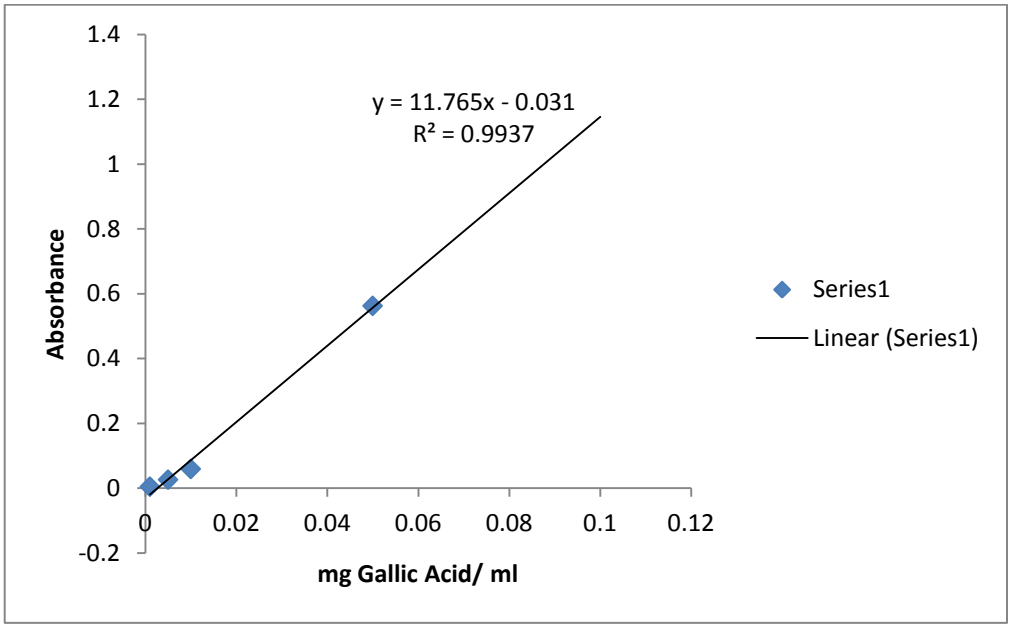

Figure 3. 4: Calibration plot of gallic acid (mg/g) against total phenolic content

3.4.4 Total Flavonoids Content

Total flavonoid content (TFC) was measured by the aluminum chloride colorimetric assay (Abouzid and Elsherbeiny, 2008). Diluted crude extracts (0.2 ml) or

standards solution of quercetin (0.0025- 0.5 mg/ml) was added to centrifuge tube containing 4.8 ml ultrapure water. $NaNO_2$ (0.3 ml, 5%) was added and mixed using a vortex mixer. After 5 minutes, 0.3 ml 10% $AlCl_3$ was added. At the 6^{th}minutes, 2 ml 1M NaOH solution was added and the total volume was made up to 10 ml with ultrapure water. The solution was mixed well and the absorbance was measured against prepared reagent blank at λ = 414 nm using a calibrated UV-Vis (Chang et al., 2002). Total flavonoid content of the leaves was expressed as mg quercetin equivalents per gram dry weight (mg QE/g DW) by comparing with the calibration curve for quercetin (Figure 3.5) using Eq 3.2 (Pan et al., 2012).

Total flavonoids content (mg/g) = $\frac{\boldsymbol{Y}\times\boldsymbol{N}\times\boldsymbol{V}}{\boldsymbol{W}}$ Eq. (3.2)

where Y – the sample fluid concentration of total flavonoids calculated by regression equation, mg/ml; N-dilution; V – extract volume, mL; W – quantity of *O. stamineus* powder, g.

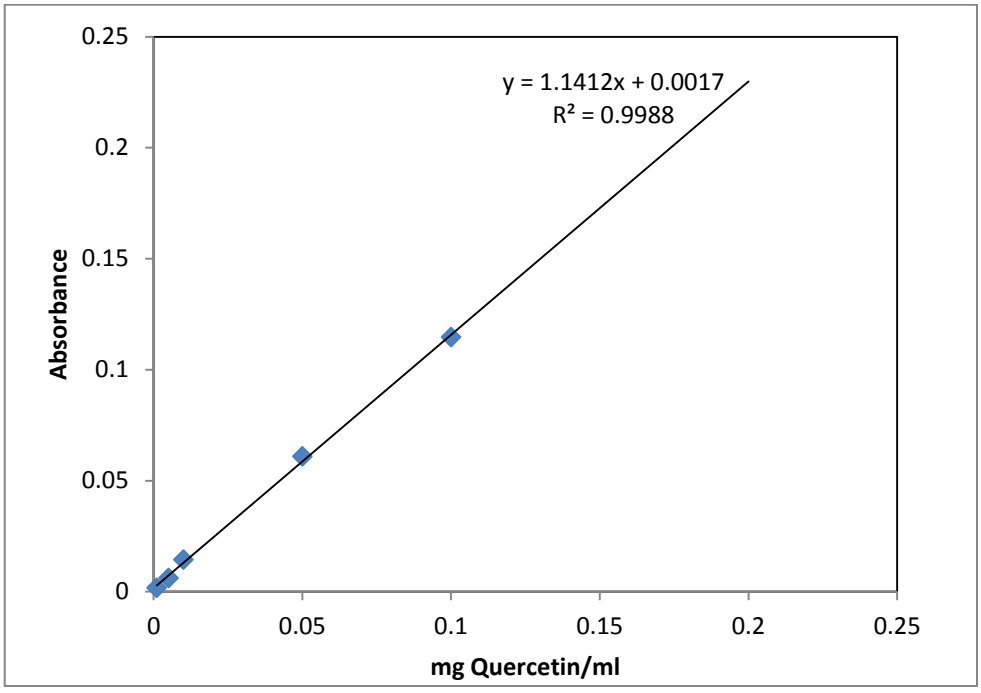

Figure 3. 5: Calibration plot of quercetin (mg/g) against total flavonoids content

3.4.5 Ultra performance liquid chromatography (UPLC)

O. stamineus extract major component such as rosmarinic acid, sinensetin and eupatorin can be performed on a Waters Acquity UPLC H-Class (Milford, MA) fit with Acquity UPLC HSS T3 column (2.1 × 75 mm, 1.8 µm) and Acquity UPLC HSS T3 VanGuard column guard (2.1 × 5 mm, 1.8 µm). There is photodiode array detector in UPLC system and it is connected to a computer, for running Water Empower 2 software. The mobile phase consists of 2 types of solvent. Solvent A is for water:trifluoroacetic acid (TFA) (20:0.001; v/v) and solvent B for acetonitrile (ACN):TFA (20:0.001; v/v). The temperature will be at room temperature. Before the sample inject to the UPLC system, it will be filter with 0.2 µm nylon membrane filter. The peak will be detected. Each of phenolic compounds will be identify by retention time from the peak area. Prepare the eight solutions of different concentration to construct the calibration model. The linear regression fitted will be constructing and inspect (Pang, 2013). The peak for rosmarinic acid was detected at 340nm 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.

