MICROWAVE EXTRACTION AND MICROENCAPSULATION OF POLYPHENOL FROM *PHYLLANTHUS NIRURI*

NGUANG SUOK LING

MASTER OF SCIENCE

UNIVERSITI MALAYSIA PAHANG

UNIVERSITI MALAYSIA PAHANG

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Date :

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NGUANG SUOK LING

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ABSTRAK

Dukung anak, mengandungi pelbagai komponen bioaktif yang menyumbang dalam bidang perubatan. Ia mempunyai sifat antioksidan, anti radang, anti kancer dan dapat mngubati Hepatitis B dan jangkitan usus penyakit kuning. Pengekstrakan ialah kaedah yang biasa digunakan untuk mendapatkan komponen bioaktif daripada bahan-bahan tumbuhan. Hasil komponen bioaktif itu bergantung kepada kaedah pengekstrakan, pelarut dan keadaan pengekstrakan. Dalam karya ini, kaedah pengekstrakan ultrasonik dan kaedah pengekstrakan gelombong mikro dikaji kerana kekurangan penyelidikan sebelum ini mengenai pengekstrakan ultrasonik dan pengekstrakan gelombang mikro telah disediakan dalam kesusasteraan. Dalam pendapatan kajian ini, didapati bahawa hasil komponen bioaktif adalah sangat bergantung kepada kekutuban pelarut yang digunakan dalam pengekstrakan, hasil tertinggi phyllanthin (4.56mg Phy/g DW) telah diperolehi dengan menggunakan 20% akueus Isopropanol manakala hasil tertinggi quercetin (10.14mg Que / g DW) telah diperolehi dengan menggunakan 20% etanol berair dan hasil tertinggi asid Gallic (15.44mg GAE / g DW) telah diperolehi dengan menggunakan air. Kekutuban pelarut meningkatkan pengeluaran kedua-dua komponen hydroxylated dan methoxylated dari *P. niruri*. Daripada analisis reka bentuk komposit pusat, pengekstrakan gelombong mikro pada kuasa pengeluaran di 250W, masa pengekstrakan dalam 2.47 minit hingga 5.72 minit dan kepekatan etanol daripada 36.58% hingga 76.31% mampu untuk mendapatkan hasil optimum pengekstrakan polifenol dengan kebaikan 91.70%. Pengekstrakan gelombong mikro disediakan pengekstrakan yang cepat tanpa ketara menjejaskan hasil pengekstrakan. produk tepung sering dikehendaki kerana jangka hayat yang lebih panjang, mudah untuk penggunaan dan mudah pengangkutan / pengendalian. Proses membuat serbuk sering dijalankan di dalam pengering semburan pada suhu tinggi (180° C). Untuk mengurangkan degradasi komponen bioaktif semasa pengeringan semburan, kaedah pemikrokapsulan diperkenalkan. WPI dan MD serbuk semburan encapsulation telah mengadakan pengekalan polifenol yang baik dari *P. niruri*. Pemikrokapsulan menggunakan campuran WPI dan MD pada nisbah 1: 9 menyumbang pengekalan tertinggi phyllanthin (84,33%), asid Gallic (88,93%) dan quercetin (88.39%) diikuti oleh MD dan WPI pengkapsulan. Pemikrokapsulan menggunakan campuran WPI dan MD pada nisbah 1: 9 dicadangkan kerana ia menyediakan pemeliharaan yang lebih baik daripada polifenol semasa pengeringan semburan yang bertentangan dengan merangkumi protein tunggal WPI dan MD. Keputusan analisis oleh UPLC menggambarkan asid Gallic dan quercetin adalah lebih mudah terdedah kepada pencemaran haba daripada phyllanthin semasa pengeringan semburan. Kajian yang lebih mendalam terhadap ujian toxic dicadangkan dengan mengunakan binatang untuk memastikan *P. niruri* extrak tidak membahayakan dalam ACCU lab yang diiktirafkan.

ABSTRACT

Phyllanthus Niruri (ver. name: Dukung Anak) contains miscellaneous bioactive compounds which contribute in various medical effects such as antioxidant, anti-inflammatory, anti-cancer and treating Hepatitis-B, jaundice intestinal infection. Extraction is the most common method to obtain the bioactive component from the plant materials. The yield of bioactive component in the extract is dependent on the solvent used, extraction method and condition. In this work, ultrasonic assisted extraction and microwave assisted extraction method were studied as there's limited work on ultrasonic assisted extraction and microwave assisted extraction were available in the literature. From the finding of this work, it is found that the yield of the bioactive component is highly dependent on solvent polarity used in the extraction, the highest yield of phyllanthin (4.56mg Phy/g DW) was obtained using 20% aqueous Isopropanol whereas the highest yield of quercetin (10.14mg Que/g DW) was obtained by using 20% aqueous ethanol and highest yield of gallic acid (15.44mg GAE/g DW) was obtained by using water. The polarity of solvent enhances the extraction of both hydroxylated and methoxylated compounds from the *P. niruri*. From the central composite design analysis, microwave assisted extraction at extraction power at 250W, extraction time ranged from 2.47 minutes to 5.72 minutes and ethanol concentration will ranged from 36.58% to 76.31% able to obtain the optimum yield of polyphenol extraction with the desirability of 91.70%. Microwave assisted extraction provided a fast extraction without significantly compromising the extraction yield. Powdered product is often desired due to its longer lifespan, convenient for consumption and easier transportation/ handling. The process of powder making is often carried out in a spray dryer at high temperature (180°C). To minimize degradation of the bioactive compounds during spray drying, microencapsulation method was introduced. WPI and MD encapsulation spray powder had performed a good polyphenol retention from *P. niruri*. Microencapsulation using mixture of WPI and MD at the ratio 1:9 delivered highest retention of phyllanthin (84.33%), gallic acid (88.93%) and quercetin (88.39%) followed by MD and WPI encapsulation. Microencapsulation using mixture of WPI and MD at ratio 1:9 is suggested as it provides a better preservation of polyphenol during spray drying as opposed to single protein encapsulate of WPI and MD. The results analysis by UPLC illustrates gallic acid and quercetin are more susceptible to thermal degradation than phyllanthin during spray drying. It is recommended to perform a toxicity analysis of *P. niruri* extract in ACCU accredited lab before it can undergo clinical trial.

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LIST OF SYMBOLS

%	Percentage
°C	Degree Celsius
hr	Hour
λ	Wavelength
ε'	Dielectric constant
kHz	KiloHertz
wt	Weight
tanδ	Dissipator factor
cm	Centimeter
mm	Milimeter
µm	Micrometer
kg	Kilogram
g	gram
ml	Mililiter

LIST OF ABBREVIATIONS

AA	Antioxidant activity
ANOVA	Analysis if variance
BHA	Butylated hydroxyanisole
CCD	Central composite design
DE	Dextrose equivalent
DoE	Design of experiment
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
EtOH	Ethanol
Eup	Eupatorin
FESEM	Field emission scanning electron microscopy
GA	Gallic acid
H20	Water
HPLC	High performances liquid chromatography
i.d.	Internal diameter
MAE	Microwave assisted extraction
MD	Maltodextrin

Min	Minute
PHY	Phyllanthin
QUE	Quercetin
RSM	Response surface methodology
Sec	Second
TFC	Total flavonoid content
TPC	Total phenolic content
UAE	Ultrasonic assisted extraction
UPLC	Ultra performances liquid chromatography
WPI	Whey protein isolate

CHAPTER 1

INTRODUCTION

1.1 Background

Phyllanthus niruri, locally termed as Dukung anak (Family: Phyllanthaceae) is an annual herb that grows about 50cm tall and having a smooth bark on the ascending branches with small flowers and tiny fruits that filled with seed. It is often growing in tropical areas and wet rainforest conditions which spreads rapidly throughout the tropical and subtropical countries such as Malaysia and India. In India, *P.niruri* is a common herbs used to heal problems such as stomach, genitourinary system, liver, kidney and spleen (Murugaiyah & Chan, 2007; Moreira et al., 2013). Traditionally, *P. niruri* act as traditional hearbs in many countries due to its well-known of its curative properties. Historically it can increase the appetite, relieve inflammations and fever (Cuto et al., 2013). In terms of health, it helps in restricting the growth of hepatitis B virus found in blood stream, having antifungal, anti-viral and hypoglecemic action and useful in the treatment of liver disease such as jaundice and liver cirrohosis (Markom et al., 2007). It also helps to remedy fatty liver and liver damage. *P. niruri* is diuretic and thus, it is used widely in urinary tract infections and bacterial infections like cyctitis and protastitis. Besides, the active constituents in the plant do also exhibit anticancer, antioxidant and anti-inflammatory properties that were influenced by the presence of valuable polyphenols (Tang et al., 2011).

From the analysis done by ministry of health of Malaysia (MoH), 2015, genitourinary system disease & Hepatitis B has reached 12.94% in 2014 with the increment of 8.22%. Even though *P. niruri* can be easily obtained in large scale all around Malaysia, but the awareness of the functionality of the medical effect toward genitourinary system disease & Hepatitis B in Malaysia was low and no much the

research had been done toward the extraction method and the method in construct the better storage and shelf life of the bioactive components extracted from *P. niruri*.

Microencapsulation technology is referring the process that surrounds the particle or the droplet by coating a layer or embedded a homogenous or heterogeneous matrix to form small capsules with varies function of properties. With the aid of the microencapsulation, the particles are coated and the physical barrier among the core compound and other component were formed as well. In neutracetical industry, microencapsulation is done by liquid droplets, solid particles or gas compounds which entrapped into thin films of a food grade microencapsulation agent. Single or double layered wall might form after the core compound composed into the single or several ingredients. Retention of the core compound is governed by its chemical functionality, solubility, polarity and volatility. According to Gouin, (2004), microencapsulation technology had been widely used in food industry. The encapsulation method was first developed in year 1930s and the first encapsulation agent used was gum acacia (Shahidi and Han, 1993).

HPLC and UV-VIS spectrophotometer analyst are the common analyst chose to analyse the components in phytochemicals. No further or other equipment such as Ultraviolet-Visible spectroscopy (UV-Vis), Liquid Chromatography-Mass Spectrometry Quadrupole-Time of Flight (LCMS-Q-TOF) and Ultra Performance Liquid Chromatography (UPLC). It is essential to test the sample using various methods, i.e. UV-vis, LCMS-Q-TOF and UPLC to cross validate the result which is necessary for accurate identification and quantification of polyphenol content. No powder form of extracted polyphenol from *P. niruri* had been commercialized in the market. In addition, powder based product is more desirable and convenience to store, consume and having longer shelf life.

1.2 Problem statement

The most important factor that affects the yield and recovery of the bioactive components from plant materials is based on the type of extraction method. Previous extraction method performed by Markom et al. (2007) was soxhlet extraction, the extraction time consumed up to 3 hours to obtain 150mL volume of extract. Another method of extraction performed by Tripathi et al. (2006) was maceration that consumes

10 hours for the extraction process. Both method performed by previous researcher were the conventional and traditional method which normally required high temperature and long duration to obtain the extract. Furthermore, the high temperature setting will cause the thermal degradation of the polyphenol due to the heat exposure for a prolonged period. (Akowuah and Ismail, 2010). Another researcher, Muhamad et al. (2015) and Mercali et al. (2013) had reported the degradation of active compound such as Vitamin A & E, polyphenol and antioxidant after gone through the exposure of high temperature for long period of time. In order to reduce the probability of thermal degradable of the bioactive components during the extraction, shorter time and reduction of the exposure under high temperature extraction method is more preferable. Hence, ultrasonic assisted extraction (UAE) and microwave assisted extraction (MAE) had been introduced in this study to overcome the thermal degradation issue. One of the objective of this research study is to investigate the performance of ultrasonic assisted extraction and microwave assisted extraction in extracting polyphenol from *P. niruri*.

Bioactive components from the plant materials that involving solid-liquid extraction and mass transfer process involving the solvent, liquid transport to the inner part of the solid plant materials. Solubility of the solute which release from solid matrix to the external bulk phase of the plant. Ultrasonic assisted and microwave assisted extraction able to reduce on the limitation of the mass transfers for both internal and external transport. Moreover, with the aid of the ultrasonic wave, cell membrane of the plant can be breakable which reduce the limitation of inner mass transfer. There are limited optimization literature studies of UAE from *P. niruri* by response surface methodology (RSM) as per current published journal and there is no previous work on optimization extraction of bioactive components from *P. niruri* by using MAE available. Therefore, optimisation by RSM on UAE and MAE methods were performed in this work. Of course one factor at time (OFAT) and tow level factorial study (2LF) must be performed before the RSM is conducted.

A success extraction is very subjective toward the type of solvent used. In the extraction process, solvent will diffuse into the plant material and solubilize compounds with similar polarity (Ncube et al., 2008). Current solvent extraction and processing of *P.niruri* were performed by water or methanol extraction. In present study, only extraction of lignans from *P.niruri* had been conducted (Murugaiyah and Chan, 2007).

From the previous phytochemicals extraction process conducted by Barbara et al, (2015) and Poh-Hwa et al, (2011), methanol is the solvent added to aid the polyphenol extraction. Solvent type might affect the recovery and purification of the bioactive components yield after the extraction process, where the Food and Drug Administration (FDA) approved chemical as the solvent extraction is another concern in the consideration for the solvent extraction where the end product from *P. niruri* will be consumed and applicable in nutraceuticals industry. For the study of the effect of polarity of solvent in extraction can refer section 2.3.1. Hence, ethanol, isopropanol and water were used as the solvent study in this research work. In order to achieve the aim this study, another objective on the elucidation on the combined effect of various type of solvents and method of extraction on *P. niruri* polyphenols was added.

The most effective way to produce the nutraceutical or functional food in liquid form is by extraction method from *P. niruri*. Due to the limitation of the storage of the phenolic rich solution in longer period of time frame, solid particle pharmaceutical dosage storage in the form on tablet or capsule is more desirable and preferable in prolonged the shelf life of the phenolic rich bioactive components. Besides that, end product in solid particle pharmaceutical dosage is easier to handle compared to the liquid solution product. For the time being, there's no method in the producing the phenolic rich solution from *P.niruri* in solid particle pharmaceutical form had been established yet. The method and formulation of microencapsulation on the phenolic rich solution that available now on other ingredient can refer section 2.5 on the type of the wall material as well as the drying method. For the current technology in producing the powder through the hot spray is prone to thermal degradation of the polyphenol. The wall material of polysaccharide and protein used for microencapsulation for bioactive components extract from *P. niruri* had not been study for the retention of the active components after the spray drying. Therefore, another aim of this research is to minimise thermal degradation *P. niruri* during spray drying via microencapsulation.

Spray drying is a method producing the dry powder from liquid or slurry solution via the liquid was dry rapidly in instantaneous hot air flow. Typical inlet hot air temperature ranging from 150˚C until 220˚C for the co-current flow spray drying for the purpose of ensuring the efficiency of the instantaneous evaporation of the water content in the liquid droplet. On the other hand, the temperature ranges for outlet temperature

ranged from 80˚C to 120˚C. When the polyphenols, vitamin, flavonoid, and quinine exposure of high temperature over the long period, it will face the thermal degradation whereby the nutrient contain will be facing the degradation as well. From the previous researcher study (Verbeyst et al., 2010; Xie et al., 2010; Miranda et al., 2010) proof that vitamin E and A, antioxidant, anthocyanin from tomato, strawberry, and blackberry reported having the high bioactive component degradation over a period of time exposure of high temperature. Another objective of this research study is to maximize the bioactive components retention in order to retain high nutritional value of the product. Microencapsulation technology was used to maximise the bioactive component retention after spray drying.

1.3 Objectives

This study boards on the following objectives:

i. To investigate the performance of ultrasonic assisted extraction and microwave assisted extraction in extracting polyphenol from *P. niruri* with the aid of design of experiment method i.e. OFAT, 2LF and RSM.

ii. To develop a quantification and qualification method of *P. niruri* polyphenols using UPLC.

iii. To minimise bioactive degradation of *P. niruri* during spray drying via microencapsulation.

1.4 Scope of this research

The following are the scope of this research

i. Extraction study of bioactive compound by using various solvents, extraction methods (both UAE and MAE) and process condition by response surface methodology.

ii. Performing a proximate analysis (total phenolic content, total flavonoids content and antioxidant content) of the plant leaves extracts.

iii. Performing UPLC analysis to quantify polyphenolic compounds of the plant leaves extracts.

iv. Producing a high quality with minimal degradation of the bioactive components solid powder from *P. niruri*. Extract using encapsulation agents such as whey protein isolate (WPI) and maltodextrin.

v. Performing physical analysis on the *P. niruri*-based powder such as moisture content, morphology, and particle size distribution.

1.5 Main Contribution of this work

The following is the main contribution of this work:

i. A new method to extract polyphenols from *P. niruri* via MAE has never been attempted before and hence the results presented in this thesis represent a major advancement.

ii. The microencapsulation of *P. niruri* polyphenols has never been published before. It provides a higher retention of the bioactive components after spray drying that enable a production of a high quality extract with low degradation of nutrition. Therefore, this research study is noteworthy knowledge advancement in the research for *P. niruri*.

iii. A UPLC-PDA analysis of *P. niruri* polyohenols developed for the first time by this research work.

1.6 Summary

This chapter introduces users to the UMP *Microsoft Word* Thesis Template in *.dotx* format and the basic features of *Microsoft Word* that need to be enabled first before the *.dotx* template can be used. Once these important features have been enabled, your computer should be ready and the template can now be applied to your document. You can familiarize yourself with the styles used in this *.dotx* template. Some of the styles will be explained further in Chapter 2.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

This chapter presents the review on the previous work or study related to the method of extraction (UAE & MAE), literature study that related on the extraction (factors), microencapsulation, spray drying, and the analytical method. Moreover, the bioactive components from *P. niruri* are also discussed in this chapter.

2.2 Introduction

There were various study on the medical effect from *P. niruri* had been studied and published (Murugaiyah and Chan, 2007; Poh-Hwa et al., 2011; Moreira et al., 2013; Cuto et al., 2013). Phytochemical studies from *P. niruri* (Sousa et al., 2016) by illustrated the major active components in *P. niruri* are phyllanthin, gallic acid, quercetin, corilagin, niranthin quercetin-3-hexoside.

Previous researcher reported the active component contained from *P. niruri* yield the various medical effects. There are antitumor, anti-Hepatitis B (Markom et al., 2007), antioxidant, anti-hyperalgesic, anti-inflammatory and antiallodynic behaviour (Kassuya et al., 2006; Ichoo et al., 2011; Islam et al., 2008). With the present of these medical effects, it is beneficial toward the society and medical field where the Hepatitis B case reported by Ministry of health was increasing from year to year.

Thus, isolation of the bioactive components, toxicity test and biological study of *P. niruri* is well studied based on the literature available on it. On the other hand, there is limited literatures been developed on better and fast extraction and there is no microencapsulation of *P. niruri* had been developed and studied to retain the longer shelf

life of the extract. Table 2.1 illustrates the summary of some previous work obtained from the literature.