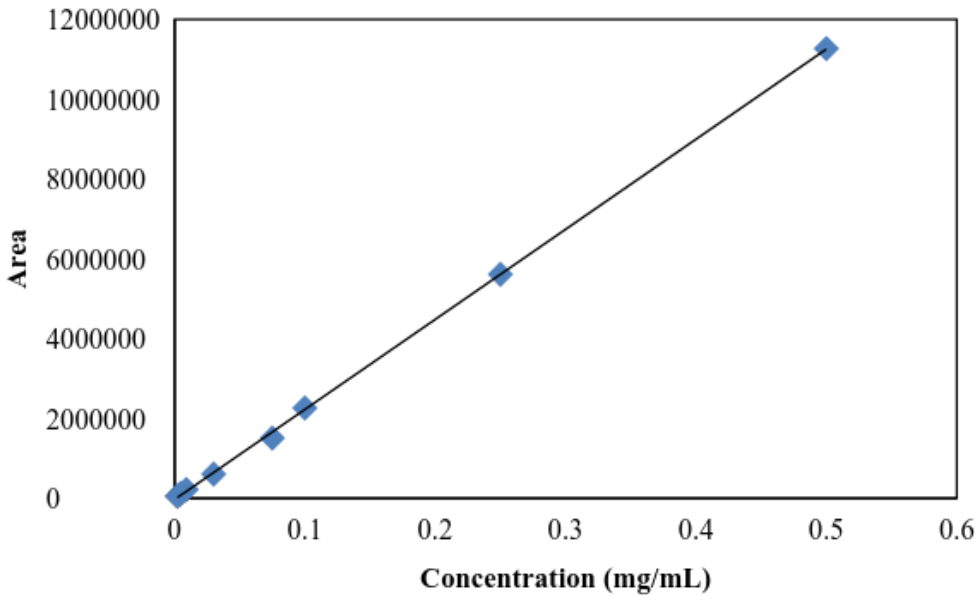

Figure 3. 6: Standard calibration curve for rosmarinic acid

3.5 Experimental design and statistical analyses

The experimental design was implemented with the aid of the Design Expert software (Stat-Ease Inc., Minneapolis, US, Version 8.0.4). The response surface methodology (RSM) was conducted to determine the MAE optimises extraction process variables for maximum recovery of TPC and TFC. The 2 level factor design model was used to determine the significant variables while the Box behnken design model is to develop the quadratic polynomial. The independent variables such as extraction time (30s to 360s), power (25W to 250W), solid to liquid ratio (0.1 g/ml to 10 g/ml) and ethanol concentration (40% to 100%) were chosen based on previous studies. The Box behnken design model was used because it is suitable for the continuous process. This model required 17 experiments to obtain the higher yield of extraction.

3.6 Summary

The experimental methods for phenolic compound extraction and quantification have been develop to make sure the result is repeatable and then it can verified using statistical analysis. Qualification and quantification of analysis using UPLC is the fast and reliable analytical method.

CHAPTER 4

4 RESULTS AND DISCUSSION

4.1 Overview

This chapter presents the results and discussion of the extraction of phenolic compounds from Misai kucing leaves using microwave assisted extraction (MAE) by respond surface methodology (RSM) to optimise the extraction yield. In this part, the percentages of solvent (ethanol), time of extraction and power irradiation have been discussed to get the better extraction yield. The bioactive component such as rosmarinic acid were analysed using UPLC. In this chapter also, total phenolic content (TPC), total flavonoids content (TFC) and antioxidant activity (AA) were examined.

4.2 Introduction

Misai kucing is well-known herbal plant that contains lots of active components such as rosmarinic acid, sinensetin and eupatorin which can be recovered by using extraction process. The yields of bioactive compounds are depending on the extraction methods, type of solvent, pH, solid-to-liquid ratio and size of particles Misai kucing leaves. Most of the previous research performed the extraction by using maceration process likes Akowuah et al. (2005) and accelerated solvent extraction by Pouralinazar et al. (2012). Conventional extractions such as maceration and soxhlet extraction are normally performed at high temperatures for several hours. The latest method to extract plant have been developed such as microwave-assisted extraction, ultrasonic-assisted extraction and supercritical extraction (Tabaraki and Nateghi, 2011; Zhang et al., 2011) which are better extraction method compared to conventional method. In the present study, the response surface methodology (RSM) was examined for optimization variables such as extraction time, temperature, ethanol concentration and solid-to-liquid

ratio to get the maximum yield of total phenolic compounds. The 2 level factorial designs with 2 replicate were used to determine the most significant effect of extraction while Box-behnken design is used to develop the quadratic polynomial.

4.3 UPLC quantification of phenolic compounds

HPLC method has been widely used by researcher to analyse the active component in the O. stamineus extract (Akowuah et al., 2005; Yam et al., 2013) but most of them concern about the analysis time which it is required around 19 to 30 minutes. They always desired for the faster analysis method. Recently, ultra-performance liquid chromatography (UPLC) has offer the fast analysis method to overcome the HPLC problem. However, there is no previous study on UPLC application to O. stamineus.

HSS T3 column was used for the separation. The polar compound of active component, rosmarinic acid was found less retained in the C-18 column (Akowuah et al., 2005; Yam et al., 2008) which, it was not good in separation term. HSS T3 column is design to improve the polar compound and ensuring the better separation of both polar and non-polar compound.

The active component was identified by the retention time and UV spectra of the standard. Active component were quantified by comparing peak areas with the results of calibration series using standard obtained from Sigma-Aldrich. The calibration equation for Rosmarinic acid was $y = 2.25 \text{ X } 10^7x - 2.33 \text{ X } 10^4$. The linear regression coefficient was 0.9997 which is good linearity.

The standard Rosmarinic acid was accurately weighed and then dissolved in appropriate volume of acetonitrile (ACN) to produce corresponding stock standard solutions. The stock standard solution later was diluted with 20% ACN to different concentrations and analyzed by UPLC to plot standard calibration curve (Figure 3.7). 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 3.7. The analysis time is less

than 7 minutes as shown in figure 4.1, which is about three times faster than the previous reported method such as Akowuah et al. (2005) and (Yam, et al., 2012). Figure 4.2 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 4.1.

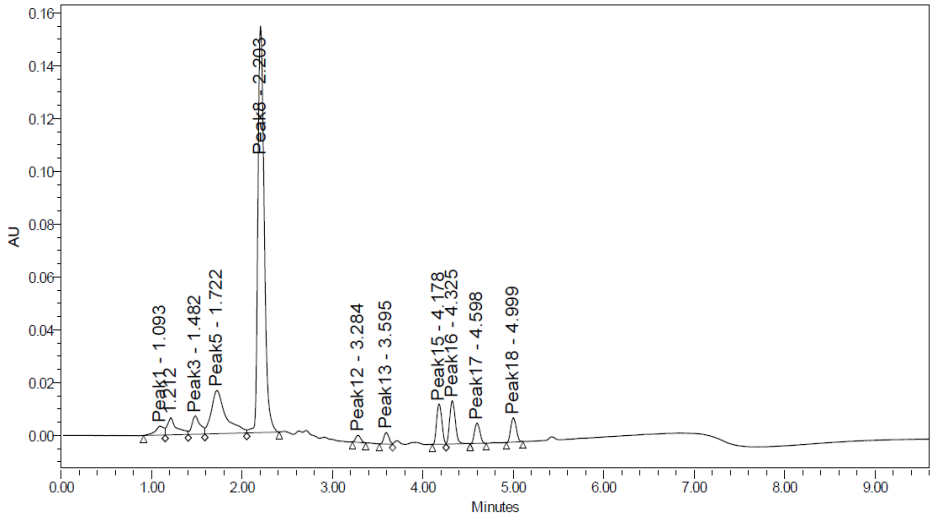

Figure 4. 1: Identification of Rosmarinic Acid Peak from extract

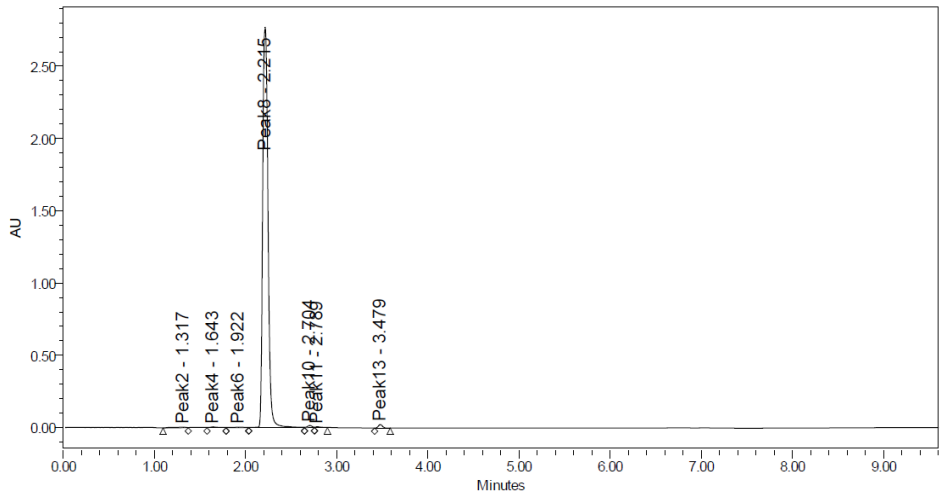

Figure 4. 2: Standard of Rosmarinic Acid

4.4 Effects of variables on extraction method

4.4.1 Influence of ethanol concentration

The recovery of the phenolic compounds from the extraction of Misai kucing leaves is with respect to concentration of ethanol. The yield of the phenolic compounds increased with increasing the amounts of the ethanol concentration in the extraction. Based on the Figure 4.6, the highest yield of rosmarinic acid was obtained at 66.6 % ethanol. The pure ethanol (100 %) is not good for rosmarinic acid extraction and the pure water also was not good for rosmarinic acid extraction. Aqueous 66.6 % ethanol is the best concentration for extracting rosmarinic acid. Hence, percentage of ethanol is the major contribution in the main effect with 10.30 % (Table 4.3). The solubility of the bioactive component in different solvent was directed by structural characteristic. The components that highly methoxylated compounds were more stable in lower polarity solvent. Similar finding was reported elsewhere such as Akowuah et al. 2005. Low-polar solvent (ethanol) with strong polar solvent (water) can be blended together in any proportion (Zhang et al., 2007). The polarity of the complex solvent will increase continuously when the water is added into ethanol. Yield of extraction will be increased with increasing water content according to the "like dissolves like" because of phenolic compounds molecules is polar (Zhang et al., 2008). Solvent with high polarity solvent like water can absorb more microwave energy which it makes the water molecule rotates as they try to align with the alternating electric field of the microwave and it is rapidly heating.

4.4.2 Influence of microwave power

The effects of microwave power on the recovery of phenolic compounds from O. stamineus leaves were investigated at ranging from 25 to 250 W with various concentration of solvent ranging from 40 to 100 % at different extraction time which is 0.5 to 6 minutes. Besides the ethanol content, the power also is the main contributor to produce higher yield of rosmarinic acid extraction. Higher power (250W) improve rosmarinic acid extraction yield while lower power 25W produced small amount of rosmarinic acid yield. The result is significant with previous work by (Chen and Spiro, 1995; Upadhyay et al., 2012) revealing the concentration of extracts increase with

increasing the microwave power. The optimum power was found out at 112.42 W by using two level factorial designs. Reduced recovery of rosmarinic acid could be due to the thermal degradation of the phytochemical is at higher power levels. In the plant cell, heat that generated by microwave may be too strong to breakdown the phytchemical that were not recovered at higher microwave power.

4.4.3 Influence of extraction time

Generally, longer extraction time increased rosmarinic acid extraction yield at lower TS (0.1 g/ml) because longer time needed for rosmarinic acid to diffiuse in the solvent although there is the risk of the degradation of extracted compound. Since solvent is not saturated at lower TS so the extraction process continues and become higher at longer time. However, if the extraction times are too long, there is no improvement on the extraction yield. In rosmarinic acid case, the extraction yield will be decreased after 90 minutes. The optimum concentrations of active components extracted are affected by the equilibrium concentration of active compound before reduction occurred (Thoo et al., 2010; Spigno et al., 2007). In this study, the recovery of rosmarinic acid was examined by three parameters which are microwave power (25 – 250 W), extraction time (0.5 – 6 min) and concentration of solvent (40 – 100 %). The solid to liquid ratio are ranging from 0.1 to 10 %.

4.4.4 Influence of liquid- to-solid ratio

The solid to liquid ratio is an important parameter to extract the maximize yield. In industrial of extraction process, it is important to maximize the extraction of yield but need to minimize the consumption of solvent (Spigno & Faveri, 2009). The lower total solid (TS) will produce higher rosmarinic acid per DW of material but higher TS contain more rosmarinic acid and hence it is affect by reduced its solubility in solvent and lower extraction yield.

4.5 Two level factorial analysis

The two level factorial design methods have identified the optimum parameter to run the extraction method as shown in Table 4.1. There are four variables used in this extraction part which are microwave power, extraction time, total solid and concentration of ethanol. These variables were optimised by using Box-behnken design model. In two level factor modesl, the concentration of ethanol was the major contribution for the main effect with 10.30% of contribution followed by extraction time with 5.66% as shown in table 4.3. The largest combined effect was between extraction time and concentration of ethanol with 19.96% of contribution followed by total solid and concentration of ethanol with 15.37% of contribution. In figure 4.4 have shown there are interactions between extraction time and concentration of ethanol with the highest contribution effect with 19.96%. In figure 4.3 have shown the values of rosmarinic acid that almost accurate to the straight line.

Table 4. 1: The RSM Two level factorial design parameter

Run	TS	Power	Time	%etoh	RA
1	10	25	6	40	3.4448
2	10	250	6	100	1.8063
3	0.1	25	6	100	3.4657
4	10	25	0.5	40	1.7795
5	10	25	0.5	100	3.5186
6	10	250	0.5	40	1.6279
7	10	250	6	40	2.0758
8	0.1	25	6	40	0.8752
9	0.1	250	6	100	0.9977
10	10	25	6	100	0.3430
11	10	250	0.5	100	2.6988
12	0.1	250	0.5	40	1.3164
13	0.1	25	0.5	100	2.7768
14	0.1	25	0.5	40	1.2323
15	0.1	250	0.5	100	3.0292
16	0.1	250	6	40	1.2108

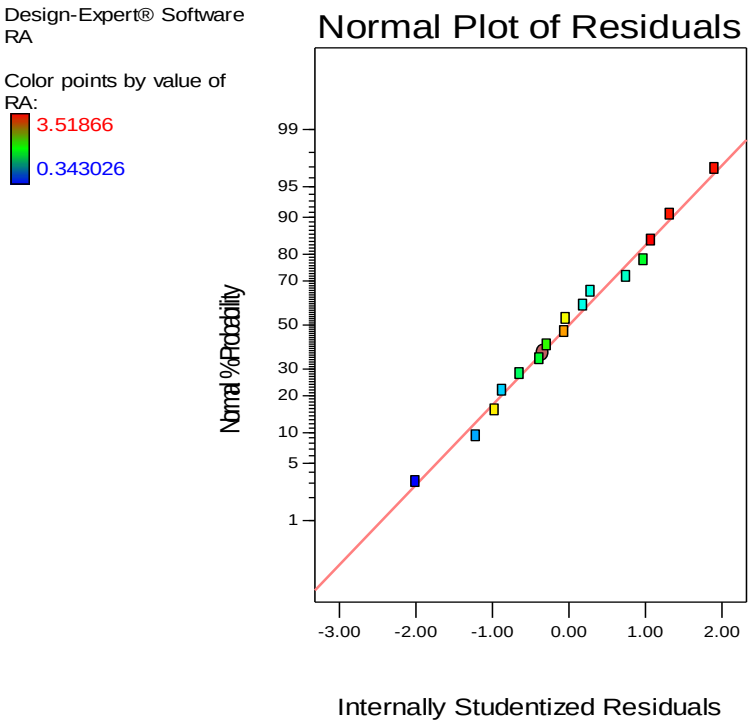

Figure 4. 3: Normal plot of residual for Rosmarinic acid

Table 4. 2: ANOVA for Response Surface Quadratic Model
Analysis of variance table of RA