Table 2.1 Previous work on *Phyllanthus Niruri.*

Author	Study	Remarks
Tara et al. (2010)	Biological activity	Report P.Niruri enhances steroid suppressed wound healing & supportive therapy to treat chronic wounds.
Markom et al. (2007)	Extraction	Optimize the yield by solvent extraction, supercritical fluid extraction and pressurized water extraction. Reported solvent extraction by soxhlet is the best method for gallic acid and ellagic acid extraction.
Murugaiyah and Chan (2007)	HPLC method development	Simple and sensitive HPL-fluorescence detection method for the simultaneous determination of four lignans (phyllanthin, hypophyllanthin, phyltetralin & Niranthin) in *P. Niruri* has been developed.
Poh-Hwa et al. (2011)	Extraction	Optimize the extraction using both water and methanol solvent for the extraction. Methanol extract showed better inhibition potential than water extract.
Islam et al. (2008)	Biological activity	Phyllanthin and hypophyllanthin isolated from Phyllanthus family exhibited antitumor activities against Ehrlich Ascites Carcinoma in Swiss albino mice.

2.3 Extraction method for flavonoids and phenolic content in plant

Medical plant or aromatic herbs have been identified and used in many traditional treatments for many kind of diseases. It is well known on the constituent of many chemical compounds for the purpose of biological functions. Due to the natural product of medical plant or aromatic herbs, the content of valuable molecules on it is proven on health benefits which prompted the new development on the recovery process on it. To obtain the active components (polyphenol) from the plant, extraction process was introduced. The most traditional ways to extract the polyphenol were conventional

method i.e. maceration (Poh-Hwa et al., 2011) and soxhlet extraction (Murugaiyah & Chan, 2007).

Both maceration and soxhlet extraction also known as the conventional method that is most common to be found in industry. Even though both of these methods of extraction were be used in industry, it associated with time consuming under heating. Extraction under heating at long period will induce thermal degradation of the bioactive components. Oxidation of the active components might occurred during these conventional extraction methods as well.

In order to reduce the thermal degradation of bioactive components, other better polyphenol extraction method were developed and studied. In most recent years, supercritical liquid extraction (SFE), ultrasonic assisted extraction (UAE), microwave assisted extraction (MAE) and pressurized water extraction (PWE) were introduced by Markom et al. (2007). Both SFE and PWE performed under high temperature so that the solvent is maintained in liquid form by increasing the diffusivity of the solvent which required to operate at high pressure. SFE and PWE operate under high temperature and pressure that induce the thermal degradation of the bioactive components so UAE and MAE are selected as the most efficient extraction methods that reduce the extraction time, increase the quality of the product extract and increase the yield of the extraction. Even though both UAE and MAE were expressed more in lab scale, some of the industry had used it for the industrial application, especially MAE.

2.3.1 Ultrasonic assisted extraction

With the aid of the ultrasonic extraction, it induces the breakdown of the cell membrane which allows the acceleration of the diffusion of the solvent through the membrane. Apart from that, disrupt of the cell wall structure enhance the facilitate of the release of the cell content into the extract (Falleh et al., 2012). In short, ultrasonic assisted extraction able to consolidate the higher diffusion rate of the cell content into extract. By referring the Figure 2.1, disrupt of the cell membrane after the ultrasonic assisted extraction can be clearly seen.

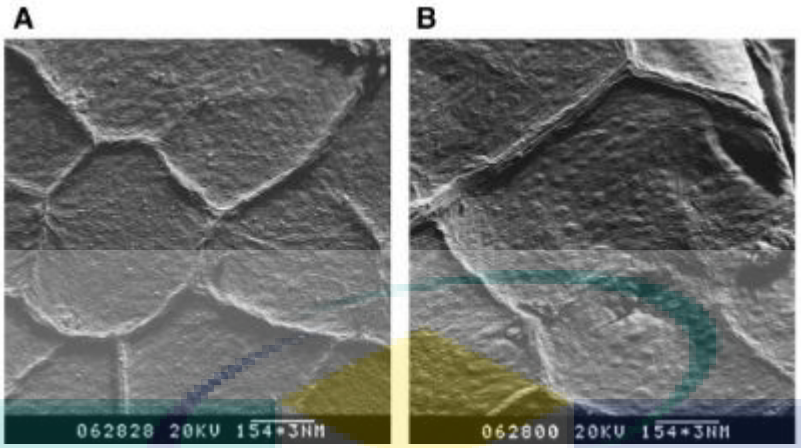


Figure 2.1 Scanning electron micrographs (100X) of Epimedium leaf sample. (A) Untreated leaf; (B) After ultrasonic assisted extraction by Zhang et al. (2009).

It is more preferable and suggested on the introduction of the ultrasonic assisted extraction in the extraction of the polyphenol extraction especially for the plants or herbs extraction of bioactive component. Based on the previous finding by Wang and Weller (2006), there are several factors such as sonication power, time and frequency will affecting the recovery rate of the cell content. Other than that, there are several researchers (Mediani et al., 2015; Murugaaiyah, V and Chan. K. L, 2007; Chen et al., 2015; Dong et al., 2016 and Fang et al., 2014) had study on the effect of temperature, solid content, type of solvent and its concentration toward the extraction yield. From the studied done by Sousa et al. (2016), the high power of 500W, ultrasonic intensity of 301W/cm2, time of 7 minutes and solid content of 40ml/g are the optimum factors to obtain the highest yield of phenolic content by UAE. Fang et al. (2014) reported the optimum time for the extraction was 7.5-12.9 minutes, optimum ethanol concentration was 43-47%, optimum power at 56-85 W, solid liquid ratio of 36-48ml/g.

The mechanism of the ultrasonic assisted extraction can be illustrated by the scanning electron micrograph on the cell structure. After undergo the significant ultrasonic assisted extraction, we can clearly see the disruption of the cell wall structure in the Figure 2.1. These phenomena will enhance the mass transfer of the solvent into the plant materials and soluble the cell content into the solvent. Another advantage of the ultrasonic assisted extraction is the disruption of the cell membrane as well. From the transmission electron micrograph shown in Figure 2.2 we can see that the chloroplast of the leaf was greatly destroyed after gone through UAE. This cavitation of UAE also

introduces more cell content to be extracted out from the leaves which also enhance the mass transfer of solvent into the chloroplast as well.

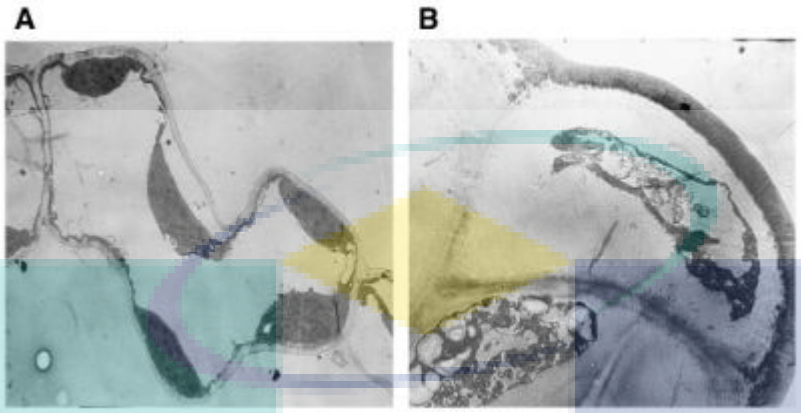


Figure 2.2 Transmission electron micrographs (3000×) of Epimedium leaf samples from (A), untreated leaf; (B), after ultrasonic-assisted extraction by Zhang et al., (2009).

2.3.2 Microwave assisted extraction

Microwave assisted extraction is the extraction process different with from the conventional method especially in solid-liquid extraction. This is because microwave assisted extraction will yield the changes in the cell structure by electromagnetic waves. With the aid of the MAE, it accelerates the extraction process and extraction yield due to synergistic combination of heat and mass transfer in the parallel direction (Chemat et al., 2009). By comparing MAE and conventional extraction, mass transfer for conventional method is from inside (cell content) to outside (solvent) while the heat is transferred from heating medium(outside) to the interior of the sample (cell content). Heat is dissipated volumetrically inside the irradiated medium in MAE. Energy transfer by two mechanisms. First is dipole rotation and second is ionic conduction through reversals of dipoles and displacement of charged ions present in the solute and the solvent by microwave heating (Routary and Orsat, 2011). These two mechanisms occur simultaneously during the MAE process. Electrophoretic migration of ions when electromagnetic fields applied and the resistance of the solution to the flow of ion results in the friction that heat the solution applied the ionic conduction principle. Dipole rotation refers to the rearrangement of dipoles with the applied fields (Eskilsson and Bjorklund, 2000). Energy transfer toward the materials during the MAE process are deliverd directly to the interior (cell content) through the molecular interaction with electromagnetic fields

through conversion of electromagnetic energy into heat energy (Thostensin and Chou, 1999).

There are several factors affecting the yield of MAE. The efficiency of the extraction refracted to the operating condition selected. The parameters that affecting the extraction yield are the solvent composition, solid liquid ratio, extraction temperature and time and microwave power. The most important factor affecting the MAE yield is solvent selection because the proper solvent will provide the better extraction process. The solvent selection was done by comparing the solubility of the compounds of interest, solvent penetration and the its interaction with the sample matric and also its dielectric constant together with the mass transfer kinetic of the extraction process (Chen et al., 2008). High selectivity toward the solutes is preferable for the extraction process. Polar solvent (ethanol, water and methanol) that presents of high dielectric constant and dielectric loss are sufficiently to be heated up by microwave. On the other hand, non polar solvent such as hexane, and chloroform has reported on the low efficiency of heating when exposed to microwave.

Table 2.2 Physical constants and disspation factors for solvents usually used in microwave-assisted extraction (MAE) (Zlotorzynski 1995 and Jassie et al., 1997).

Solvent	Dielectric constant ε'	Dissipator factor tan ∂ ($\times 10^{-4}$)	Boiling point (˚C)	Viscosity (cP)
Acetone	20.7	5555	56	0.30
Acetonitrile	37.5		82	
Ethanol	24.3	2500	78	0.69
Hexane	1.89		69	0.30
Methanol	32.6	6400	65	0.54
2-Propanol	19.9	6700	82	0.30
Water	78.3	1570	100	0.89
Ethyl acetate	6.02	5316	77	0.43

The combination of the solvent types in MAE based on the polarity of the targeted compounds will result the better extraction yield. Higher water concentration would reduce the extraction yield due to high water concentration increase the mixture polarity to a degree which resulting the no longer favourable for the extraction. These finding had been proven by Song et al. (2011) on the ethanol concentration of 60-80% in water is optimal compared to the pure water. The amount of the solvent must be sufficient to immerse the entire sample to guarantee the complete irradiation. By comparing the

conventional extraction and microwave extraction, conventional extraction required large amount of solvent in order to get the better recovery of the extract. However, for MAE, many studied (Talebi et al., 2004; Pan et al., 2003), the optimal solid to solvent ratio for MAE is 1mg/10ml to 1g/20ml. In addition, small amount of solvent is sufficient for MAE to extract the compounds of interest because large solvent volume required more energy and time to condense the extraction solution for the purification process.

Another major operating condition of MAE that affects the extraction yield is microwave power and extraction temperature and time, microwave radiation, water content and contact surface area. High microwave power can bring the high temperature in the system which resulting low extraction yield. It is known that high power will induce the temperature of the system to increase whereby the microwave power control the amount of energy provided to the matrix that converted heat energy into dielectric materials. There is the interrelation on the high temperature to the solvent power in controlling the viscosity and surface tension, facilitating the solvent to solubilize solutes, and improving the matrix wetting and penetration (Mandal et al., 2007; Li et al., 2010; Khaejeh et al., 2009). Extraction time required for MAE is a much shorter (few minutes) compared to conventional method which required few hours to complete the extraction. Irradiation time influenced by the dielectric properties of the polar solvent where longer exposure toward heat gradient increase the risk of thermolabile constituents (Mandel et al., 2007).

Instead of the dipole and ionic conduction mechanism was performed by MAE, another mechanism of the cell rupture by MAE was studied by Wang and Weller, (2006) that the internal pressure was generated when the matrix is heated and evaporated able to break down the cell structure of the plant. Occasionally, when the cell structure was broken, it enhances the extraction of the cell content to the solvent. This mechanism had confirmed by the researcher Zhang et al. (2011) by comparing the light micrographs of Epimedium leaf on untreated leaf and after microwave irradiation. From the Figure 2.3, some of the chloroplasts were damaged due to microwave irradiation as well.

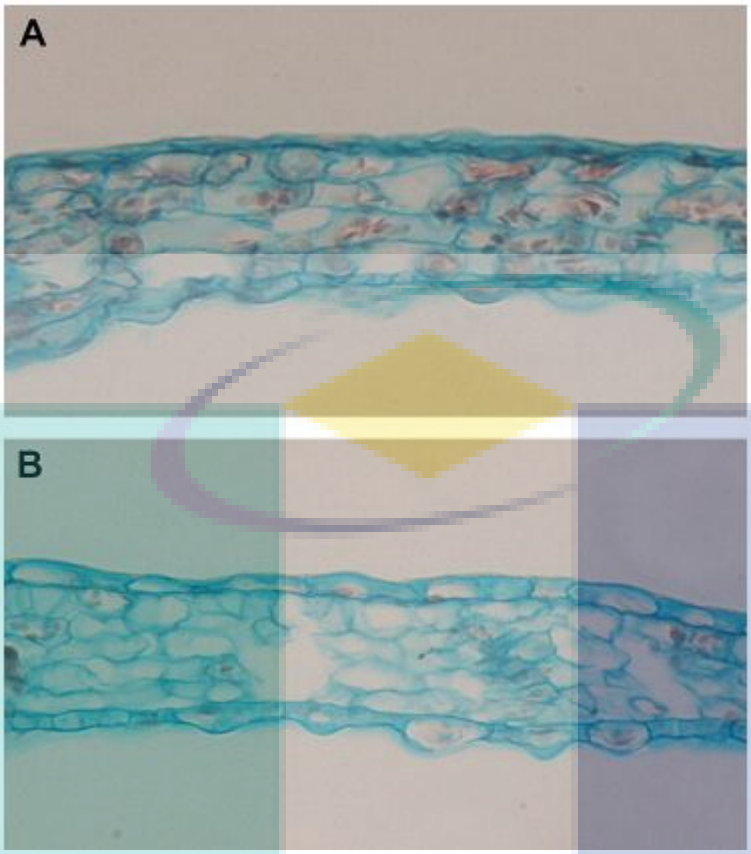


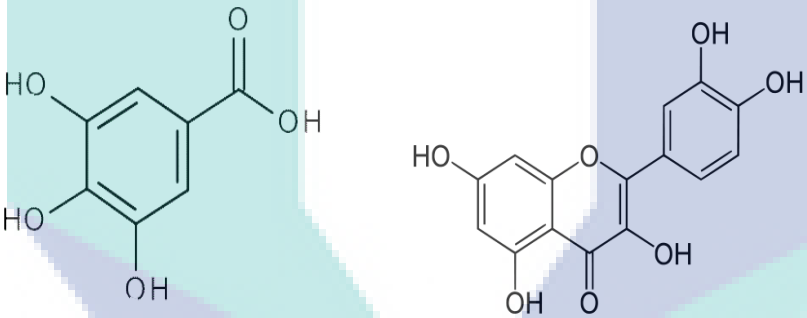
Figure 2.3 Light micrographs of Epimedium leaf samples: (A) untreated leaf sample; (B) leaf sample after microwave irradiation by Zhang et al., (2011).

2.4 Bioactive compounds from *phyllanthus niruri*

There are three categories of the compounds from the plant can be classified. First category defined the primary metabolites which consist of the important function in cell metabolism. For example, compound involving in the cell respiration and reproduction activities are classified as primary metabolites. Amino acid, nucleic acid and sugar are the common compound to be classified as first category due to its function in reproduction of the cell. Second category defined the compounds which play the roles in constructing the cell structure. Examples of second category compounds are cellulose, lignin and protein. The compounds that contribute to the plant adaption and interact with the ecosystem that limit to specific plants are belonging to third category. These substances in this category are belonging to secondary metabolites. The functions of secondary metabolites are protecting the plant from the pathogens such as phytoalexins, anti –germinative or toxic for other. In short, secondary metabolites behave as antibiotic, antifungal and antiviral. In the past, secondary metabolites had been wisely use in

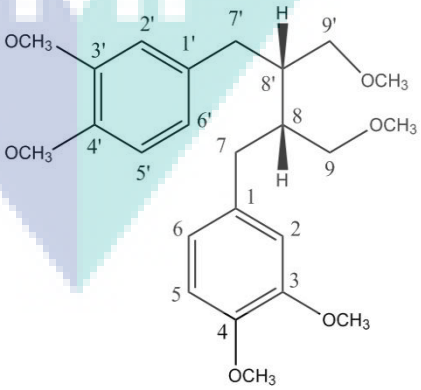
traditional medicine due to its characteristic and its useful biological activities. In the recent years, it is used in cosmetic, foods, pharmaceutical and even nutraceutics industry.

P. niruri is an annual herb, widely found in tropical and subtropical countries. In Brazil, it is well known medicine that used to treat genitourinary disorder for the elimination of kidney stone (Barros et al., 2006). These medical properties are associated with some of the active components such as lignin, alkaloids, triterpenes, and polyphenols such as quercetin, rutin, corilagin, and gallic acid. The major components consist in the *Phyllanthus Niruri* plant is polyphenols such as polymethoxylated flavonoids, phyllanthin, gallic acid, and quercetin (Maity et al., 2013 and Patel et al., 2011). From the clinical and pre-clinical trials had confirmed the medical properties of *P. niruri* (Nikam et al., 2011 and Notka et al., 2004). Phyllanthin compound is the identical of the *P. niruri*. The selected target compound for this study will be phyllanthin, gallic acid and quercetin. The structures of all these three components are illustrates in Figure 2.4.



Gallic Acid

Quercetin



Phyllanthin

Figure 2.4 Active components in *Phyllanthus Niruri* extract.

2.5 Spray Drying

Herbal product is often found in liquid form after extraction. It is difficult to prolong the shelf life of the herb product in liquid form as the storage condition such as storage temperature and moisture content allows the liquid to turns into rotten. Thus, in order to prolong the shelf life of the herb product, the idea to remove the water content by introducing heat drying is one of the practices to obtain the chemical and biological stability of the product and also inhibit the degradation of the active components. Spray drying system contains a spray chamber and cyclone. The sprayer in the spray chamber will atomize the concentrated solution to be contacted completely with the hot air in the spray chamber. At this process, micro liquid drops of the concentrated solution were dried and turns into dry powder immediately. The dry powder formed mixed up with air and separated by centrifugal force in the cyclone where dry powder will accumulate at the bottom while the exhaust gas will be released out from the top of cyclone. The advantages of producing the dry powder by spray drying are rapid drying rate, short residence time and we have the wide range of operating temperature can be chosen.