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	9	10	0.9	0.68	0.7177	not significant
A-TS	0.36	1	0.36	0.27	0.6254	
B-Power	0.45	1	0.45	0.34	0.5864	
C-Time	0.88	1	0.88	0.67	0.4508	
D-%EtOH	1.61	1	1.61	1.22	0.3203	
AB	0.053	1	0.053	0.04	0.8495	
AC	1.40E-03	1	1.40E-03	1.06E-03	0.9753	
AD	2.4	1	2.4	1.81	0.2358	
BC	0.12	1	0.12	0.093	0.7726	
BD	0.014	1	0.014	0.01	0.9224	
CD	3.12	1	3.12	2.36	0.1854	
Residual	6.61	5	1.32			
Cor Total	15.62	15				

Table 4. 3: The effects of parameter

	Term	Stdized Effects	Sum of Squares	% Contribution
	Intercept			
M	A-TS	0.30	0.36	2.29
M	B-Power	-0.33	0.45	2.86
M	C-Time	-0.47	0.88	5.66
M	D-%EtOH	0.63	1.61	10.30
M	AB	0.11	0.053	0.34
M	AC	-0.019	1.400E-003	8.966E-003
M	AD	-0.77	2.40	15.37
M	BC	-0.18	0.12	0.79
M	BD	-0.059	0.014	0.089
M	CD	-0.88	3.12	19.96
e	ABC	0.44	0.78	5.00
e	ABD	0.60	1.44	9.22
e	ACD	-0.66	1.76	11.25
e	BCD	0.066	0.017	0.11
e	ABCD	0.81	2.62	16.77
	Lenth's ME	1.70		
	Lenth's SME	3.46		

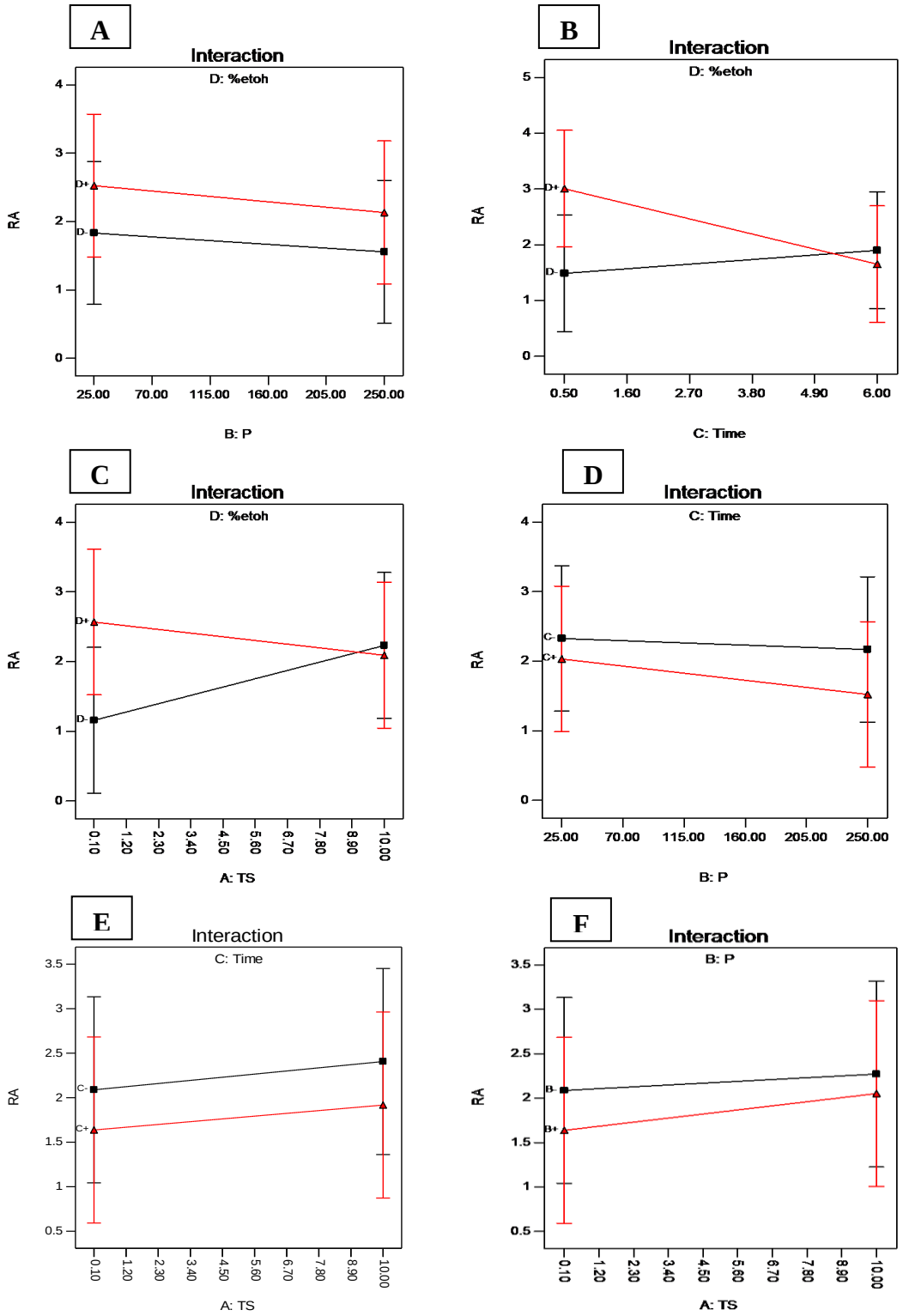

Figure 4. 4: The interaction between parameter on Rosmarinic acid (A) Interaction between %EtOH and power; (B) interaction between %EtOH and time; (C) interaction between %EtOH and total solid; (D) interaction between time and power; (E) interaction between time and total solid; (F) interaction between power and total solid

4.6 Optimisation of MAE conditions

4.6.1 Modelling and fitting the model using response surface methodology (RSM)

Box-behnken design is a model to develop the quadratic polynomial. In table 4.4 have shown the parameters used to optimize the phenolic compound. The parameters used still same as two level factorial design.

Table 4. 4: The RSM box-behnken design parameter

Run	%etoh	Power	Time	RA
1	40	137.5	6	19.9859
2	100	137.5	6	22.3777
3	70	250	6	15.7837
4	100	250	3.25	19.1007
5	70	25	6	20.4378
6	70	137.5	3.25	32.2057
7	40	25	3.25	21.8877
8	100	25	3.25	18.9352
9	40	137.5	0.5	16.9293
10	100	137.5	0.5	13.9655
11	70	137.5	3.25	21.2243
12	70	137.5	3.25	22.5790
13	70	25	0.5	18.4766
14	70	137.5	3.25	22.8984
15	40	250	3.25	20.7007
16	70	250	0.5	17.6029
17	70	137.5	3.25	23.7802

Table 4. 5: ANOVA for Response Surface Quadratic Model
Analysis of variance table of RA

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	161.29	9	17.92	1.29	0.3778	not significant
A-%EtOH	3.28	1	3.28	0.24	0.6421	
B-Power	5.36	1	5.36	0.39	0.5545	
C-Time	16.85	1	16.85	1.21	0.3076	
AB	0.46	1	0.46	0.033	0.8613	
AC	7.17	1	7.17	0.52	0.4962	
BC	3.57	1	3.57	0.26	0.628	
A^2	18.06	1	18.06	1.3	0.2921	
B^2	22.48	1	22.48	1.61	0.2444	
C^2	72.58	1	72.58	5.21	0.0563	
Residual	97.43	7	13.92			
Lack of Fit	20.56	3	6.85	0.36	0.7884	not significant
Pure Error	76.88	4	19.22			
Cor Total	258.72	16				

Final Equation in Terms of Coded Factors:

$$\mathbf{RA} = +24.54 - 0.64*A - 0.82*B + 1.45*C + 0.34*A*B + 1.34*A*C - 0.95*B*C - 2.07*A^2 - 2.31*B^2 - 4.15*C^2 \quad \text{(Eq 4.1)}$$

As shown in Table 4.5, the "Model F-value" of 1.29 implies the model is not significant relative to the noise. There is a 37.78 % chance that a "Model F-value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case there are no significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The "Lack of Fit F-value" of 0.36 implies the Lack of Fit is not significant relative to the pure error. There is a 78.84% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good which we want the model to fit. The results indicated that the model could work well for the prediction of TPC extract from O. stamineus leaves. Figure 4.5 have shown the normal plot of residual of Rosmarinic acid. The figure 4.6 shown the response surface methodology for

rosmarinic acid between concentration of ethanol versus extraction time. The optimum variables have shown in Table 4.6 which are the concentration of ethanol is optimum at 66.6% while microwave power is optimum at 112.42 at 3.75 minutes. The yield of rosmarinic acid was found at 24.7971 mg RA/g DW with desirability of 0.594. In Figure 4.5 have shown the normal plot of the rosmarinic acid for box-behnken design which are the points are not so accurate compared to two level factorial design. The optimisation of the rosmarinic acid have found only one solution with desirability 0.594.

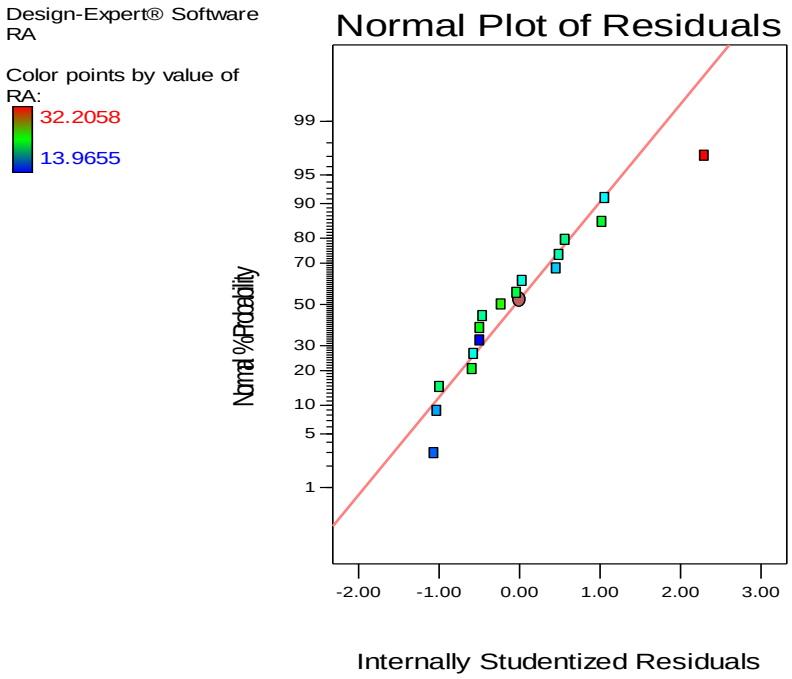

Figure 4. 5: Normal plot of residual of Rosmarinic acid for Box behnken

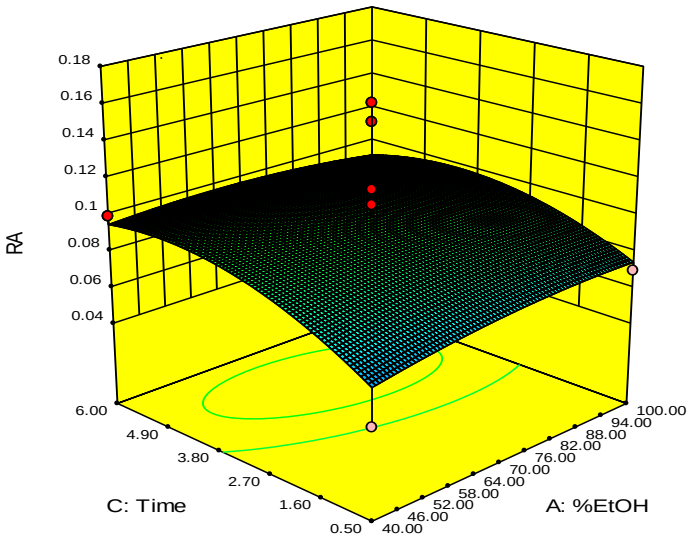

Figure 4. 6: Response surface methodology for RA (time vs. %EtOH)

Table 4. 6: Optimisation of RA

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A:%EtOH	is in range	40	100	1	1	3
B:Power	is in range	25	250	1	1	3
C:Time	is in range	0.5	6	1	1	3
RA	maximize	13.9655	32.2058	1	1	3

Solutions

Number	%EtOH	Power	Time	RA	Desirability	
1	66.6	112.42	3.75	24.7971	0.594	Selected

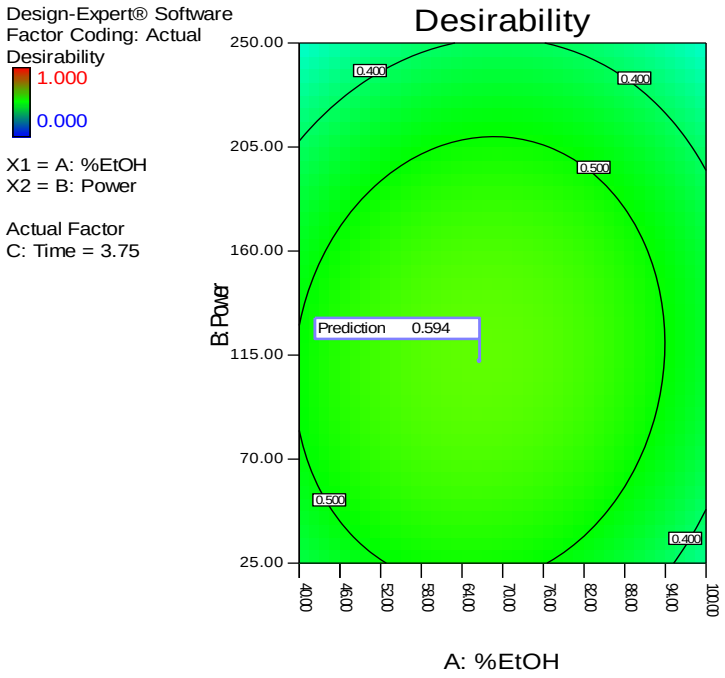

Figure 4. 7: Desirability vs %EtOH and power

The experimental design and corresponding response data for the total phenolic content (TPC) and total flavonoid content (TFC) from O. stamineus leaves extract are presented in Table 4.7 and 4.8. The regression coefficients of the intercept, linear, quadratic and interaction terms of model were calculated and given in Table 4.7 and 4.8. The regression coefficients of the intercept, linear, quadratic and interaction terms of the model were calculated using the least square technique (Zhang et al., 2013).