As the operating temperature of spray dryer operates at the high temperature, it is well known that bioactive components such as flavonoid, vitamins, antioxidant and protein will suffer from the degradation when expose to high temperature which means that low nutritional value will retain after the exposure of high temperature. From the previous finding buy Anandharamakrishnan et al. (2008), they found that there is the protein denaturation up to 60% when increase the operating temperature of a scale spray dryer. Another finding by Miranda et al. (2010), thermal degradation of other components such as vitamin and antioxidant also reported at the end of their research study. Even though producing dry powder is good in prolonging the shelf life, low retention of nutritional value of the active components after exposure to high temperature will be another problem yield. There is one solution by introducing microencapsulation to overcome this problem by reducing the thermal degradation rate of the active components (Young et al., 1993 and Krishnaiah et al., 2012). Further details regarding on the microencapsulation will be discussed in section 2.6.

To enhance the encapsulate performance and efficiency, proper operating condition of spray dryer should be studied. Solution feed rate toward the atomizer need to adjust accordingly to ensure the spray droplets are at the desired drying level before

contact to the surface of drying chamber. It is important to have the air inlet temperature so that the final moisture content and the drying rate are controllable. These had been confirmed by the studied done by Fernandes et al. (2012), when the air inlet temperature set from 140-160˚C, it tends to produce the spray powder with high moisture content which also adhere on the surrounding wall of the chamber.. Precious studied also showed other operating condition such as the outlet temperature ranged from 52-130˚C while inlet temperature ranged from 120˚C to 200˚C. For the case of maltodextrin and whey protein isolate, the outlet temperature ranged from 80 to 130˚C while for the inlet temperature ranged from 140 to 200˚C. Table 2.3 illustrates the summary review of spray drying condition from different researcher.

Table 2.3 Review of spray drying condition.

Encapsulated ingredient	Wall material	Feed temperature(˚C)	Air inlet temperature(˚C)	Air outlet temperature(˚C)	References
Ethyl butyrate ethyl caprylate	Whey protein and lactose	5	160	80	Rosenberg and Sheu (1996)
Linoleic acid	Whey protein concentrate, whey protein isolate, maltodextrin	40	180	85	Choi et al. (2010)
Anhydrous milk fat	Whey protein and lactose	50	160	80	Young et al. (1993)
Ooregano, citronella and marjoram flovors	Whey protein and milk protein	NR	185-195	85-95	Baranauskiene et al. (2006)
Arachidonyl L-ascorbate	Maltodextrin, gum Arabic, soybean polysaccharides	NR	200	100-110	Watanabe et al. (2004)
Caraway essential oil	Milk protein/ maltodextrin/whey protein	NR	175-185	85-95	Bylaite et al. (2001)
Short Chain fatty acid	Maltodextrin/ gum arabic	NR	180	90	Teixeira et al. (2004)
Lippa sidoides	Maltodextrin and gum arabic	50	140-160	NR	Fernande et al. (2012)
Cumin oleoresin	Gum Arabic, modified starch	NR	176-180	95-105	Soottitantawat et al. (2005b)

NR= Not reported

2.6 Microencapsulation

Microencapsulation is the process where droplets (core) are coated by the microencapsulating agent. There are many coating wall materials, it can be made up from variety food grade excipients such as polysaccharides, cellulose or polymer which able to provide the physical barrier protection of the droplets from the environment conditions. The size of microcapsules can be ranged from 1μm to 800μm with the active core in it. The wall of coating maybe single layer or double layer coated with uniform or non-uniform thickness. Polymer excipients like polysaccharides, cellulose, povidone reported the good encapsulating agent due to its good solubility and encapsulation efficiency. Maltodextrin, gelatine and porous starch had been studied and specifically for the improving on the stability.

There are several method of microencapsulation can be performed. It can be done by spray drying, liposome entrapment, interfacial polymerization, spray-cooling, air suspension coating, coaceravation, co-crystallization, spray–chilling, rotational suspension separation, centrifugal extrusion, freeze-drying, extrustion, molecular inclusion and other more. (King, 1995; Gibbs et al., 1999; Gouin, 2004).

Types of microcapsules can be vary based on the physic-chemical properties of core, the composition of the coating material and the microencapsulation technique. The morphology of microcapsules can be in spherical shape, irregular shape, or microcapsules with several cores, multi-layer coating with single core and etc. For the better review on the morphology of the microcapsules can refer Figure 2.5.

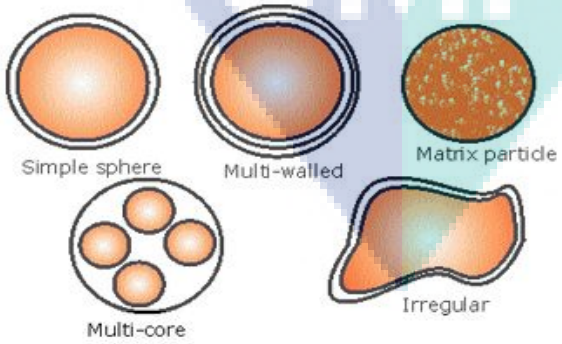


Figure 2.5 Morphology of different type of microcapsules.

Spray-drying is the one of the method which able to encapsulate active materials within a protective matrix formed from the polymer or melt more effectively (Dziezak, 1988). In terms of food industry, the spray drying is the most common technology used in order to reduce the cost and also the availability of the equipment together with the well know efficiency to protect drug or food ingredient against deterioration and volatile losses.

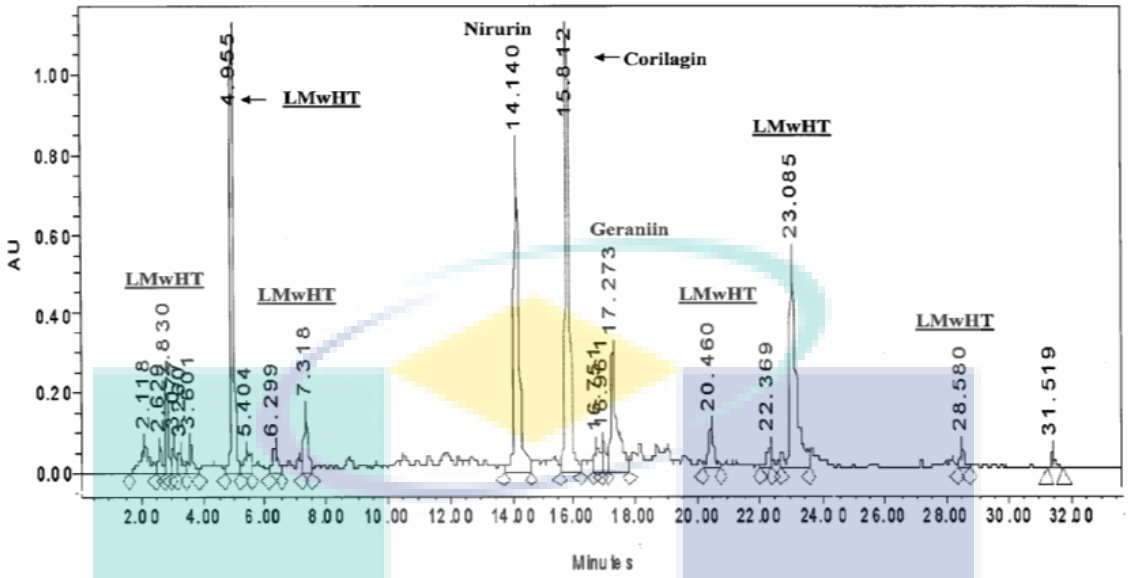
The selection of the wall materials for microencapsulation by spray drying is important by referring to both microcapsule stability and encapsulation efficiency. With the consideration of the core materials and the final product, the wall materials can be selected from various type of natural or synthetic polymers. Mircoencapsulation by spray drying able to process good properties of coating or film forming, emulsification and drying process. Moreover, the concentration of wall solutions needs to contain low viscosity according to Reineccius, (1988) and the solubility of the wall material in water must within the acceptance level (Gousin, 2004). Carbohydrate based wall material like starch and maltodextrin, protein based such as whey protein isolate and gelatin, gum based like acacia gum and gum arabic are the common encapsulating agent used in the industries. Maltodextrin and whey protein isolate are chosen as the encapsulating agent for this study due to their low viscosity at high solid content and high solubility in water.

2.7 Ultra Performance Liquid Chromatography

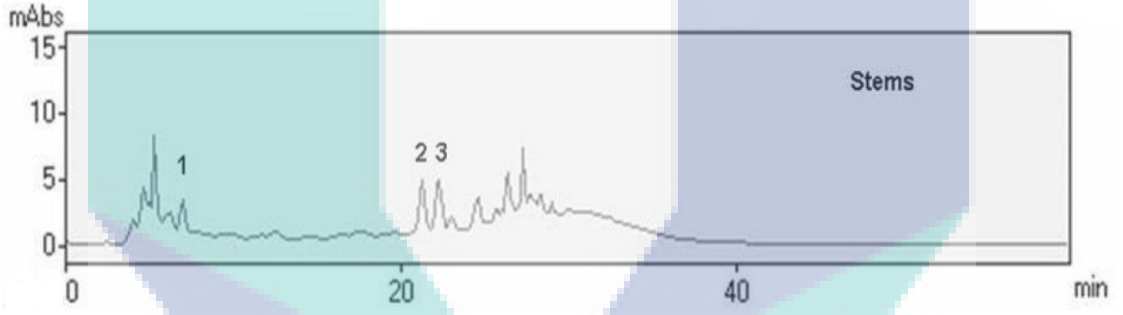
P. niruri consists of variety of active components with its medical effect. Analytical method is required for the accurate and precise quantification and qualification of these active components. Mainly the work done before this regarding the analysis of active components was done by HPLC analysis. Summary of HPLC analysis methods are presented in Table 2.3. From the literature studies, it can be seen that LC analysis is the best method to analyse the active components. Faster analysis always the more desirable and preferable by introducing ultra-performance liquid chromatography (UPLC). UPLC column usually packed with smaller particle size at 1.7 μm. It operates at higher pressure to increase speed, resolution and efficiency by comparison of conventional HPLC method (Swartz, 2005). UPLC column with high packed pressure can provide the sharper and faster separation than HPLC. Refer Table 2.4 on the review of HPLC analysis for *P. niruri* by different researcher.

Table 2.4 Review of HPLC analysis for *Phyllanthus Niruri*.

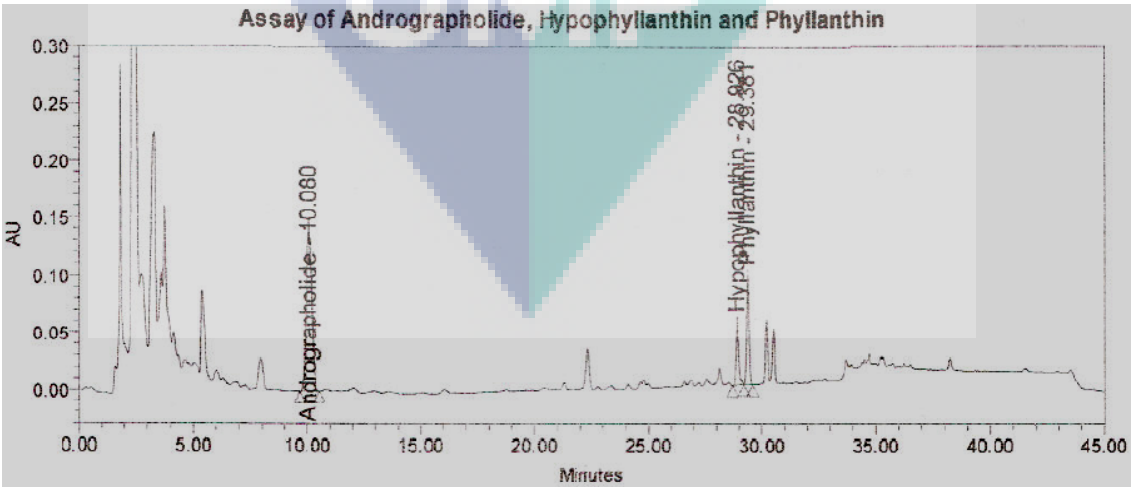
Authors	Column	Mobile Phase	Mode of column separation
Ghosal et al. (2012)	RP C18 250mm1.X 4mm id., 5um particles d	A: 0.1%phosporic acid, B: Acetronitrile	Reverse phase
Cuoto et al. (2013)	RP-18 Li Chrosher 250X4mm id, 5um particle diameter	A: 1% phosphoric acid, B: CAN: phosphoric acid 1% (w/w) (50: 50v/v)	Reverse phase
Bhope et al. (2013)	reverse-phase 250 mm × 4.6 mm, 5 µ, symmetry C8 column (Waters).	0.1% OPA (solvent A) and acetonitrile:methanol (1:1) (solvent B).	Reverse phase
Annamalai and Lakshmi. (2009)	µBondapak C18 column (25 cmx4.6 mm)	methanol : water (66:34 v/v) as the mobile phase	Reverse phase



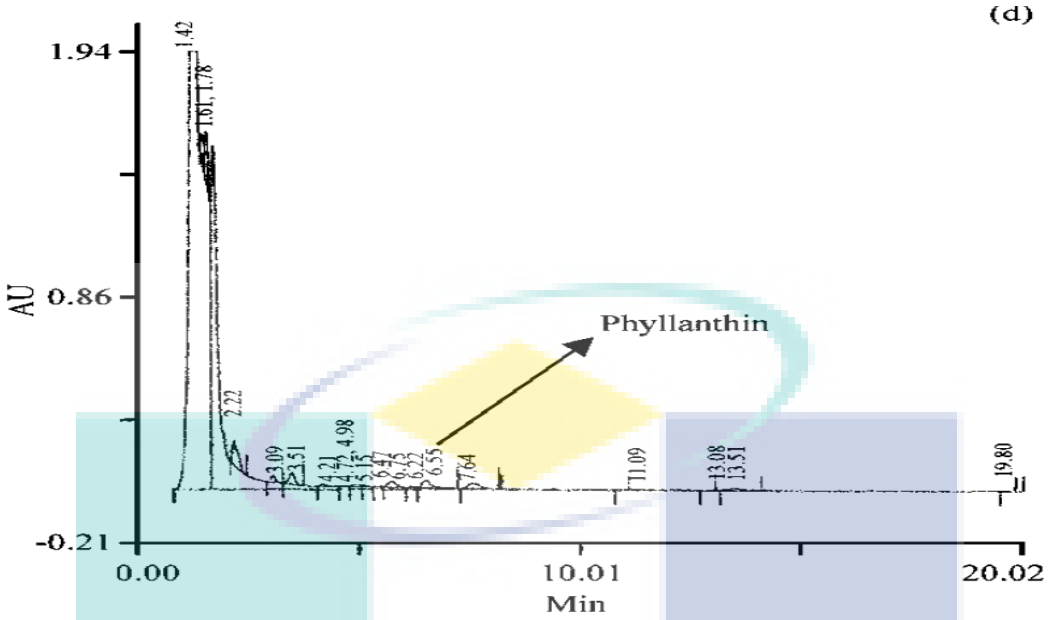
(A) Ghosal et al. (2012)



(B) Cuoto et al. (2013) 1-Gallic acid



(C) Bhope et al. (2013)



(D) Annamalai and Lakshmi. (2009)

Figure 2.6 HPLC chromatogram of *Phyllanthus Niruri* extract from various authors.

2.8 Summary

P. Niruri contain many medical effect active components such as phyllanthin, gallic acid and quercetin. A good analytical method on liquid chromatography is important to isolate and precise quantification and qualification of the active components. Based on the previous analytical method on the bioactive components from *P. Niruri* were using HPLC, limited research study on UPLC had been developed. Review from the previous work would suggested that alternative extraction methods such as UAE and MAE method of extraction would bring better extraction yields compared to conventional method of extraction. Extraction with the combined of two or more effects in both UAE and MAE extraction via response surface methodology had been employed in this research study using response surface methodology. Preservation of the extracted bioactive components may achieve via microencapsulation by spray drying process. Combined of two or more encapsulating agent had been reported better microencapsulation efficiency and thus, the method was adopted throughout this research work and bioactive components degradation was identify at the end this research study.

CHAPTER 3

METHODOLOGY

3.1 Overview

In this chapter, experimental procedures clearly described and illustrated for this research study. Experiments procedures included extraction method, proximate analysis on total phenolic, total flavonoids and antioxidant content, ultra-performance liquid chromatography (UPLC), spray drying, particle size characterization, moisture content and scanning electron microscopy (SEM). Details on the experimental methods and materials (chemicals and plant materials) are clearly presented in this chapter. Figure 3.1 below shows the flow chart of this research methodology. Extraction process by UAE and MAE were carried out by adopting response surface methodology. 2LF screening was done first before the CCD optimization. After extraction process completed, polyphenol extract identification and quantification were be done via UPLC. Second part of this research study was the microencapsulation where the polyphenol extract was mixed with the encapsulant and then viscosity was measure after the solution mixed homogenously. Emulsion was then undergo the spray drying process to form the micro particle spray dry powder. Characterization on the spray dry powder was done by screening on the moisture content, particle size distribution, surface morpology and lastly polyphenol retention before and after spray dry via UPLC.

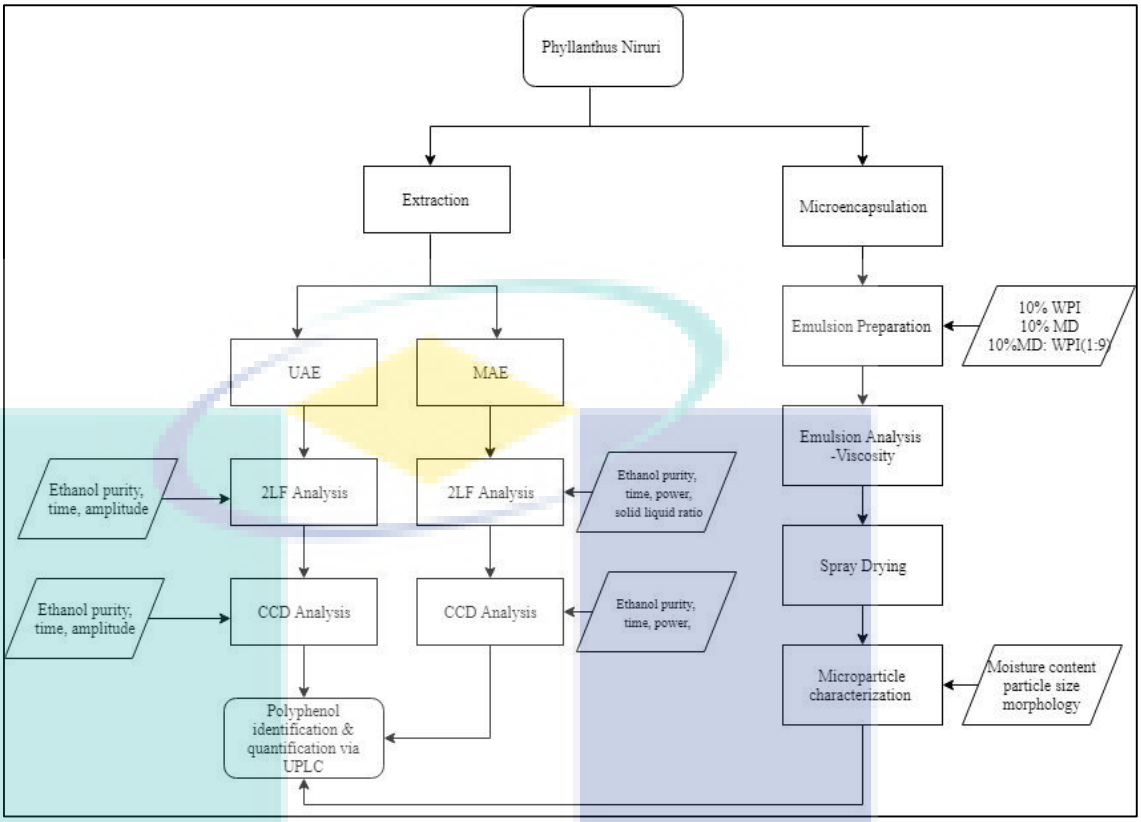


Figure 3.1 Experimental work flow.