Table 4. 7: ANOVA for Response Surface Quadratic Model
Analysis of variance table of TPC

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1451.10	9	161.23	3.98	0.0410	significant
A-%EtOH	53.01	1	53.01	1.31	0.2900	
B-Power	101.09	1	101.09	2.50	0.1580	
C-Time	129.82	1	129.82	3.21	0.1164	
AB	2.09	1	2.09	0.052	0.8268	
AC	2.93	1	2.93	0.072	0.7955	
BC	0.97	1	0.97	0.024	0.8812	
A^2	128.02	1	128.02	3.16	0.1185	
B^2	126.54	1	126.54	3.13	0.1203	
C^2	200.19	1	200.19	4.95	0.0615	
Residual	283.23	7	40.46			
Lack of Fit	41.69	3	13.90	0.23	0.8713	not significant
Pure Error	241.54	4	60.39			
Corr Total	1734.34	16				

Table 4. 8: Coefficients in term of actual for TPC

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	2.8	1	21.68	-48.47	54.07	
A-%EtOH	0.6	1	0.52	-0.63	1.83	48.16
B-Power	0.16	1	0.1	-0.08	0.40	26.04
C-Time	7.4	1	4.13	-2.37	17.16	25.49
AB	-2.14E-04	1	9.42E-04	-2.44E-03	2.01E-03	14.88
AC	0.01	1	0.039	-0.081	0.10	14.68
BC	1.59E-03	1	0.01	-0.023	0.026	6.78
A^2	-6.13E-03	1	3.44E-03	-0.014	2.02E-03	42.38
B^2	-4.33E-04	1	2.45E-04	-1.01E-03	1.46E-04	12.36
C^2	-0.91	1	0.41	-1.88	0.058	11.62

Table 4. 9: ANOVA for Response Surface Quadratic Model
Analysis of variance table of TFC

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	39522.37	9	4391.37	2.78	0.0959	not significant
A-%EtOH	12356.38	1	12356.38	7.81	0.0267	
B-Power	1531.06	1	1531.06	0.97	0.3579	
C-Time	7911.39	1	7911.39	5.00	0.0604	
AB	118.06	1	118.06	0.075	0.7926	
AC	995.13	1	995.13	0.63	0.4537	
BC	65.70	1	65.70	0.042	0.8443	
A^2	7260.06	1	7260.06	4.59	0.0694	
B^2	2693.28	1	2693.28	1.70	0.2331	
C^2	4933.25	1	4933.25	3.12	0.1207	
Residual	11069.96	7	1581.42			
Lack of Fit	1158.49	3	386.16	0.16	0.9207	not significant
Pure Error	9911.47	4	2477.87			
Corr Total	50592.32	16				

Table 4. 10: Coefficients in term of actual for TFC

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	219.51	1	17.78	177.45	261.56	
A-%EtOH	-39.30	1	14.06	-72.55	-6.05	1.00
B-Power	13.83	1	14.06	-19.41	47.08	1.00
C-Time	31.45	1	14.06	-1.80	64.69	1.00
AB	5.43	1	19.88	-41.58	52.45	1.00
AC	15.77	1	19.88	-31.24	62.79	1.00
BC	-4.05	1	19.88	-51.07	42.96	1.00
A^2	-41.52	1	19.38	-87.35	4.30	1.01
B^2	-25.29	1	19.38	-71.12	20.54	1.01
C^2	-34.23	1	19.38	-80.06	11.60	1.01

Final Equation in Terms of Coded Factors:

$$\mathbf{TFC} = +219.51 - 39.30*A + 13.83*B + 31.45*C + 5.43*A*B + 15.77*A*C - 4.05*B*C - 41.52*A^2 - 25.29*B^2 - 34.23*C^2 \quad \text{(Eq 4.2)}$$

The experimental results given in the Table 4.7 and 4.9 are using analysis of variance (ANOVA). In Table 4.7 shows that, the Model F-value of 3.98 implies the model is significant. There is only a 4.10% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case there are no significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The "Lack of Fit F-value" of 0.23 implies the Lack of Fit is not significant relative to the pure error. There is a 87.13% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good and it need the model to fit. The results indicated that the model could work well for the prediction of TPC extract from O. stamineus leaves.

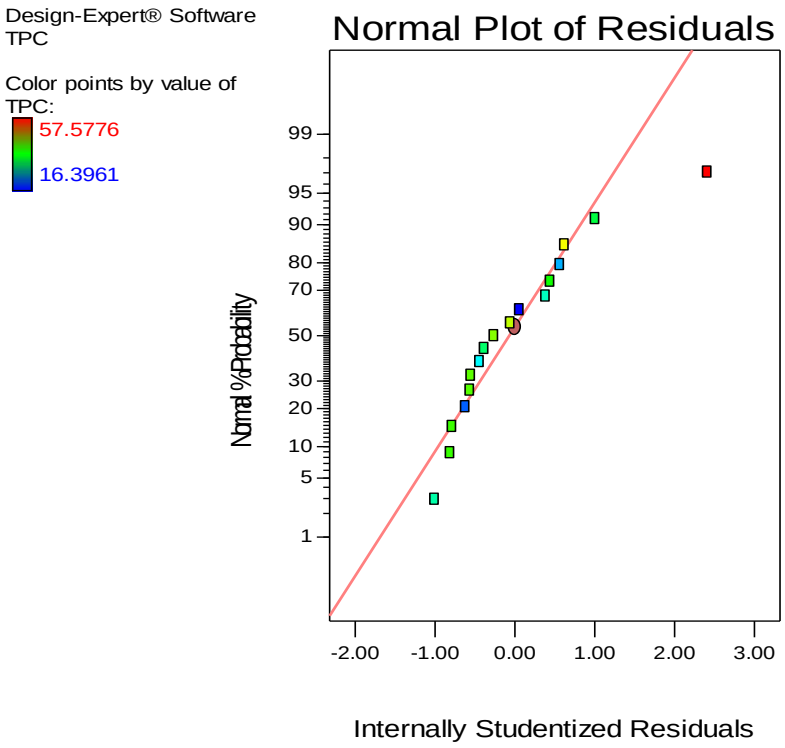

Figure 4. 8: Graph of normal plot of residual for TPC

However, in Table 4.9 show that the Model F-value of 2.78 implies there is a 9.59% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The "Lack of Fit F-value" of 0.16 implies the Lack of Fit is not significant relative to the pure error. There is a 92.07% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good and it need the model to fit. The results indicated that the model could work well for the prediction of TFC extract from O. stamineus leaves. In table 4.11 have shown the table of optimisation of total phenolic content (TPC) and total flavonoid content (TFC). The optimum value of TPC is 48.51 mg GAE/g DW while TFC is 233.243 mg QE/g DW with 54.68% of ethanol, 170.47 W of microwave power at 4.34 minutes of extraction time with 0.74 desirability. Only one solution was

found.