3.2 Chemicals

Ethanol, sodium nitrate, sodium hydroxide, Folin & Ciocalteu reagent, butylated hydroxyanisole (BHA) and HPLC gradea cetonitrile from Merck (Darmstadt, Germany). Gallic acid, trifluroacetic acid and quercetin were obtained from Fisher Scienctific (Pittsburgh, PA). Aluminium hexachloride, 2,2-diphenyl-1-picrylhydrazyl, HPLC grade dimethyl sulfoxide were obtained from sigma Aldrich (St/ Louis, MO). A lactose-free whey protein isolate (WPI powder with 99% of undenatured protein was obtained from Ultimate Nutrition (Fleetwood, UK). Maltodextrin of 10 DE was obtained from San Soon Seng Food Industries (Malaysia).

3.3 Plant Material

Dried aerial plant of *P. niruri* that been voucher (voucher number KLU46618) specimen deposited at the Herbarium of Rimba Ilmu, Institute of Science Biology, University of Malaya, Kuala Lumpur was obtained from Malaysia Herbal Shop,

Selangor. Dried plant had been grinded and sieved. Every particle size sieved had been analysed to obtained accurate comparison. Figure 3.2 below shows the *P. niruri* plant.

Figure 3.2 *Phyllanthus Niruri* Plant.

3.4 Extraction Method

Ultrasonic assisted extraction (UAE) and microwave assisted extraction (MAE) had been chosen as the extraction method in the research study.

3.4.1 Ultrasonic Assisted Extraction (UAE)

Powdered *P. niruri* was weighted 2wt.% and mixed with solvent in 250mL sealed Erlenmeyer flask. UAE was carried out by using the sonicator Q700 with a microtip probe of 13mm diameter and had been employed in the response surface methodology in study the optimization of the extraction time, temperature and solvent concentration. According to the 2 level factorial studies, with three independent variables, ethanol concentration (A), time (B) and amplitude (C) as shown in Table 3.2. Selection of a factor is important in defining a research problem and the result of experimental research greatly depends on it. From literature, synthesizing of the total phenolic content, total flavonoid content, antioxidant activity, phyllanthin, quercetin and gallic acid were mainly affected by the factors listed in Table 3.1. The low and high value were chosen accordingly to past studies

(Sousa et al., 2016; Nguang et al., 2017). Total 8 experiments points had been carried out.

Table 3.1 Factors and their designated low and high value.

Factor	Units	Low value (-1)	High value (+1)
A: Ethanol purity	%	20	80
B: Time	minutes	3	9
C: Amplitude	%	20	90

Table 3.2 Experimental design matrix for UAE.

Standard	Run	Factors		
		EtOH Purity %	Time Minutes	Amplitude %
8	1	80	9	90
4	2	80	9	20
5	3	20	3	90
1	4	20	3	20
3	5	20	9	20
7	6	20	9	90
2	7	80	3	20
6	8	80	3	90

After the screening of 2 level factorial, optimization of the independent variable had been done according to central composite design (CCD). The CCD coupled with steepest ascent method were employed to optimize the significant factors in polyphenol extraction yield. The factors are the ethanol purity, time, and amplitude.

Having three factors screened in 2 level factorial, all factors were selected for optimization. These three factors are ethanol purity, time and amplitude have the positive effect on polyphenol extraction. The level with the highest response for polyphenol extraction was chosen as the center point for the new optimization design. The three selected factors for optimization using RSM using CCD with new levels and the center

point of the design is shown in Table 3.3 and the experimental design matric for optimization was tabulated as Table 3.4.

Table 3.3 Levels of extraction condition variables tested in CCD.

Factor	-1	0	+1
A: Ethanol Purity	15	22.5	40
B: Time	7	11	15
C: Amplitude	75	82.5	90

Table 3.4 Experimental design matric for optimization.

Standard	Run	Ethanol Purity (%)	Time (min)	Amplitude (%)
16	1	48.52	11.00	82.50
20	2	15.00	15.00	75.00
11	3	27.50	11.00	95.11
15	4	27.50	11.00	82.50
4	5	27.50	17.73	82.50
1	6	40.00	7.00	75.00
7	7	27.50	11.00	82.50
2	8	27.50	11.00	82.50
19	9	27.50	11.00	69.89
14	10	15.00	15.00	90.00
13	11	27.50	4.27	82.50
5	12	27.50	11.00	82.50
9	13	27.50	11.00	82.50
8	14	40.00	7.00	90.00
10	15	15.00	7.00	75.00
17	16	6.48	11.00	82.50
6	17	27.50	11.00	82.50
12	18	15.00	7.00	90.00
18	19	40.00	15.00	75.00
3	20	40.00	15.00	90.00

3.4.2 Microwave Assisted Extraction (MAE)

Powdered *P. niruri* to solvent ratio (2g/100ml) had been weight and mixed in 250ml conical flask. 5 ml of plant material had been placed in a test tube for MAE using CEM Explorer SP 48 microwave reactor (Matthews Nc, USA). The parameters were

determined based on literature and equipment limitation. According to the 2 level factorial studies, with three independent variables, ethanol concentration (A), time (B), power (C) and solid to liquid ratio (D) as shown in Table 3.5. Selection of a factor is important in defining a research problem and the result of experimental research greatly depends on it. From literature, synthesizing of the total phenolic content, total flavonoid content, antioxidant activity, phyllanthin, quercetin and gallic acid were mainly affected by the factors listed in Table 3.5. The low and high value were chosen accordingly to past studies (Pang et al., 2016; Nguang et al., 2017).

Table 3.5 Factors and their designated low and high value.

Factor	Units	Low value (-1)	High value (+1)
A: Time	Minutes	1	6
B: Power	W	30	250
C: Ethanol Purity	%	30	80
D: Solid liquid ratio	g/ml	0.025	0.2

Table 3.6 Experimental design matrix for MAE.

Standard	Run	Factors			
		Time minutes	Power W	EtOH purity %	Solid Liquid ratio g/ml
4	1	6	250	30	0.025
5	2	1	30	80	0.025
11	3	1	250	30	0.200
10	4	6	30	30	0.200
15	5	1	250	80	0.200
16	6	6	250	80	0.200
6	7	6	30	80	0.025
2	8	6	30	30	0.025
13	9	1	30	80	0.200
9	10	1	30	30	0.200
7	11	1	250	80	0.025
1	12	1	30	30	0.025
14	13	6	30	80	0.200
12	14	6	250	30	0.200
3	15	1	250	30	0.025
8	16	6	250	80	0.025

After the screening of 2 level factorial, optimization of the independent variable had been done according to central composite design (CCD). The CCD coupled with steepest ascent method were employed to optimize the significant factors in polyphenol extraction yield. The factors are the ethanol purity, time, and amplitude.

Having four factors screened in 2 level factorial, three factors were selected for optimization. These three factors are ethanol purity, time and amplitude which have the positive effect on polyphenol extraction. The level with the highest response for factor reaction time was chosen as the center point for the new optimization design. The three selected factors for optimization using RSM using CCD with new levels and the center point of the design is shown in Table 3.7 and the experimental design matric for optimization was tabulated as Table 3.8.

Table 3.7 Levels of extraction condition variables tested in CCD.

Factor	-1	0	+1
A: Ethanol Purity	30	55	80
B: Time	1	3.5	6
C: Power	30	140	250

Table 3.8 Experimental design matric for optimization.

Standard	Run	Ethanol Purity (%)	Time (Min)	Power (W)
16	1	55	3.5	140
6	2	80	1	250
15	3	55	3.5	140
11	4	55	1	140
7	5	30	6	250
8	6	80	6	250
3	7	30	6	30
10	8	80	3.5	140
20	9	55	3.5	140
9	10	30	3.5	140
12	11	55	6	140
4	12	80	6	30
5	13	30	1	250
19	14	55	3.5	140
2	15	80	1	30
13	16	55	3.5	30
18	17	55	3.5	140
14	18	55	3.5	250
17	19	55	3.5	140
1	20	30	1	30

3.5 Microencapsulation and Spray Drying

3.5.1 Encapsulating agent solution preparation

The *P. niruri* extracts was encapsulated by three types encapsulating materials, which consist of WPI, maltodextrin and the mixture of blended WPI and maltodextrin at the ratio of 1: 9 (w/w). The solution of encapsulating agent was prepared by dissolving it with ultrapure water and then it was stirred using magnetic stirrer to homogenize and rehydrate purpose. The encapsulating solution had been diluted by using ultrapure water in order to acquire the desired concentration which shown in Table 3.9. The crude extract was then mixed with the prepared encapsulating solution by magnetic stirrer under the temperature of 40ºC for 30 minutes to ensure the well mixing of the solution.

Table 3.9 Encapsulating agent and its concentration.

Encapsulating agent	Concentration of encapsulating agent (wt%)
WPI	10
Maltodextrin	10
Blend of WPI and Maltodextrin (1:9)	10

3.5.2 Spray Drying Process

The crude extraction solution with the mixture of encapsulating agent were spray dried by using the lab scale spray dryer (Lab Plant, SD06A, UK) with the co-current flow shown in the Figure 3.3. Internal diameter of the nozzle/atomizer is 0.5mm and the airflow rate can be adjustable accordingly to get the desirable air velocity for the spray dry process for the solution. The air velocity had been set at 4m/s constantly during the experiment and the inlet temperature was set at 180ºC. Liquid solution was feed into an electrical heater and then flew with the feed spray in the main drying chamber. The main chamber was made of thick transparent glass with the internal diameter of 21.5cm and a total height of 50cm, and dished shaped at the bottom. The length between the tip of the atomizer and the axis of the side exit tube is 47cm. To recover the powder particle from the spray chamber, the air/powder cyclone was used for the separation of powder and the exhaust gas. Dried powder samples was collected at the bottom of the cyclone with the schott bottle attached.

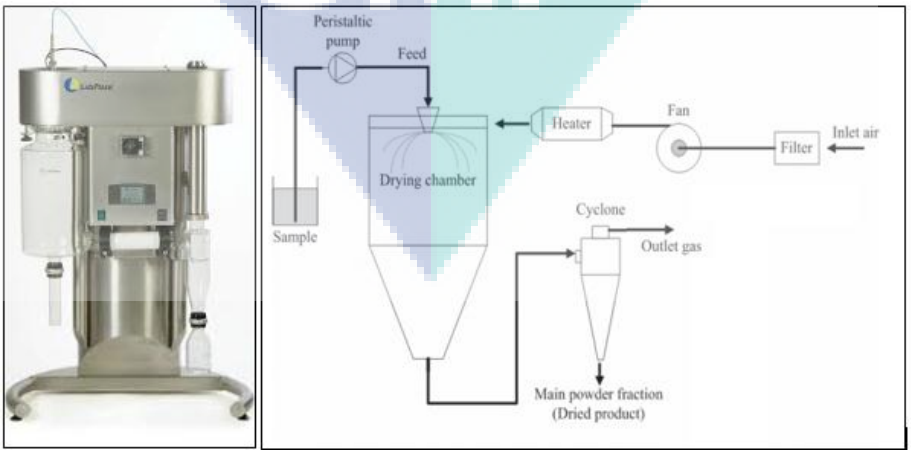


Figure 3.3 Spray Dryer and Schematic diagram of spray dryer.

3.6 Analysis

3.6.1 Total Phenolic Content

Singleton's method was used to determine the total phenolic content (TPC) using the Folin–Ciocalteu reagent (Trabeksi et al., 2010). Firstly, add the sample aliquot of 0.125ml to a centrifuge tube that containing 0.5 ml of ultrapure water and 0.125 ml of the Folin–Ciocalteu reagent. Then 1.25ml of 7% Na2CO3 solution was added after 3 minutes, and then make up the final volume to 3 ml with ultrapure water. The solution was mixed well and incubated for 60 min in the dark. Absorbance against the prepared blank reagent at λ = 760 nm was measured using a calibrated ultraviolet–visible spectroscopy (Hitachi U-1800, Japan). TPC 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 in Figure 3.4 using the equation (3.1) (Pan et al., 2012)

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

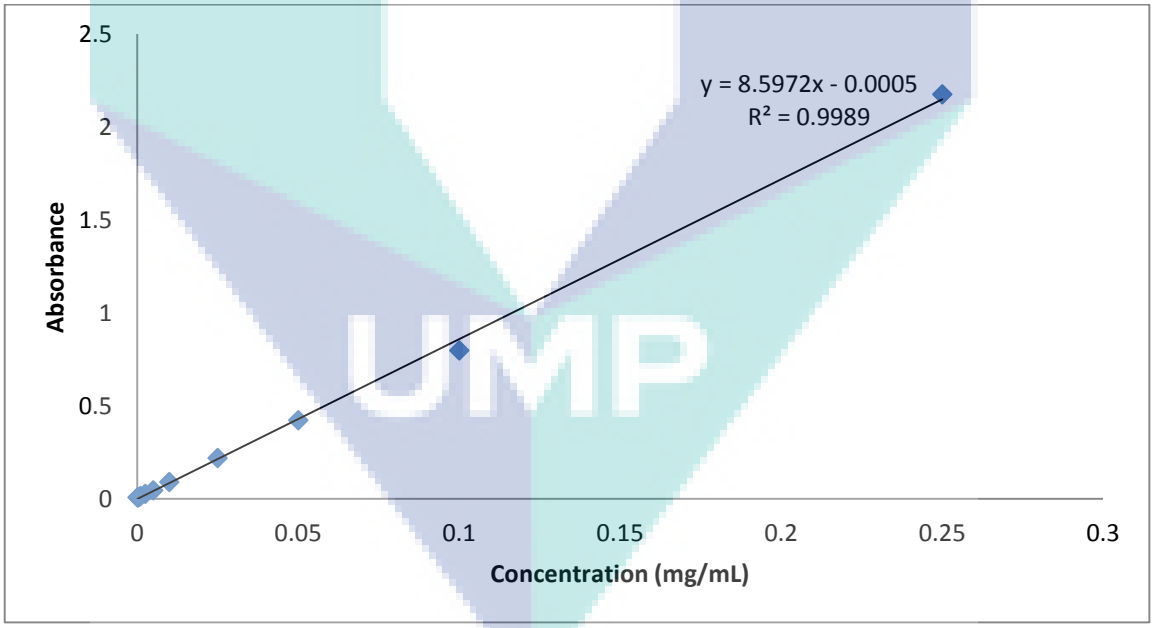


Figure 3.4 Standard Calibration Curve of Gallic acid.

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

3.6.2 Total Flavonoids Content

Total flavonoids content was measured by aluminium chloride colorimetric assay. (Abouzid and Elsherbeiny, 2008). 0.2mL of crude extract or standards solution of quercetin was added to a centrifuge tube containing 4.8ml ultrapure water. Then, 0.3ml of 5% NaNO2 was added into it and then mixed well. After 5 minutes, 0.3ml of 10% AlCl3 was added and mixed well. At 6th minutes, 2ml 1M NaOH solution was added and make the final volume of 10 ml with ultrapure water. The absorbance against prepared reagent blank at λ=414nm was measured using a calibrated UV-Vis. (Chang et al., 2002). Total flavonoid content was expressed as mg quercetin equivalents per gram dry weight (mg QE/g DW) by comparing the calibration curve for quercetin (Figure 3.5) using equation (3.2) (Pan et al., 2012).

$$\text{Total flavonoid content}\left(\frac{\text{mg}}{\text{g}}\right) = \frac{Y \times N \times V}{W} \qquad 3.2$$

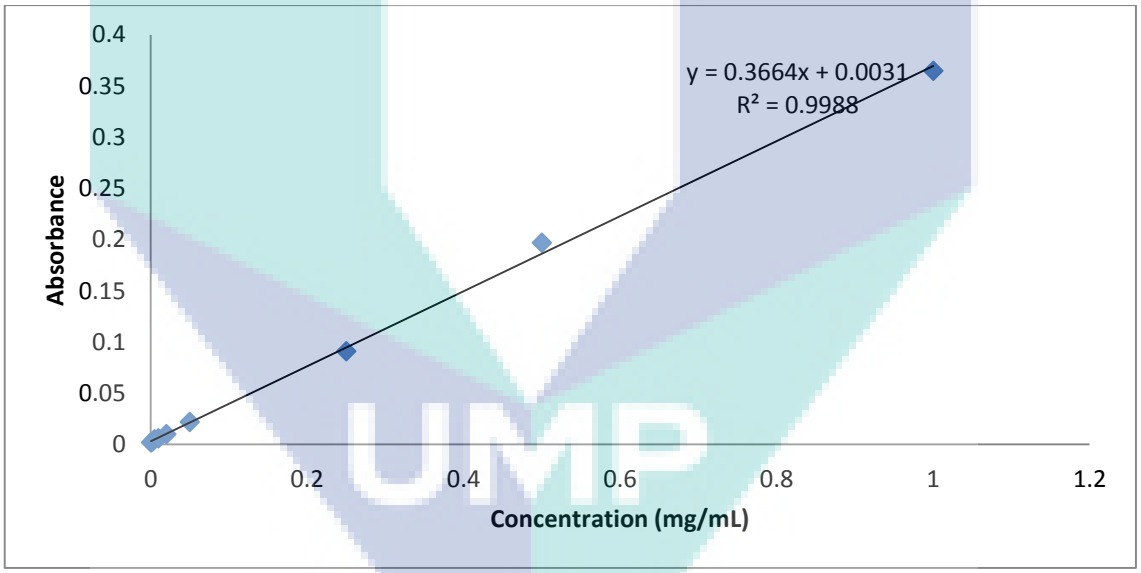


Figure 3.5 Standard Calibration Curve of Quercetin.

3.6.3 Antioxidant Activity

Antioxidant activity was measured by using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging method by Thoo et al. (2010). 0.1ml extract or absolute methanol (as control) was added into 3.9 ml of methanolic DPPH (60μM). The solution was mixed equally for 1 minute and then incubate in the dark for 60 minutes at room

temperature. The absorbance was measured at λ=515nm using a calibrated ultraviolet-visible spectroscopy (Hitachi U-1800, Japan). The activity was calculated based on the percentage of scavenged DPPH as follows:

$$Radical\ scavenging\ activity(\%) = \frac{Ac - As}{Ac} \times 100 \qquad 3.3$$

where Ac is the absorbance of the control at 60 minutes, and As is the absorbance of the sample at 60 min. Measurements were calibrated to a standard curve of prepared butylated hydroxyanisole (BHA) at concentration from 0.5 to 125ug/ml. (Figure 3.6) and expressed as mg BHAg-1 DW.

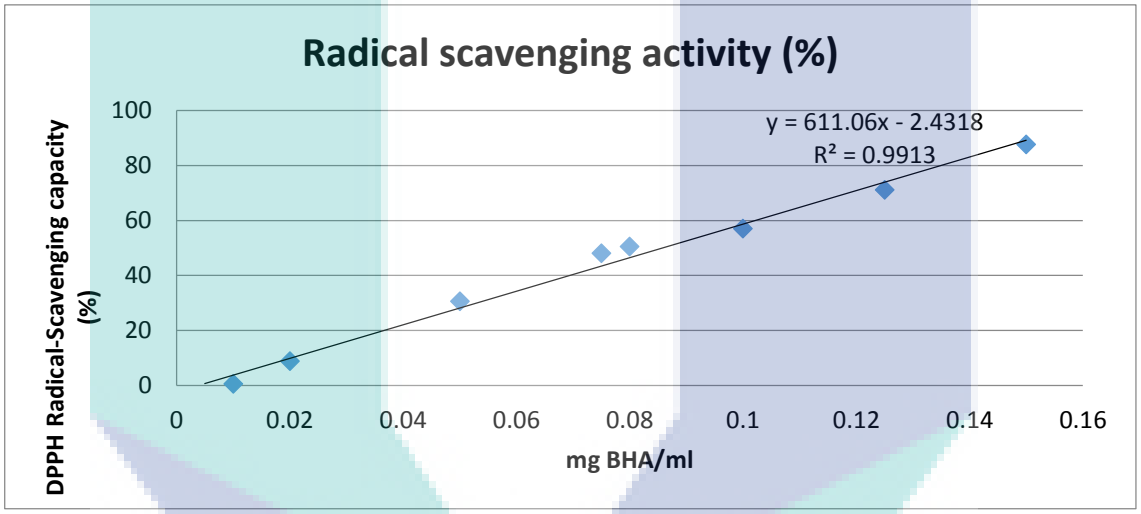


Figure 3.6 Standard Calibration Curve of Antioxidant Activity.

3.6.4 Ultra performance liquid chromatography

The stock solution of phyllanthin (10 mg/ml) was prepared in 99% ethanol, whereas quercetin (1 mg/ml) were dissolved in DMSO and gallic acid (1 mg/ml) were dissolved in ultrapure water. The three analytical standards were further diluted to develop an eight points standard calibration curve as shown in the Figure 3.7, Figure 3.8 and Figure 3.9. Qualitative and quantitative determinations the major constituents from the *P. niruri* extract (Phyllanthin, Gallic acid and Quercetin) are determine and quantify by Waters Acquity UPLC H-Class (Waters, USA) fitted with Merck RP-18 column (2.1mm × 100mm, 2.0µm). The UPLC system is equipped with photodiode array detector (Waters, USA) and connected to a computer running Water Empower 2 software. The

mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% ACN in water). The following gradient elutions, were: 0-2.5 min, 5-40%B; 2.5-5.0 min, 40-50% B; 5.0-15.0 min, 50-95% B then reconditioning the column with 5%B isocratic for 5 min. The temperature was maintained at room temperature wuth injection volume of 5µl and a flow rate at 0.3 mL/min. The sample was filtered with 0.2µm PES membrane filter (Cronus, UK) before injecting into UPLC system. The sample was filtered with 0.2 µm PES membrane filter before injected into the UPLC system. The peaks for phyllanthin (10.0-10.2min), gallic acid (3.10-3.30 min) and quercetin (14.4-14.7 min) were detected at 278 nm.

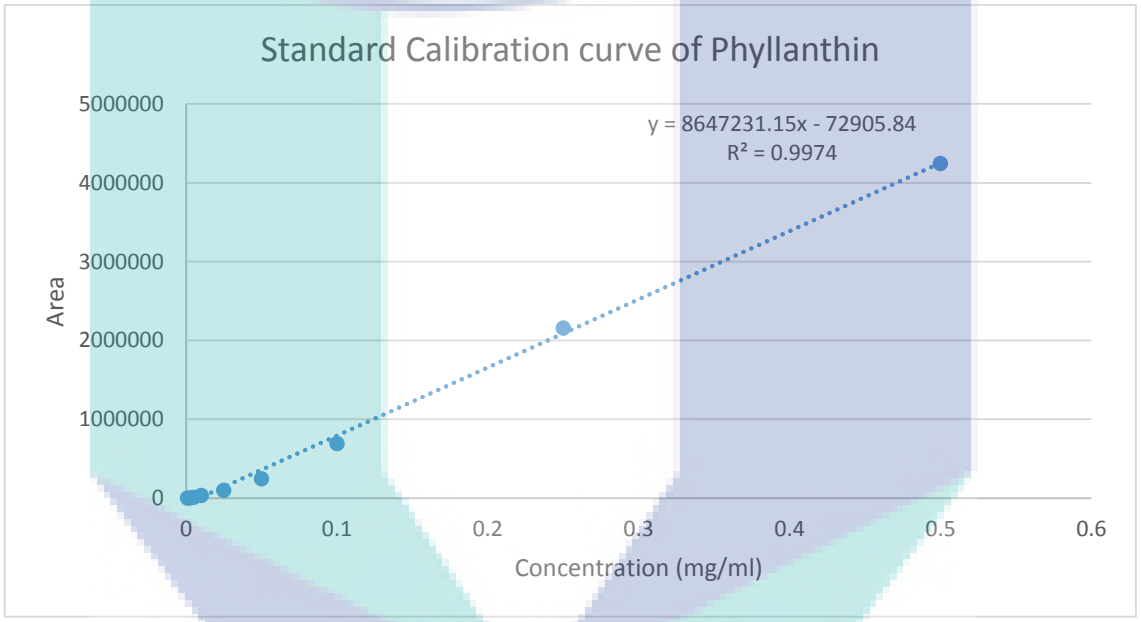


Figure 3.7 Standard Calibration curve of Phyllanthin.

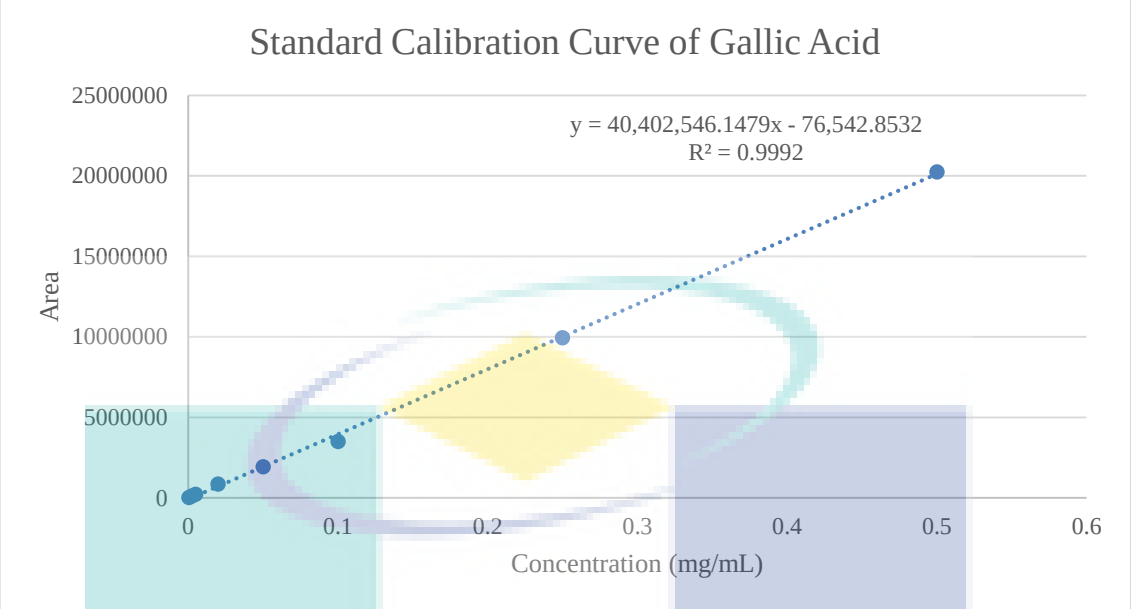


Figure 3.8 Standard Calibration curve of Gallic Acid.

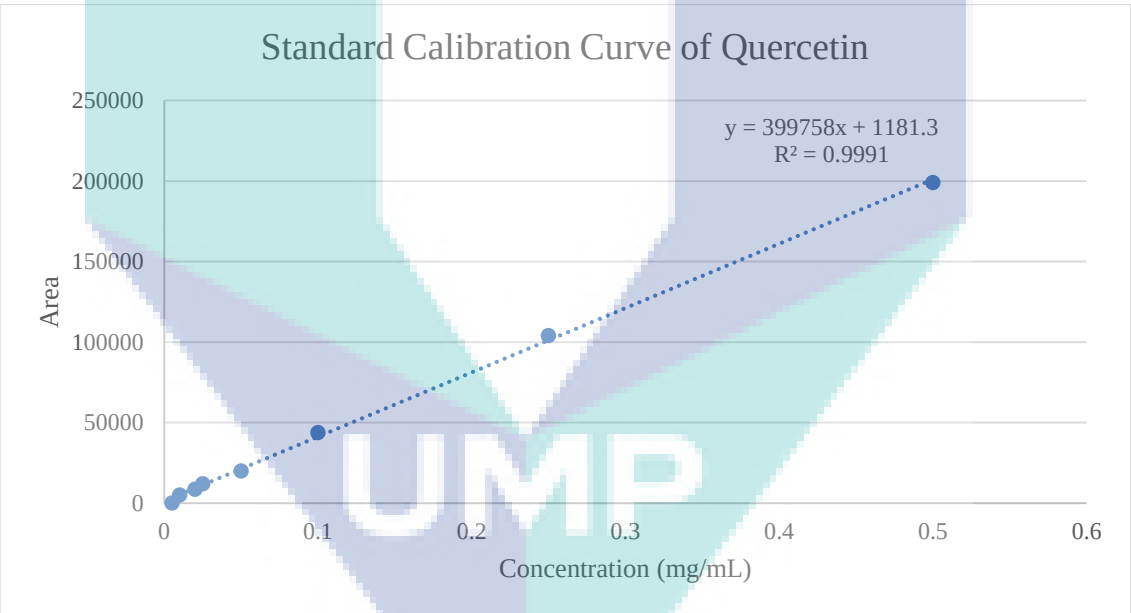


Figure 3.9 Standard Calibration curve of Quercetin.

3.6.5 Scanning electron microscopy (SEM)

The morphologies of the spray-dried particles were evaluated with a scanning electron microscope (Hitachi TM3030, Japan). The dried powder was mounted on specimen stubs with double sided adhesive carbon tapes and coated with platinum. The

specimen was coated with platinum and was examined at 15 kV with a magnification ranging from 500× to 2000×.

3.6.6 Particle size analysis

Particle size distribution of spray dry powder was measured using Malvern laser diffraction particle size analyser (Malvern 2000, Malvern Instruments Co., Worcestershire, UK) equipped with Scirocco 2000; an automated dry powder dispersion unit for the Mastersizer 2000 particle size analyser.

3.6.7 Moisture content analysis

Moisture content of the spray dried powder is important to take as the consideration to calculate the exact amount of sample needed for the comparison with initial solution. Moisture content was measured by using a moisture analyser (AND MS-70, Japan). The sample was heated at 105˚C then the initial and final mass will be calculated automatically by the moisture content analyser on the spray dried powder samples. Final reading shown on the equipment on the moisture content had been recorded.

3.7 Statistical Analysis

Each test had been repeated triplicated with a new batch of *P. niruri* and the data analysis of variance (ANOVA) was performed by in Microsoft Excel 2010 and tested a least significant difference (LSD) to compare the means with a confidence interval of 95%. All the samples extract was well preserved under -80°C in the freezer.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Overview

This chapter presents all the results and discussion of this research study regarding the extraction of polyphenols from *Phyllanthus Niruri* by using ultrasonic assisted extraction (UAE) and microwave assisted extraction (MAE). Solvents were assessed for its extraction capability by determine the yield of active components extraction, antioxidant activity, total phenolic content and total flavonoid content. Bioactive components (phyllanthin, gallic acid and quercetin) were studied and analysed by UPLC. After the suitable solvent was identified, effect of solvent ratio, extraction time and ultrasonic amplitude were further study by response surface methodology to determine the optimum setting. The optimum of solvent ratio, extraction time and microwave power will also being studied and identified at the end of this chapter.

4.2 Introduction

Phyllanthus Niruri contains a lot active components such as phyllanthin, gallic acid and quercetin which are beneficial toward the human genitourinary system. It can be recovered via extraction. Different solvent, method and condition yields different amount of bioactive components extracted.

Extraction process required solvent in the solid for the dissolution of the solute, hence, solvent acts an important player during the extraction process to obtain a complete extraction with high yield. In the extraction process, solvent will diffuse into the inner surface of the plant material (solid) to solubilize the compounds with the similar polarity (Ncube et al., 2008). Extract may widely vary with respect to its phenolic, flavonoid

and antioxidant activity by various solvent. Hence, the combined effect of different extraction method and solvent was one of the aims of this research study.

4.3 UPLC Quantification of polyphenol

The bioactive components from *P. niruri* were identified by means of the retention time and UV spectra of the authentic standard (phyllanthin, gallic acid and quercetin). From chromatogram in Figure 4.1, all the bioactive components were well separated within 16 minutes retention time. Figure 4.2 to Figure 4.4 shows that the UV spectra of the three target compounds in extracts and standard is well matched. The retention time where the peak of the target compound is found also match well. Therefore, confirming the presence of phyllanthin, gallic acid and quercetin in the extracts. The active components were quantified by comparing the peak areas of the sample with the results of a calibration series developed using the external standard. The calibration curves of the studied phenolic compounds showed good linearity where $R^2 > 0.997$ in the range of 0.08 – 500 µg/L concentration. The UPLC method developed for the first time in this study was capable of producing a faster and accurate qualitative and quantitative analysis of polyphenols from *P. niruri* extract. The time of analysis was about 16 minutes as shown in Figure 4.1, which is over twice faster than other reported methods, e.g. 38 min reported by both Murugaiyah & Chan (2007) and Sousa et al. (2016).

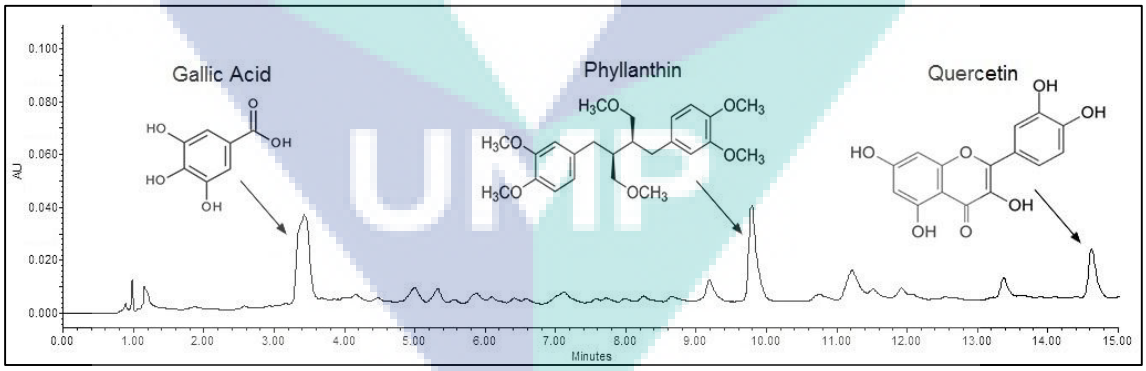


Figure 4.1 UPLC chromatogram of *P. niruri* extract and chemical structures of the markers.

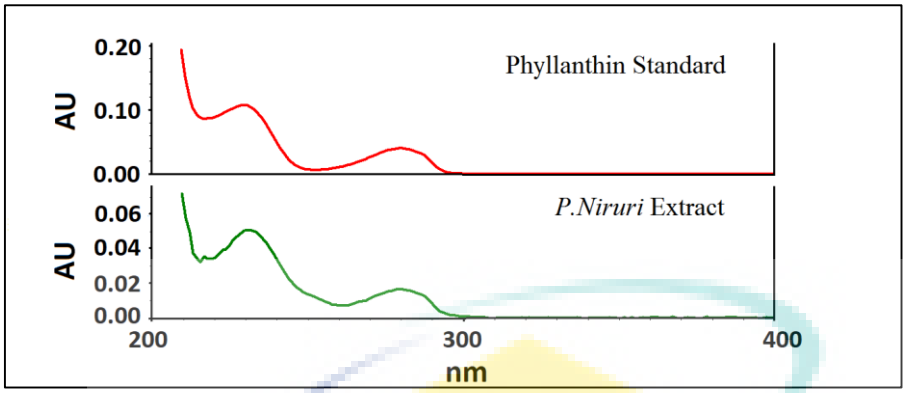


Figure 4.2 Identification of phyllanthin by matching UV spectra of sample to standard in Empower software library.

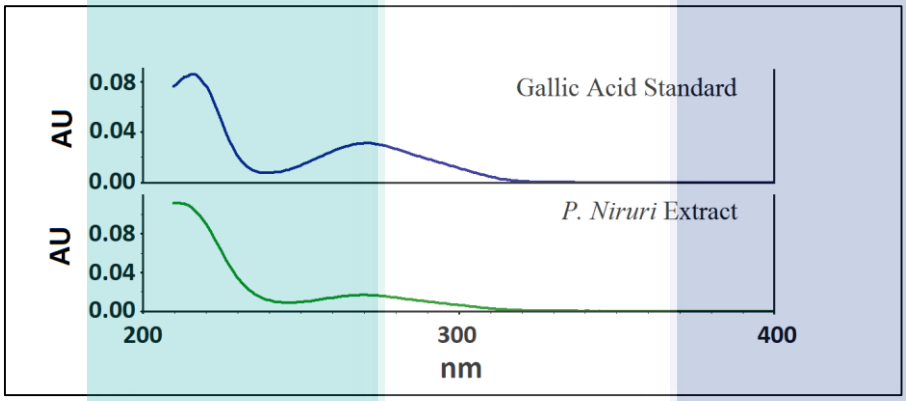


Figure 4.3 Identification of gallic acid by matching UV spectra of sample to standard in Empower software library.