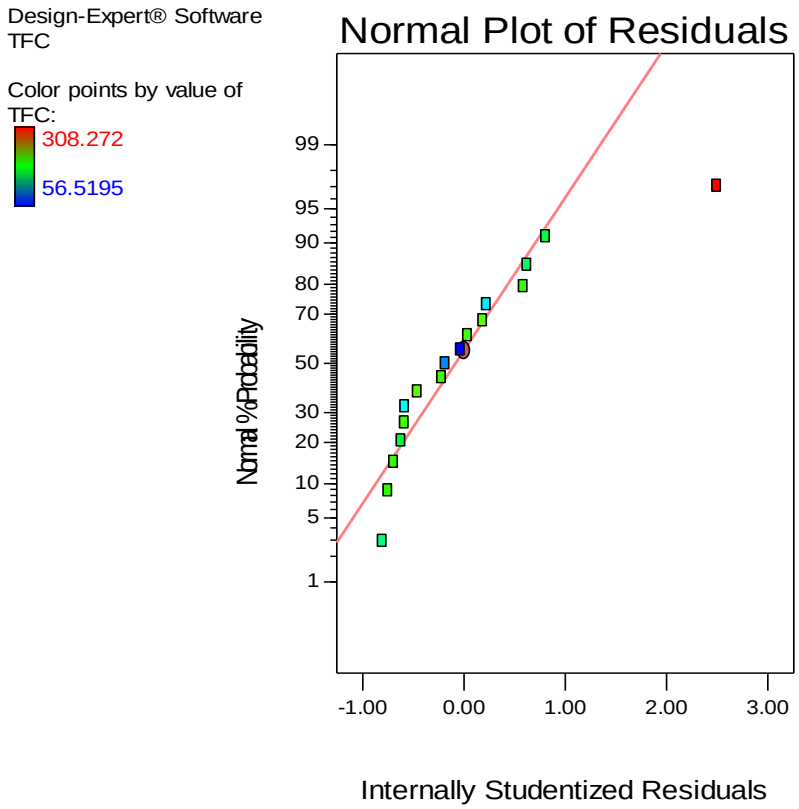

Figure 4. 9: Graph of normal plot of residual for TFC

Table 4. 11: Optimisation of TPC and TFC

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A:%EtOH	is in range	40	100	1	1	3
B:Power	is in range	25	250	1	1	3
C:Time	is in range	0.5	6	1	1	3
TFC	maximize	56.5195	308.272	1	1	5
TPC	maximize	16.3961	57.5776	1	1	5

Solutions

Number	%EtOH	Power	Time	TFC	TPC	Desirability	
1	54.68	170.47	4.34	233.243	48.51	0.74	Selected

4.6.2 Analysis of response surfaces

The investigation of interactive effects of the independent variables and their mutual interaction on the extraction recovery of phenolic compounds, three dimensional response surface of multiple non-linear regression models were plotted in Figure 4.9 and Figure 4.10. The figure shown were generated by plotting the response using z-axis against two independent variable which are ethanol concentration and microwave power while the other two independent variables such as irradiation time and solid to liquid ratio are kept at zero level (Hayat, et al., 2009).

Figure 4.10 (A-B) and Figure 4.11 (A-B) describes the interactions between the amount of ethanol concentration with the other two factors which are microwave power and extraction time on the recovery of total phenolic content. The recovery of TPC from O. stamineus leaves extract increased from 18 to 49 mg GAE/g DW by decreasing the ethanol concentration from 70 to 55% and increase the microwave power with 25 to 170W while the recovery of TFC from O. stamineus leaves extract also increased from 100 to 233 mg QE/g DW with the same factors as TPC. The highest peak recovery of TPC was at 48.51 mg GAE/g DW with 54.68 % (ethanol concentration) and 170.47 W (extraction power) and the highest peak recovery of TFC was 233.243 mg QE/mg DW with the same factors. However, additional of ethanol concentration and microwave power will cause negative effects. The best point of balance should be find for the maximum extraction rate of phenolic compound between extraction power and ethanol concentration. The recovery of TPC and TFC are depends on ethanol concentration because of its quadratic and linear effects. The suggestion to increase the TPC yield is the ethanol concentration because of phenolic compounds are more soluble in ethanol and water (54.68%). Ethanol is function as facilitate an increase in the extraction yield while water could enhance the swelling of the plant cell, which is increase the contact surface area between plant matrix and solvent, thus, increasing in the extraction yield (Hayat, et al., 2009).

In Figure 4.10 (C) and Figure 4.11 (C) are shows the quantity of TPC and TFC increased when the two parameters are increased which are extraction time and extraction power. Higher microwave power with longer extraction time result in

continuous higher temperature in extraction system. The higher temperature ang longer extraction time could enhance the solubility of phenolic compounds and decrease the viscosity of extraction solvent. Moreover, high temperature also lead to degradation of phenolic compounds. The interaction effect of extraction time and microwave power had a significant influence on the acquired ratio of TPC ($p > 4.1$) but not significant on the acquired ratio of TFC ($p > 9.59$). However, the recovery of the TPC and TFC can be overcome with the microwave energy which was found to be function of the interaction effect of extraction time and microwave power.

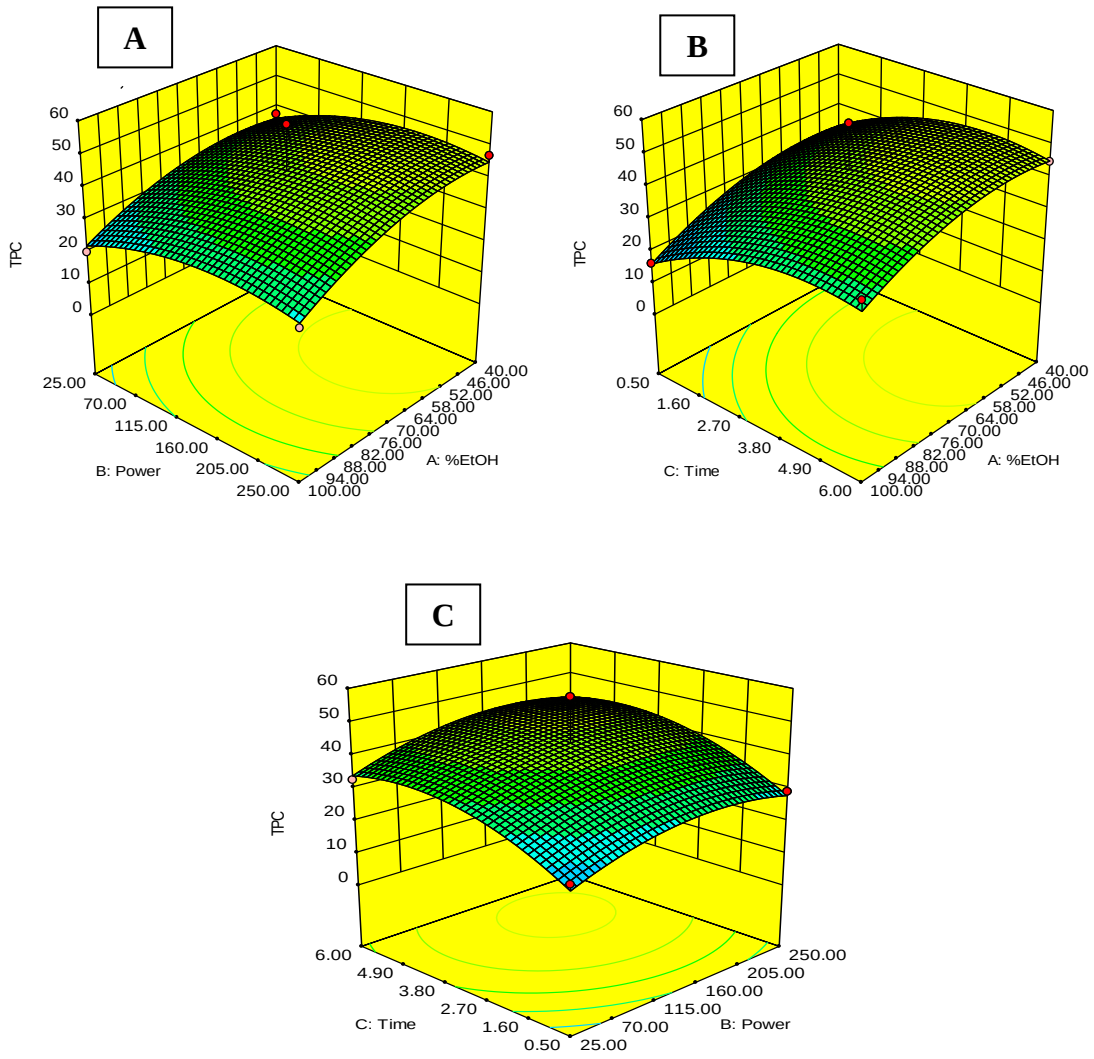

Figure 4. 10: Response surface analysis for the TPC yield from O. stamineus leaves extract with microwave-assisted extraction with respect to ethanol concentration and microwave power (A); ethanol concentration and extraction time (B); extraction time and microwave power (C)