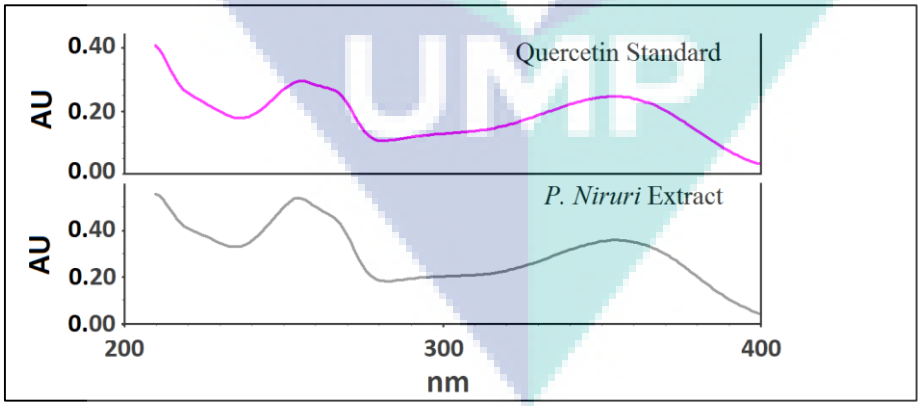


Figure 4.4 Identification of quercetin by matching UV spectra of sample to standard in Empower software library.

4.4 Influence of solvent type to the polyphenols extraction

Solvent type is the one of the factor affecting the extraction of the polyphenol. The yield of polyphenol extraction using solvent of different polarities such as water, 20% ethanol, 40% ethanol, 60% ethanol, 80% ethanol, 100% ethanol, 20% isopropanol, 40% isopropanol, 60% isopropanol, 80% isopropanol and 100% isopropanol was studied for UAE. In order to get a fair comparison, all extraction was carried out at equivalent solid to solvent ratio. Solvent polarity and its structural characteristic affect the solubility of bioactive component in a different solvent. It was found that highly methoxylated compounds such as quercetin, which is a lipophilic compound shows good stability in lower polarity solvent (Cutoto etal., 2013 & Ghosal et al., 2013). Similarly, Annamalai & Lakshimi, (2009) reported that the amount of sinensetin and eupatorin, which is highly methoxylated compounds, found to be extracted at higher extracted amount at lower polarity solvent, isopropanol extract.

On the other hand, a highly hydroxylated compounds such as gallic acid, which is hydrophilic is much easily soluble in water. Result from this work shows that gallic acid has the higher extraction yield in water compared to quercetin. The result in Table 4.1 shows that aqueous alcoholic solvent (20% of either ethanol and isopropanol) has a higher simultaneous extraction yield of phyllanthin, (4.41 mg Phy/g DW), gallic acid (13.19 mg GA/g DW) and quercetin (10.14mg Que/g DW) compared to pure solvent such as 100% ethanol, 100% isopropanol and pure water. The results suggest that polarity of the solvents used affect the efficiency of the polyphenol extraction. Result shows that, a mixture of lower and high polarity solvent produced a higher extraction yield of both target components. For instance, solvent with a lower polarity index such as the isopropyl alcohol has a better efficiency to extract a wider range of phenolic content Poh-Hwa et al., (2011). The major components present in Phyllanthus species are active hydrolysable tannins that can be extracted using the ethanol-water mixture as the components are semipolar compounds such as ellagitannins and gallotannins from Tian et al., (2017), which is in good agreement to the result obtained in this work.

Table 4.1 Effect of solvent on polyphenols extraction from *Phyllanthus Niruri.*

Solvent Type	Total Phenolic content(mg GAE/g DW)	Total Flavonoid content (mg QE/g DW)	Antioxidant activity (mg/g)	Bioactive component		
				Phyllanthin (mg Phy/g DW)	Gallic Acid (mg GAE/g DW)	Quercetin (mg Que/g DW)
Ethanol	23.481±0.078	32.172±0.369	69.061±3.428	4.431±0.146	0.992±0.088	7.349±0.730
Isopropanol	5.740±0.589	12.303±0.056	11.403±0.966	4.313±0.043	1.226±0.011	8.557±0.843
Water	33.432±1.082	38.390±1.742	118.835±4.221	0.551±0.290	15.445±2.436	3.191±0.539
20% Ethanol	40.264±0.461	49.579±1.041	115.290±2.106	4.410±0.038	13.195±0.368	10.124±4.519
40% Ethanol	42.549±0.943	60.741±0.214	127.853±5.226	4.415±0.132	9.860±0.125	5.483±0.314
60% Ethanol	40.631±0.215	53.302±1.094	123.347±5.662	4.452±0.050	9.201±0.102	6.124±0.206
80% Ethanol	37.513±0.739	48.211±0.942	121.095±8.494	4.513±0.196	4.422±0.221	6.746±1.148
20% Isopropanol	42.968±0.128	60.748±3.159	135.265±7.334	4.561±0.388	3.061±0.336	9.107±2.545
40% Isopropanol	44.551±0.078	61.990±1.609	145.417±0.738	4.344±0.013	2.674±0.323	8.509±1.437
60% Isopropanol	41.380±1.797	57.029±1.004	123.341±4.567	4.339±0.014	2.341±1.190	9.632±1.128
80% Isopropanol	33.121±0.63	49.575±0.672	99.340±2.690	4.308±0.083	2.218±0.139	8.453±0.455

4.5 Factorial Analysis on Ultrasonic Assisted Extraction (UAE)

2^{4-1} factorial design with three parameters were studied for UAE. 8 experiments were tabulated for UAE factorial design. Fractional factorial experimental design and the result for UAE was tabulated in Table 4.2. Response was analysed by examining the model fitting, interpreting the model graphically, finding the optimum point, and performing a model validation.

4.5.1 Effect of Solvent purity, Time and Amplitude on UAE

The effect of solvent concentration, time and amplitude on the polyphenol extraction yield is summarized in Table 4.2. The solvent concentration ranged from 20% to 80%, time ranged from 3 minutes to 9 minutes and amplitude ranged from 20% to 90% were studied in the factorial design.

Table 4.2 Experimental design and response for factorial analysis of UAE.

		Factors			Responses					
Standard	Run	EtOH Purity %	Time Minutes	Amplitude %	Total Phenolic Content mg GAE/g DW	Total Flavonoid content mg QE/g DW	Antioxidant Activity mg/g DW	Phyllanthin mg Phy/g DW	Gallic Acid mg GAE/g DW	Quercetin mg Que/g DW
8	1	80	9	90	32.074	41.496	4.552	80.842	4.009	97.825
4	2	80	9	20	22.420	27.850	3.094	54.790	2.803	84.179
5	3	20	3	90	30.271	31.944	5.443	80.063	3.784	88.273
1	4	20	3	20	21.373	22.391	3.140	42.704	2.672	78.720
3	5	20	9	20	28.992	37.402	5.245	80.211	3.624	93.731
7	6	20	9	90	36.960	45.590	7.596	92.638	4.620	101.919
2	7	80	3	20	13.638	12.839	1.528	30.724	1.705	69.168
6	8	80	3	90	25.502	27.850	2.810	67.953	3.188	84.179

Table 4.3 to Table 4.8 show the percentage contributions of each factor on the yield of extraction for total phenolic content, total flavonoid content, antioxidant activity, phyllanthin, gallic acid and quercetin. The main factor A, B and C played the major contribution in the gallic acid and quercetin extraction, which contributes more than 90% compared to the interactive factors. The suggested best condition for main factor in UAE to maximize total phenolic content, total flavonoid content, antioxidant activity, phyllanthin, gallic acid and quercetin yield are at 20% ethanol concentration, 9 minutes

extraction time and 90% amplitude. The optimum condition was achieved by setting the ethanol concentration, time and amplitude in range, whereas maximising the total pheolic content, total flavonoid content, antioxidant activity, phyllanthin, gallic acid and quercetin yield. The desirability of the optimum solution is 0.997, which is closer to the maximum value of 1.0, indicating that the solution is close to the optimum condition for phyllanthin, gallic acid, quercetin, total phenolic content, total flavonoid content and antioxidant activity extraction. Three experimental runs were performed on the optimum point obtained from the two-level factorial study. It was found that the predicted (from two-level factorial) and actual response (experiment) are in good agreement with deviation of less than 10%. The result indicates that the optimisation model based on two-level factorial study is sufficiently accurate to predict the phyllanthin, gallic acid, quercetin, total phenolic content, total flavonoid content and antioxidant activity extraction yield. It was found from 2LF analysis that, none of the factors A, B, and C has a combined interactive effect on the yield of total phenolic content, total flavonoid content, antioxidant activity, phyllanthin, gallic acid and quercetin.

Table 4.3 Sum of squares and the percent contribution for each term for Phyllanthin.

Term	Sum of Squares	% Contribution
A-EtOH Purity	1.118818	19.666
B-Time	1.67572	29.454
C-Amplitude	2.82252	49.612
AB	0.002254	0.040
AC	0.042778	0.752
BC	0.024066	0.423

Table 4.4 Sum of squares and the percent contribution for each term for Gallic acid.

Term	Sum of Squares	% Contribution
A-EtOH Purity	11.140	43.440
B-Time	7.160	27.830
C-Amplitude	6.840	26.590
AB	0.110	0.440
AC	0.460	1.780
BC	0.006	0.024

Table 4.5 Sum of squares and the percent contribution for each term for Quercetin.

Term	Sum of Squares	% Contribution
A-EtOH Purity	469.82	14.48
B-Time	946.94	29.18
C-Amplitude	1597.99	49.23
AB	21.54	0.66
AC	22.77	0.70
BC	162.99	5.02

Table 4.6 Sum of squares and the percent contribution for each term for Total Phenolic Content.

Term	Sum of Squares	% Contribution
A-EtOH Purity	73.8677	20.059
B-Time	107.0918	29.080
C-Amplitude	182.7852	49.635
AB	0.158725	0.043
AC	3.098404	0.841
BC	1.020561	0.277

Table 4.7 Sum of squares and the percent contribution for each term for Total Flavonoid Content.

Term	Sum of Squares	% Contribution
A-EtOH Purity	93.11	11.81
B-Time	410.62	52.07
C-Amplitude	269.09	34.12
AB	0.1725	0.018
AC	14.90	1.89
BC	0.93	0.12

Table 4.8 Sum of squares and the percent contribution for each term for Antioxidant Activity.

Term	Sum of Squares	% Contribution
A-EtOH Purity	71.09	19.390
B-Time	108.93	29.710
C-Amplitude	182.43	49.750
AB	0.14	0.037
AC	2.68	0.730
BC	1.22	0.330

Table 4.9 Suggested best condition for factors in UAE for maximizing all responses.

Factors	Phyllanthin	Gallic Acid	Quercetin	Total Phenolic Content	Total Flavonoid Content	Antioxidant Activity
A-Ethanol Purity (%)	20	20	20	20	20	20
B-Time (Min)	9	9	9	9	9	9
C-Amplitude (%)	90	90	90	90	90	90

4.5.2 Optimization on the polyphenol extraction

A CCD with a total of 20 experiments that include 7 runs for factorial design, 7 runs for axial points and 6 repetitive runs at the central point were performed. The CCD experimental design and responses is shown in Table 4.10. The values of regression coefficients were calculated, the response variable and the test variables were fitted to the second-order polynomial equation. The model equation in coded form is given as follows:

second-order polynomial equation. The model equation in coded form is given as follows:

$$Phyllanthin = 4.25 + 0.33 * A + 0.33 * B + 0.00760 * C - 0.00023 * A * B - 0.031 * A * C + 0.092 * B * C - 0.033 * A^2 + 0.055 * B^2 + 0.021 * C^2 \quad (4.1)$$

$$Gallic\ Acid = 7.75 + 0.65 * A + +1.11 * B + 0.30 * C + 0.33 * A * B - 0.18 * A * C + 0.26 * B * C + 0.25 * A^2 + 0.29 * B^2 - 0.34 * C^2 \quad (4.2)$$

$$Quercetin = 13.59 + 1.60 * \mathrm{A} + 0.44 * \mathrm{B} - 0.25 * \mathrm{C} + 0.43 * A * B - 0.53 * A * C - 0.67 * B * C - 0.095 * -0.25 * B^2 + 0.056C^2 \quad (4.3)$$

$$Total\ Phenolic\ Content = 42.08 + 3.09 * A + 3.40 * B - 0.082 * C - 0.27 * A * B - 0.036 * A * C + 0.63 * B * C - 0.47 * A^2 + 0.39 * B^2 + 0.06 * C^2 \quad (4.4)$$

$$Total\ Flavonoid\ Content = 69.24 + 3.44 * A + 3.75 * B - 0.11 * C - 0.30 * A * B - 0.040 * A * C + 0.70 * B * C - 0.65 * A^2 + 0.27 * B^2 - 0.12 * C^2 \quad (4.5)$$

$$Antioxidant\ Activity = 93.49 + 11.00 * A + 2.99 * B - 1.75 * C + 2.98 * A * B - 3.62 * A * C - 4.62 * B * C - 0.65 * A^2 - 1.75 * B^2 + 0.38 * C^2 \quad (4.6)$$

Table 4.10 Experimental design and response for optimization.

Standard	Run	Ethanol Purity (%)	Time (min)	Amplitude (%)	Phyllanthin (mg Phy/g DW)	Gallic Acid (mg GAE/g DW)	Quercetin (mg Que/g DW)	Total Phenolic Content (mg GAE/g DW)	Total Flavonoid Content (mg QE/g DW)	Antioxidant Activity (mg/g)
16	1	48.52	11.00	82.50	4.446	8.207	14.996	44.462	64.585	103.091
20	2	15.00	15.00	75.00	4.231	7.214	14.569	42.310	62.433	74.894
11	3	27.50	11.00	95.11	3.841	6.114	14.043	38.414	58.537	88.443
15	4	27.50	11.00	82.50	3.859	7.524	19.092	38.588	58.711	90.640
4	5	27.50	17.73	82.50	4.696	10.254	16.139	46.963	67.086	89.908
1	6	40.00	7.00	75.00	4.266	8.125	14.954	42.659	62.782	91.739
7	7	27.50	11.00	82.50	4.690	7.414	16.994	46.905	67.028	91.739
2	8	27.50	11.00	82.50	4.115	8.820	18.645	41.147	61.270	96.133
19	9	27.50	11.00	69.89	4.458	6.365	14.741	44.578	64.701	104.189
14	10	15.00	15.00	90.00	4.440	8.781	14.269	44.404	64.527	80.753
13	11	27.50	4.27	82.50	3.789	5.786	15.053	37.890	58.013	90.640
5	12	27.50	11.00	82.50	4.103	8.082	19.616	41.031	61.154	95.767
9	13	27.50	11.00	82.50	4.283	7.937	17.875	42.834	62.957	92.837
8	14	40.00	7.00	90.00	4.208	7.948	18.433	42.078	62.201	101.626
10	15	15.00	7.00	75.00	3.370	6.044	14.635	33.703	53.826	68.303
17	16	6.48	11.00	82.50	3.551	7.598	18.422	35.506	55.629	83.683
6	17	27.50	11.00	82.50	4.225	6.898	18.388	42.252	62.375	93.204
12	18	15.00	7.00	90.00	3.638	7.465	12.931	36.378	56.501	78.190
18	19	40.00	15.00	75.00	4.708	9.737	18.113	47.079	67.202	124.696
3	20	40.00	15.00	90.00	5.214	11.500	15.711	52.139	72.262	101.626

4.5.2.1 Effect of ethanol purity, extraction time and amplitude in polyphenol extraction

The effect of the three factors, i.e., ethanol concentration, extraction time and amplitude on total phenolic content, total flavonoid content, antioxidant activity, phyllanthin, gallic acid and quercetin extraction were analysed using RSM. Contour plot were generated to study the interactive effect of the variables to the response. The effect of non-interaction factors ethanol concentration (A), time (B) and amplitude (C) on polyphenol extraction is depicted in Figure 4.6 for total phenolic content, total flavonoid content antioxidant activity, phyllanthin, gallic acid and quercetin. The result obtained from CCD study agrees to the two-level factorial analysis.

The relationship between the response and experimental variables is shown in Figure 4.6. It can be observed that longer extraction time (>13 min) and higher sonication amplitude (ranging from 80 to 87%) gave a higher yield of gallic acid. Higher ethanol concentration (40%) is also favorable for gallic acid extraction. Similarly, Phyllanthin also getting the same trend as gallic acid where longer extraction time (>13min) and ethanol concentration at 40% and high amplitude (90%) is favourable for phyllanthin extraction. Quercetin extraction also favors longer extraction time (>13 min) and higher ethanol concentration (40%), in a similar manner to that of gallic acid. However, the best sonication amplitude for quercetin extraction is lower (75 to 78%) than that of gallic acid. Earlier, Nguang et al., (2017) also found that 40% aqueous ethanol yielded the highest yield of total polyphenol and total flavonoid from *P. niruri*.

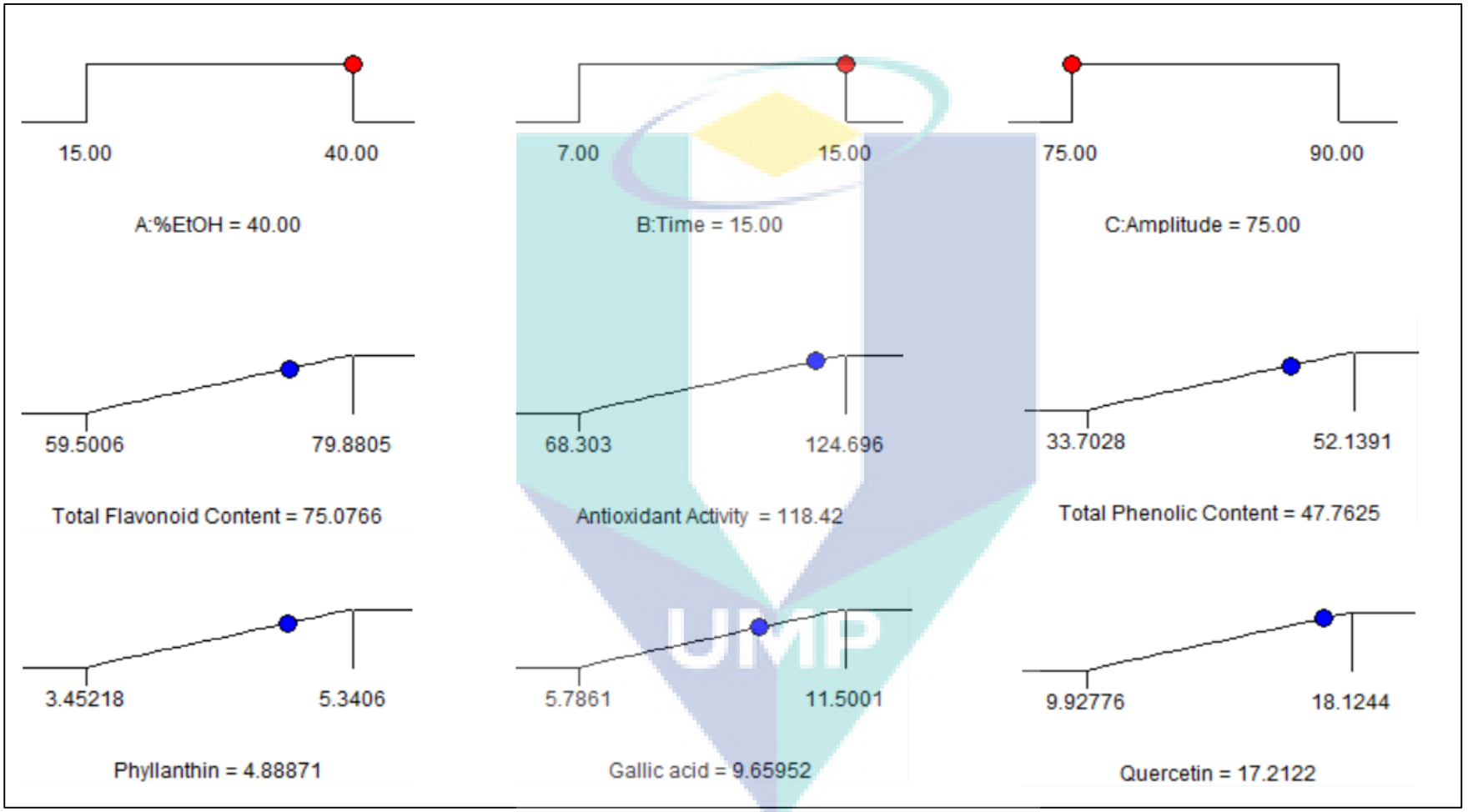


Figure 4.5 Correlation of range of variables and predicted optimum condition for simultaneous maximum polyphenols recovery using the CCD model.