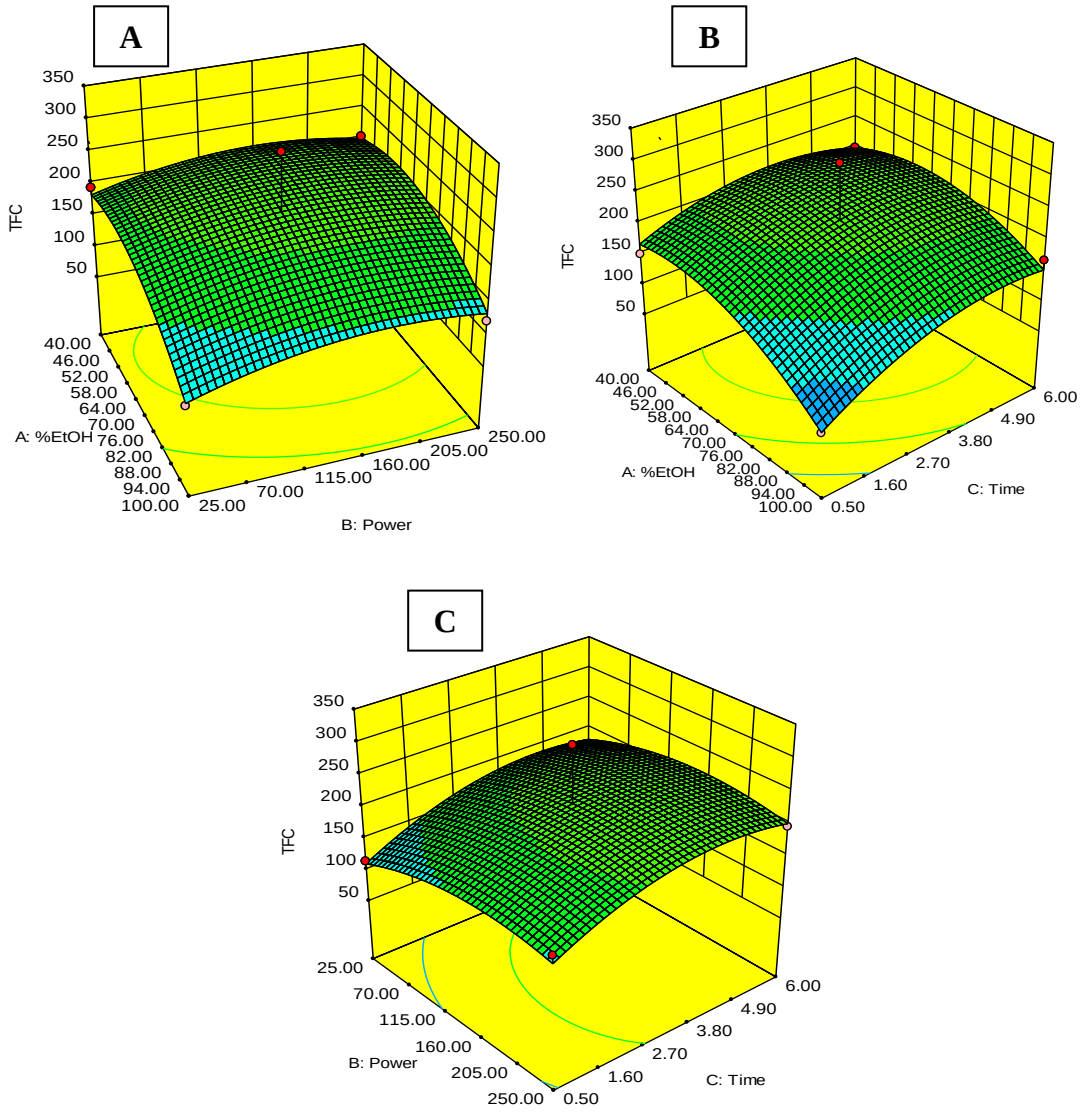

Figure 4. 11: Response surface analysis for the TFC yield from O. stamineus leaves extract with microwave-assisted extraction with respect to ethanol concentration and microwave power (A); ethanol concentration and extraction time (B); microwave power and extraction time (C)

4.7 Summary

The highest phenolic content of 48.51 mg GAE/g DW was obtained using 55% aqueous ethanol while highest flavonoid content was obtained at 233.243 mg QE/g DW. The highest yield of rosmarinic acid of 24.7971 mg RA/g DW was obtained using 66% aqueous ethanol. Aqueous solvent provides bigger range of polarity compared to the pure solvent and enhances simultaneous extraction of both hydrophilic (hydroxylated) and lipophilic (methoxylated) compounds. The yield of rosmarinic acid was found at 66.6% of ethanol with 112 W of the microwave power for 3.75 minutes the extraction time. Both TPC and TFC were found at 54.68% of ethanol with 170 W of the microwave power for 4.34 minutes the extraction time. This work may be useful for obtaining higher phenolic compounds extract from O. stamineus.

CHAPTER 5

5 CONCLUSION

5.1 Conclusion

Microwave assisted extraction method is the best method to extract the bioactive compounds. It is the fastest method and can produce higher extraction rate without damage the quality of extraction. The experimental methods for bioactive compound extraction and quantification have been develop to make sure the result is repeatable and then it can verified using statistical analysis. Qualification and quantification of analysis using UPLC is the fast and reliable analytical method. The RSM have been used to get the optimum yield of extraction. The models of RSM that have been used to quantify the yield are two level factorial design and Box-behnken design.

The highest phenolic content of 48.51 mg GAE/g DW was obtained using 55% aqueous ethanol while highest flavonoid content was obtained at 233.243 mg QE/g DW. The highest yield of rosmarinic acid of 24.7971 mg RA/g DW was obtained using 66% aqueous ethanol. Aqueous solvent provides bigger range of polarity compared to the pure solvent and enhances simultaneous extraction of both hydrophilic (hydroxylated) and lipophilic (methoxylated) compounds. The yield of rosmarinic acid was found at 66.6% of ethanol with 112 W of the microwave power for 3.75 minutes the extraction time. Both TPC and TFC were found at 54.68% of ethanol with 170 W of the microwave power for 4.34 minutes the extraction time. This work may be useful for obtaining higher phenolic compounds extract from O. stamineus.

5.2 Future work

The solution propose in this research may need to investigated further before they can be considered for production of functional food. So, for the future study, they should consider on pharmacological test on toxicity to approve the quality of the O. stamineus extract.

Solvent extraction using 55% of ethanol was found to be the best solvent to simultaneously extract the components content from O. stamineus leaves. Moreover, ethanol is the best solvent compared to methanol and isopropanol because ethanol is safe and has been approved by Food and Drug Administration (FDA). Besides that, before considered for human consumption, toxicity analysis is needed by performed animal test in ACCU accredited lab.

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