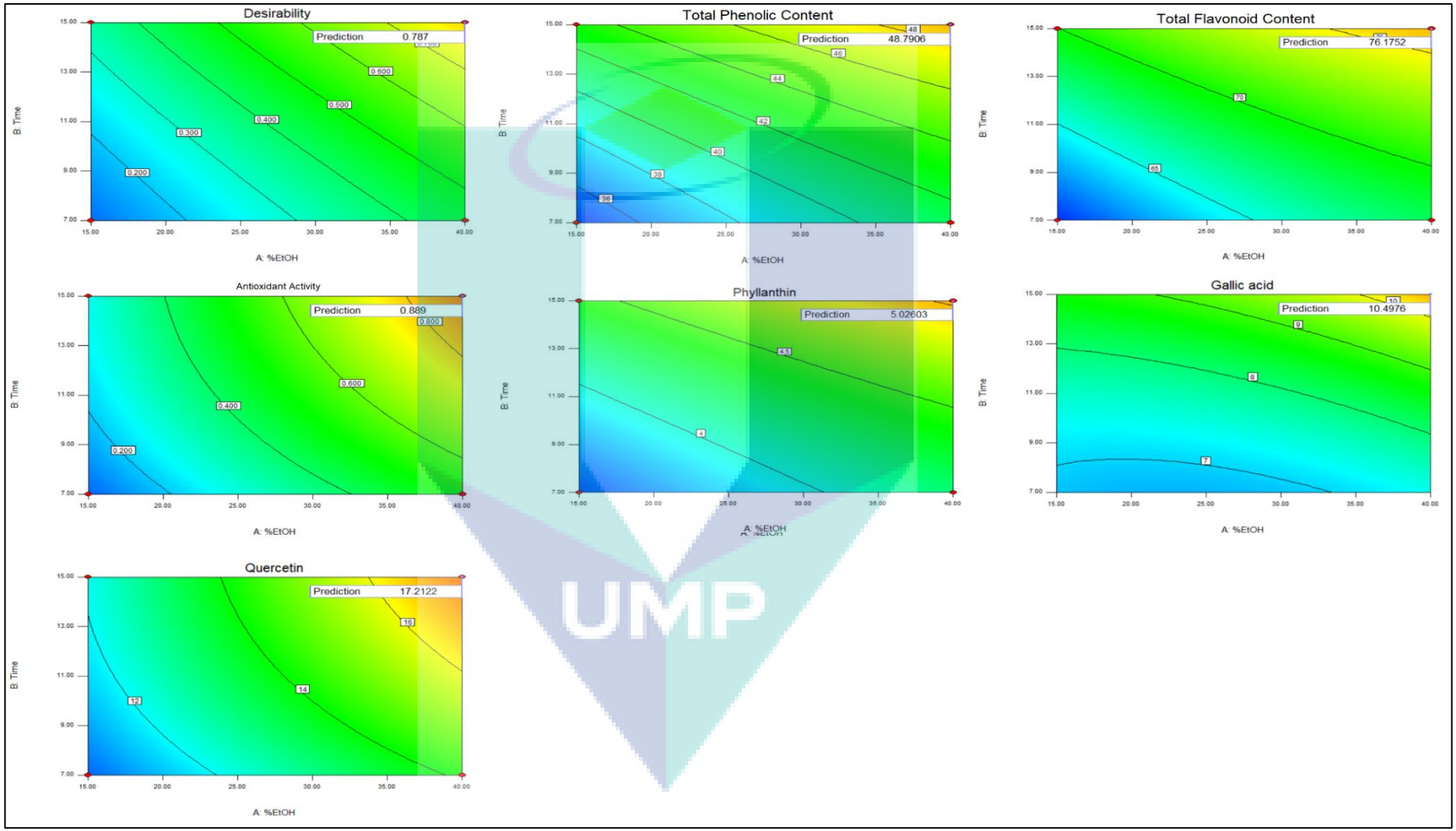


Figure 4.6 Predicted optimum condition for simultaneous maximum polyphenols recovery using CCD mode.

The optimum parameter to obtain the highest yield of phyllanthin, gallic acid and quercetin was determined from the model equation with the condition of all the parameters is kept in range. Only one response is optimized at a time. The optimum condition for phyllanthin, total phenolic content and total flavonoid content extraction was found in 40% ethanol concentration, extraction time of 15 minutes and high amplitude of 90% with the yield of 5.026mg Phy/g DW, 48.791mg GAE/g DW and 76.175mg QE/g DW respectively. The optimum condition for gallic acid extraction was found in ethanol concentration of 40%, extraction time of 15 min and sonication amplitude of 86.85% with the yield of gallic acid of 10.494 GA/g DW as shown in Table 4.11. Meanwhile, the optimum condition for quercetin and antioxidant activity extraction was found in ethanol concentration of 40%, extraction time of 15 min and sonication amplitude of 75% with the yield of gallic acid of 17.212 Que/g DW. The desirability for all the response optimization is close to unity with the value of 0.787. The model is considered good since the desirability is close to 0.8.

The suitability of the model equation to predict the desired response (phyllanthin, gallic acid, quercetin, total phenolic content, total flavonoid content and antioxidant activity yield) is tested experimentally using the optimum conditions described in Table 4.11. The result of the verification using triplicate run is presented in Table 4.12. The deviation between the predicted and measured responses was ranging from 0.139% to 4.742%. The experimental values were in good agreement with the predicted values of the model with an error less than 5%, which proved adequacy of the model for predicting the optimum yield of all the response from UAE.

Table 4.11 Condition for factors optimizing polyphenol extraction.

Factor	Phyllanthin	Gallic Acid	Quercetin	Total Phenolic Content	Total Flavonoid Content	Antioxidant Activity
Ethanol Purity (%)	40	40	40	40	40	40
Time (min)	15	15	15	15	15	15
Amplitude (%)	90	86.85	75	90	90	75

Table 4.12 Comparison between predicted and experimental value for optimum condition.

Response		Predicted Value	Experimental Value	Error
Phyllanthin	Run 1	5.026	5.241	4.102
	Run 2	5.026	4.999	0.540
	Run 3	5.026	5.105	1.548
Gallic Acid	Run 1	10.498	10.103	3.910
	Run 2	10.498	10.706	1.943
	Run 3	10.498	10.468	0.287
Quercetin	Run 1	17.212	17.524	1.780
	Run 2	17.212	17.692	2.713
	Run 3	17.212	17.236	0.139
Total Phenolic Content	Run 1	48.791	51.139	4.591
	Run 2	48.791	46.582	4.742
	Run 3	48.791	50.139	2.689
Total Flavonoid Content	Run 1	76.175	79.881	4.639
	Run 2	76.175	79.535	4.225
	Run 3	76.175	77.024	1.102
Antioxidant Activity	Run 1	118.42	123.696	4.265
	Run 2	118.42	119.203	0.657
	Run 3	118.42	120.809	1.978

4.6 Factorial Analysis on Microwave Assisted Extraction (MAE)

2^{5-1} factorial design with three parameters were studied for MAE. 16 experiments were tabulated for MAE factorial design. Fractional factorial experimental design and the result for MAE was tabulated in Table 4.13. Response was analysed by examining the model fitting, interpreting the model graphically, finding the optimum point, and performing a model validation.

4.6.1 Effect of Time, Power, Solvent purity and Solid Liquid Ratio on MAE.

The effect of extraction time, power, ethanol concentration and solid liquid ratio on the polyphenol extraction yield is summarized in Table 4.13. Extraction time ranged from 1 minute to 6 minutes, power ranged from 30 W to 250 W, solvent concentration ranged from 30% to 80%, and solid liquid ratio ranged from 0.025g/ml to 0.200g/ml were studied in the factorial design.

Table 4.13 Experimental design and response for MAE factorial analysis.

Factors						Responses					
Standard	Run	Time	Power	EtOH purity	Solid Liquid ratio	Total Phenolic Content	Total Flavonoid content	Antioxidant Activity	Phyllanthin	Gallic Acid	Quercetin
		minutes	W	%	g/ml	mg GAE/g DW	mg QE/g DW	mg/g	mg Phy/g DW	mg GAE/g DW	mg Que/g DW
4	1	6	250	30	0.025	16.197	17.877	40.694	4.049	3.239	7.989
5	2	1	30	80	0.025	6.485	5.595	18.533	1.621	1.297	3.198
11	3	1	250	30	0.200	79.299	155.704	195.437	19.825	15.860	39.112
10	4	6	30	30	0.200	75.461	118.859	162.196	18.435	15.092	37.219
15	5	1	250	80	0.200	86.511	146.152	183.592	21.628	17.302	42.669
16	6	6	250	80	0.200	67.726	110.671	138.889	16.952	13.545	33.403
6	7	6	30	80	0.025	11.661	13.783	31.906	2.915	2.332	5.751
2	8	6	30	30	0.025	8.753	8.324	23.882	2.188	1.751	4.317
13	9	1	30	80	0.200	29.748	45.169	72.407	7.437	5.950	14.672
9	10	1	30	30	0.200	64.585	91.567	133.158	16.146	12.917	31.854
7	11	1	250	80	0.025	17.186	24.700	47.189	4.296	3.437	8.476
1	12	1	30	30	0.025	16.255	16.512	42.222	4.064	3.251	8.017
14	13	6	30	80	0.200	92.327	143.422	192.762	23.082	18.465	45.537
12	14	6	250	30	0.200	81.335	120.224	173.276	20.334	16.267	40.116
3	15	1	250	30	0.025	12.824	16.512	36.491	3.206	2.565	6.325
8	16	6	250	80	0.025	18.582	26.064	47.189	4.645	3.716	9.165

Table 4.14 to Table 4.19 show the percentage contributions of each factor on the yield of extraction for total phenolic content, total flavonoid content, antioxidant activity, phyllanthin, gallic acid and quercetin. The main factor D played the major contribution in the response from the extraction, which contributes more than 80% compared to the interactive factors. The suggested best condition for main factor in MAE to maximize total phenolic content, total flavonoid content, antioxidant activity, phyllanthin, gallic acid and quercetin yield are at 250 W power and 0.2g/ml solid to liquid ratio. 30% ethanol concentration and 1 minute extraction time is the suggested best condition for gallic acid, total flavonoid content and antioxidant activity. On the other hand, 80% ethanol concentration and 6 minutes extraction time is suggested best condition for phyllanthin, quercetin and total phenolic content. The optimum condition was achieved by setting the ethanol concentration, time and amplitude in range, whereas maximising the total phenolic content, total flavonoid content, antioxidant activity, phyllanthin, gallic acid and quercetin yield. The desirability of the optimum solution is 0.917, which is closer to the maximum value of 1.0, indicating that the solution is close to the optimum condition for phyllanthin, gallic acid, quercetin, total phenolic content, total flavonoid content and antioxidant activity extraction. Three experimental runs were performed in Table 4.20 on the optimum point obtained from the two-level factorial study. It was found that the predicted (from two-level factorial) and actual response (experiment) are in good agreement with deviation of less than 10%. The result indicates that the optimisation model based on two-level factorial study is sufficiently accurate to predict the phyllanthin, gallic acid, quercetin, total phenolic content, total flavonoid content and antioxidant activity extraction yield. It was found from 2LF analysis that, none of the factors A, B, C and D has a combined interactive effect on the yield of total phenolic content, total flavonoid content, antioxidant activity, phyllanthin, gallic acid and quercetin.

Table 4.14 Sum of squares and the percent contribution for each term for phyllanthin

Term	Sum of Squares	% Contribution
A-Extraction Time	12.9200	1.259773
B-Power	22.6746	2.210898
C-Ethanol Purity	2.0094	0.19593
D-Solid liquid ratio	853.4142	83.21233
AB	25.8219	2.517773
AC	7.3533	0.71699
AD	10.8182	1.054839
BC	2.1657	0.211176
BD	4.2329	0.412739
CD	1.9684	0.191932

Table 4.15 Sum of squares and the percent contribution for each term for gallic acid.

Term	Sum of Squares	% Contribution
A-Extraction Time	2.409979	0.544241
B-Power	8.310171	1.876671
C-Ethanol Purity	5.91481	1.335731
D-Solid liquid ratio	382.0932	86.28744
AB	6.20842	1.402037
AC	5.448083	1.230331
AD	1.711244	0.386447
BC	1.412837	0.319059
BD	0.517308	0.116823
CD	5.858368	1.322985

Table 4.16 Sum of squares and the percent contribution for each term for quercetin.

Term	Sum of Squares	% Contribution
A-Extraction Time	49.13335	1.33361
B-Power	79.21287	2.150049
C-Ethanol Purity	6.948258	0.188594
D-Solid liquid ratio	3079.304	83.58053
AB	100.1029	2.717061
AC	33.87141	0.919361
AD	41.10881	1.115803
BC	7.75302	0.210438
BD	12.99206	0.352639
CD	6.798969	0.184542

Table 4.17 Sum of squares and the percent contribution for each term for total phenolic content.

Term	Sum of Squares	% Contribution
A-Extraction Time	218.6492	1.323965
B-Power	345.8176	2.093996
C-Ethanol Purity	37.46883	0.226881
D-Solid liquid ratio	13750.38	83.26136
AB	431.6884	2.613962
AC	108.0732	0.654405
AD	184.0218	1.114289
BC	39.63526	0.24
BD	60.5081	0.366389
CD	36.76022	0.222591

Table 4.18 Sum of squares and the percent contribution for each term for total flavonoid content.

Term	Sum of Squares	% Contribution
A-Extraction Time	205.3089	0.414786
B-Power	1906.905	3.852521
C-Ethanol Purity	56.33191	0.113807
D-Solid liquid ratio	40240.54	81.29797
AB	2346.852	4.741347
AC	476.7262	0.96313
AD	168.0646	0.339541
BC	37.70979	0.076185
BD	538.1792	1.087284
CD	168.0646	0.339541

Table 4.19 Sum of squares and the percent contribution for each term for antioxidant activity.

Term	Sum of Squares	% Contribution
A-Extraction Time	417.8507	0.59169
B-Power	2155.094	3.05168
C-Ethanol Purity	350.5143	0.496339
D-Solid liquid ratio	58034.17	82.1782
AB	2680.354	3.795465
AC	579.4216	0.820479
AD	433.6173	0.614016
BC	17.6644	0.025013
BD	357.7041	0.50652
CD	379.7116	0.537684

Factor D, solid liquid ratio plays major contribution toward the MAE extraction for all the responses. The decrease of the solid liquid ratio decreases the yield of the responses. This phenomenon can be explained by turmeric power which contribute mainly on the maximizing and also allows the transmission of microwave energy. Thus, it leads an effective dissolution of polyphenols. When the amount of the plant powder increases, the more the bioactive components come in contact with the solvent. Therefore, higher leaching out rates during the extraction process which increase the yield of responses. When the solid liquid ratio reaching its saturation phase, the cellular diffusion phase stops and the stabilise rate of extracted compounds resulting the decrease of the extraction yield. According to Xi (2009), it is consistent with mass transfer principles whereby the driving force during mass transfer is the concentration gradient between the solid and the bulk of the liquid, which is greater when a higher solvent-to solid ratio is used.

Solid liquid ratio plays important role in determining the extraction yield from the plant. Higher solid liquid ratio means more source of the plant material provided for the extraction process. More solid material, required adequate volume of liquid solvent for the extraction in order for complete solvent diffusion. From the previous researcher, Sousa et al. (2016), they found that at the higher liquid solid ratio (40mL/g) able to yield 27mg/g TPC, which is approximately 7mg/g TPC higher at the liquid solid ratio at 20mL/g. With the increasing of the solid material, liquid solvent required to increase so

that more diffusion of the liquid solvent into the cell. Sousa et al. (2016) also mention that at the relevant volume of the liquid solvent, it allowed the complete diffusion which also improve the permeation of the phenolic compounds. However, when the liquid solvent was further increase at the fixed solid amount, the polyphenol extraction will decrease. Same phenomena were observed by another researcher Wang et al. (2013).

Table 4.20 Condition for factors optimizing polyphenol extraction.

Factor	Phyllanthin	Gallic Acid	Quercetin	Total Phenolic Content	Total Flavonoid Content	Antioxidant Activity
Ethanol Purity (%)	40	40	40	40	40	40
Time (min)	15	15	15	15	15	15
Amplitude (%)	90	86.85	75	90	90	75

4.6.2 Optimizing on polyphenol extraction by MAE

A CCD with a total of 20 experiments which include 7 runs for factorial design, 7 runs for axial points and 6 repetitive runs at the central point were performed. The CCD experimental design and responses is shown in Table 4.21. The values of regression coefficients were calculated, the response variable and the test variables were fitted to the second-order polynomial equation. The model equation in coded form is given as follows:

$$Phyllanthin = 5.03 - 0.21 * A + 0.31 * B + 0.35 * C + 0.004 * A * B + 0.077 * A * C + 0.022 * B * C - 0.82 * A^2 - 0.19 * B^2 - 0.029 * C^2 \quad (4.7)$$

$$Gallic\ Acid = 8.25 - 2.63 * A + 2.32 * B + 2.88 * C - 0.90 * A * B - 1.11 * A * C + 0.83 * B * C + 1.02 * A^2 - 1.89 * B^2 - 2.37 * C^2 \quad (4.8)$$

$$Quercetin = 72.76 - 3.26 * A + 3.03 * B + 4.67 * C + 0.79 * A * B + 0.096 * A * C - 0.45 * B * C - 9.30 * A^2 - 2.47 * B^2 - 0.65 * C^2 \quad (4.9)$$

$$Total\ Phenolic\ Content = 61.95 - 3.21 * A + 3.14 * B + 4.98 * C + 0.73 * A * B + 0.16 * A * C - 0.52 * B * C - 10.19 * A^2 - 2.58 * B^2 - 0.25 * C^2 \quad (4.10)$$

$$Total\ Flavonoid\ Content = 92.65 + 6.70 * A + 3.72 * B + 10.05 * C - 4.03 * A * B + 5.27 * A * C - 5.89 * B * C - 8.01 * A^2 - 6.77 * B^2 + 3.78C^2 \quad (4.11)$$

$$Antioxidant\ Activity = 153.36 - 12.18 * A + 6.91 * B + 3.53 * C + 7.01 * Z * B + 9.54 * A * C - 2.30 * B * C - 14.23 * A^2 - 17.86 * B^2 + 1.47 * C^2 \quad (4.12)$$

Table 4.21 Experimental design and response for MAE optimization.

Standard	Run	Ethanol Purity (%)	Time (Min)	Power (W)	Total Phenolic Content (mg GAE/g DW)	Total Flavonoid Content (mg QE/g DW)	Antioxidant Activity (mg/g)	Gallic Acid (mg GAE/g DW)	Phyllanthin (mg Phy/g DW)	Quercetin (mg Que/g DW)
16	1	55	3.5	140	61.410	80.513	158.296	10.155	5.145	72.933
6	2	80	1	250	46.976	114.008	110.940	2.956	3.936	58.499
15	3	55	3.5	140	67.014	90.437	140.417	7.710	5.615	78.537
11	4	55	1	140	50.571	73.069	116.739	1.853	4.237	62.094
7	5	30	6	250	56.915	80.513	126.403	15.066	4.769	68.439
8	6	80	6	250	53.902	96.640	141.383	6.581	4.516	65.425
3	7	30	6	30	51.681	80.513	146.699	5.345	4.330	63.205
10	8	80	3.5	140	47.610	86.715	130.752	4.117	3.989	59.134
20	9	55	3.5	140	63.683	100.362	153.464	8.603	5.336	75.207
9	10	30	3.5	140	52.157	79.272	137.517	13.057	4.370	63.681
12	11	55	6	140	64.423	95.399	144.282	9.502	5.398	75.947
4	12	80	6	30	43.803	78.031	102.242	1.558	3.670	55.327
5	13	30	1	250	57.127	79.272	145.249	7.571	4.786	68.650
19	14	55	3.5	140	66.327	96.640	170.860	9.965	5.557	77.850
2	15	80	1	30	39.045	69.347	83.880	1.012	3.271	50.568
13	16	55	3.5	30	52.316	86.715	160.229	2.209	4.383	63.839
18	17	55	3.5	140	59.030	92.918	158.296	8.702	4.946	70.554
14	18	55	3.5	250	67.331	102.843	139.450	8.187	5.641	78.855
17	19	55	3.5	140	61.674	101.602	158.779	7.100	5.167	73.197
1	20	30	1	30	45.601	58.1828	135.101	1.444	3.820	57.125

4.6.2.1 Effect of polyphenol purity, power and extraction time on polyphenol extraction

The effect of the three factors, i.e., ethanol concentration, extraction time and power on total phenolic content, total flavonoid content, antioxidant activity, phyllanthin, gallic acid and quercetin extraction were analysed using RSM. Contour plot were generated to study the interactive effect of the variables to the response. The effect of non-interaction factors ethanol concentration (A), time (B) and power (C) on polyphenol extraction is depicted in Figure 4.8 for total phenolic content, total flavonoid content antioxidant activity, phyllanthin, gallic acid and quercetin. The result obtained from CCD study agrees to the two-level factorial analysis.

The relationship between the response and experimental variables is shown in Figure 4.8. It can be observed that extraction time and higher power (>200 W) gave a higher yield of phyllanthin, gallic acid, quercetin, total phenolic compound and total flavonoid compound. Higher ethanol concentration (>50%) is also favorable for phyllanthin, quercetin, total phenolic content and total flavonoid content extraction. Gallic acid and antioxidant activity extraction is favour in lower ethanol concentration (<40%). Similar results were reported by Li et al., (2017) on the antioxidant activity studied of microwave assisted extraction from Exotic Gordonia axillaris fruits where the optimum antioxidant capacity (TEAC) values increased to 198.16μmol Trolox/g DW at 36.89% ethanol concentration.

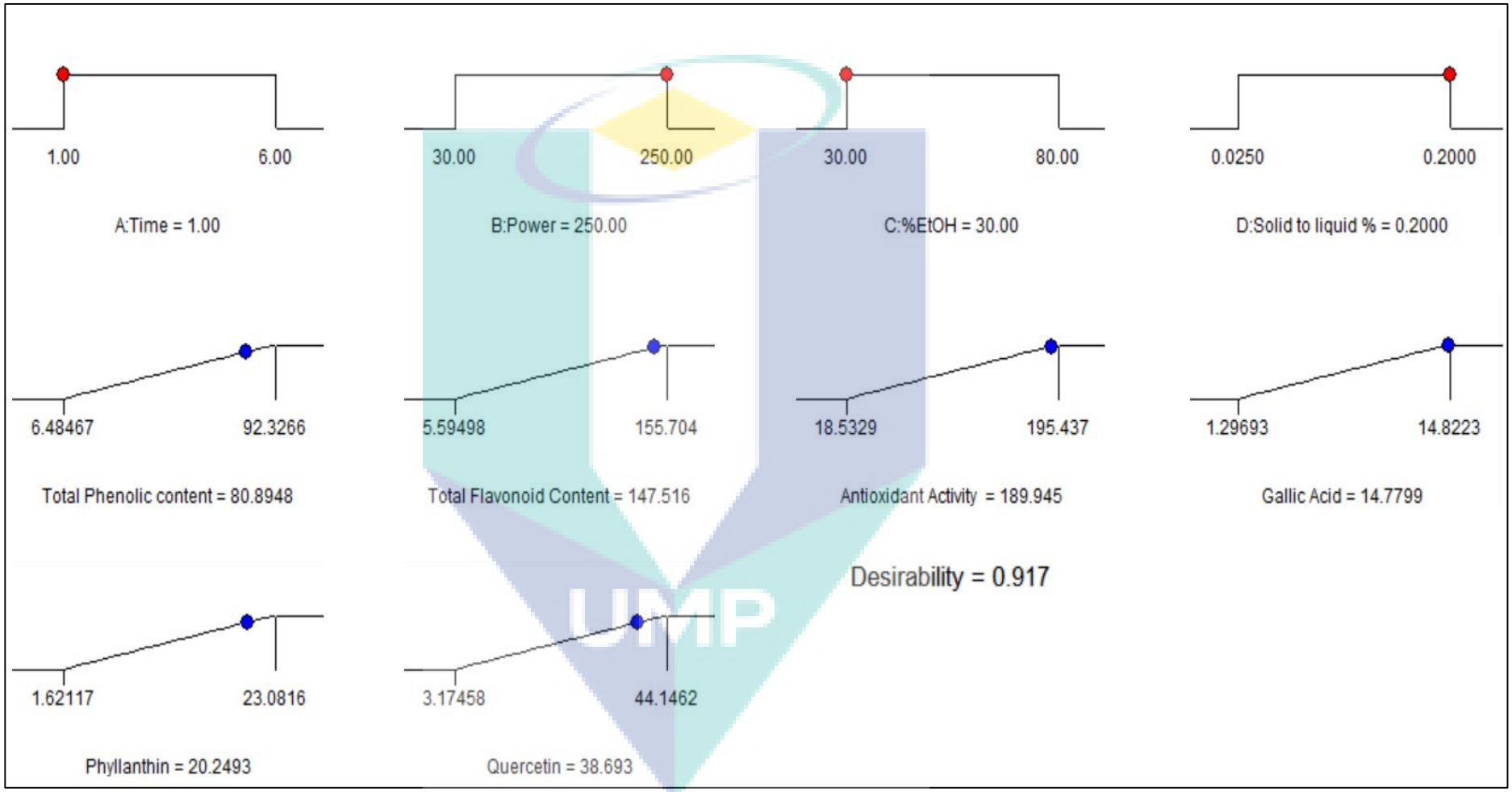


Figure 4.7 Correlation of range of variables and predicted optimum condition for simultaneous maximum polyphenols recovery using the CCD model.

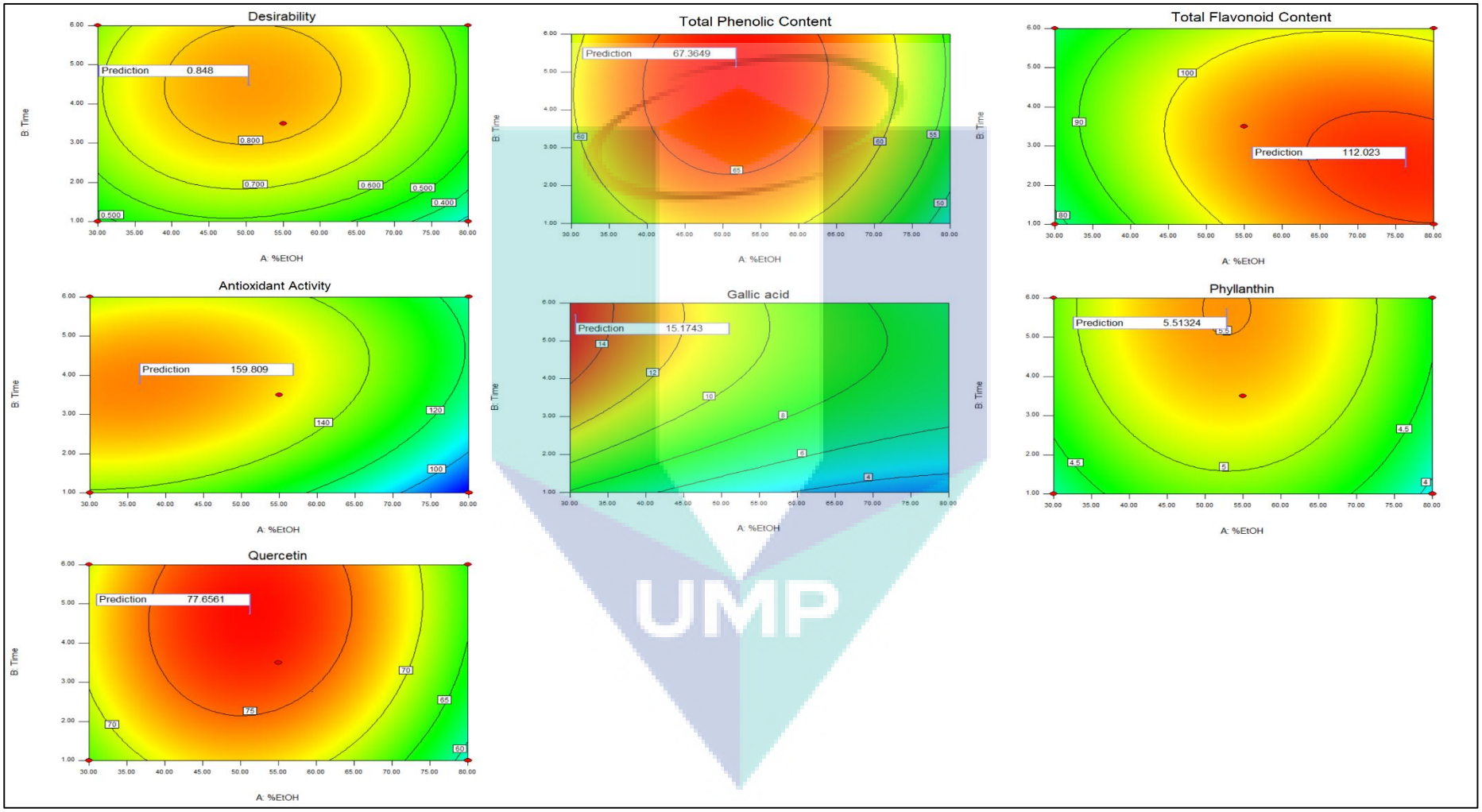


Figure 4.8 Predicted optimum for simultaneous maximum polyphenols recovery using CCD model.

The optimum parameter to obtain the highest yield of phyllanthin, gallic acid, quercetin, total phenolic content, total flavonoid content and antioxidant activity was determined from the model equation with the condition of all the parameters is kept in range. Only one response is optimized at a time. The optimum condition for Phyllanthin, quercetin and total phenolic content extraction was found in ethanol concentration from 51.08% to 52.86%, extraction time from 4.75 minutes to 5.72minutes and microwave power from 225.65W to 250W with the yield of 5.513mg Phy/ g DW, 77.656mg Que/ g DW and 67.365mg GAE/ g DW respectively as shown in Table 4.22. Meanwhile, the optimum condition for gallic acid and antioxidant activity extraction was found in ethanol concentration of 30% to 36.58%, extraction time from 3.78 minutes to 4.96 minutes. Optimum microwave power for both gallic acid and antioxidant activity was found at 250W and 30W respectively. With these optimum parameter, gallic acid and antioxidant activity yield were 15.174 mg GA/ g DW and 159.809mg/g. The desirability for all the response optimization is close to unity with the value of 0.848. The model is considered good since the desirability is close to 0.9.

The suitability of the model equation to predict the desired response (phyllanthin, gallic acid, quercetin, total phenolic content, total flavonoid content and antioxidant activity yield) is tested experimentally using the optimum conditions described in Table 4.22. The result of the verification using triplicate run is presented in Table 4.23. The deviation between the predicted and measured responses was ranging from 0.474% to 3.414%. The experimental values were in good agreement with the predicted values of the model with an error less than 5%, which proved adequacy of the model for predicting the optimum yield of all the response from MAE.

Table 4.22 Condition for factors in optimizing polyphenol extraction.

Factor	Phyllanthin	Gallic Acid	Quercetin	Total Phenolic Content	Total Flavonoid Content	Antioxidant Activity
Ethanol Purity (%)	52.86	30.03	51.29	51.08	76.31	36.58
Power (W)	250	225.65	250	247.91	250	30
Extraction time (min)	5.72	4.96	4.75	4.78	2.47	3.78

Table 4.23 Comparison between predicted and experimental value for optimum condition.

Response		Predicted Value	Experimental Value	Error
Phyllanthin	Run 1	5.513	5.331	3.414
	Run 2	5.513	5.487	0.474
	Run 3	5.513	5.764	4.355
Gallic Acid	Run 1	15.174	15.394	1.429
	Run 2	15.174	15.531	2.299
	Run 3	15.174	15.031	0.951
Quercetin	Run 1	77.656	76.905	0.977
	Run 2	77.656	77.097	0.725
	Run 3	77.656	75.364	3.041
Total Phenolic Content	Run 1	67.365	66.214	1.738
	Run 2	67.365	68.135	1.130
	Run 3	67.365	66.097	1.882
Total Flavonoid Content	Run 1	112.023	113.496	1.298
	Run 2	112.023	112.946	0.817
	Run 3	112.023	110.651	1.240
Antioxidant Activity	Run 1	159.809	154.326	3.553
	Run 2	159.809	158.040	1.119
	Run 3	159.809	160.671	0.537

4.7 Summary

The isopropyl alcohol able to yield higher polyphenol extraction compared to ethanol. When dealing in the pharmaceutical industry, it's more preferable to solvent which comply use Food and Drug Administration (FDA) standard. Hence, ethanol was chosen for the rest of the extraction study. At 40% ethanol, it yielded the total phenolic content at 42.54mgGAE/g, 60.74mg QE/g of total flavonoid content, 127.85mg BHA/g antioxidant activity, 4.41mg Phy/g of phyllanthin, 9.86mg GA/g gallic acid, and 5.48mg Que/g of quercetin. From the optimization study of UAE, it is found that the best ethanol purity is 40% of ethanol, best extraction time is 15 minutes and the amplitude is ranging from 75 to 90 %. For MAE, it is found that ethanol purity ranging from 36.58% to 76.31 % subjective to which response is required. The best power is suggested at 250W except for antioxidant activity extraction, the power is suggested to be at 30W. The extraction time for MAE is suggested to be ranging from 2.47 minutes to 5.72 minutes. By

comparing both UAE and MAE, MAE is suggested to be used or more preferable method for polyphenol extraction as it required able to obtain higher yield compared to UAE method in shorter extraction time.

CHAPTER 5

MICROENCAPSULATION

5.1 Introduction

This chapter presented the study of the microencapsulation of the polyphenols and to select the best encapsulating agent.

5.2 Moisture content

The average moisture content values (g water/g total %) of the spray-dried powders by the different encapsulating agent is shown in Table 5.1. The highest moisture content reported in Table 5.1 is 10% Whey Protein Isolate (WPI) encapsulated powder which ranged from 10.21% to 10.82% with the three repetitive screening. On the other hand, a mixture of 10% WPI and Maltodextrin (MD) at the ratio of 1:9 encapsulated powders found to content the least moisture content that ranged from 7.49% to 9.04% after three repetitive screening. The results show that increasing viscosity leads to the increment of the moisture content of the final products for all the feed concentrations. During drying the droplet expand as the water content vaporized thus creating a hollow sphere. Higher viscosity causes the droplet film to expand lesser than that of lower viscosity droplet during drying. The limited expansion causes a harder curst formation on the particle surface hence increasing the probability of water vapour entrapment inside the dried powder.

The importance of measuring the moisture content is due to the physical properties (particle flowability, bulk density, particle agglomeration of the powder) of the spray powder are highly affected by the moisture content of the spray powder.

Feed solid content may affect the moisture content of the dried powder. For instance, Goula and Adamopoulus (2004) found that a higher feed solid concentration yields a stronger crust formation due to the reduction of water content in the sample, while the solid concentration increases despite exposed to the same amount of heat. Similar observations are also reported by Anandharamakrishnan, Rielly & Stapley (2008). MD encapsulated powder, has a lower moisture content compared to WPI encapsulated powder due to higher hygroscopicity of the protein compared to maltodextrin. Earlier Schuck et al. (2012) reported that WPI powder has a 6.2% of hygroscopicity compared to 4.9% for maltodextrin powder. Table 5.1 shows the spray powders with the lowest moisture content is produced from a mixture of WPI and MD at the ratio of 1:9. However, it should be noted that the moisture content of MD and WPI:MD 1:9 is not significantly difference ($P > 0.05$), because both formulation is mainly made of maltodextrin.

Table 5.1 Physical properties of spray dry powder with different encapsulating agent.

	Without encapsulating agent	10%WPI	10%MD	WPI:MD 1:9
Total Solid Content (wt%)	2.08±0.22	10.56±0.15	10.44±1.22	10.71±0.51
Moisture Content (wt%)	8.66±3.41	10.41±0.35	8.23±0.78	7.68±1.82
Viscosity(mPas)	5.20±0.27	6.19±0.37	5.40±0.30	5.18±0.36
D_{mean}(μm)	7.30±0.62	13.48±0.83	12.67±0.75	16.68±0.14

*D_{mean} is volume weighted average.

5.3 Particle Size Distribution

The particle size analysis showed that the *P. niruri* spray-dried powders consisted of particles with a diameter ranging from 0.67 to 59.80μm (Figure 5.1). It was found that WPI encapsulated powder had a volume weighted mean D of 13.48μm, while MD encapsulated powder had a volume weighted mean of 12.67μm. On the other hand, WPI and MD mixture in the ratio of 1:9 showed highest volume weighted mean of 16.68μm. The particle size of *P. niruri* spray-dried powder encapsulated by WPI:MD 1:9 is significantly larger compared to those encapsulated by either MD or WPI. Particle size of WPI and MD encapsulated powder is not significantly different ($P > 0.05$) due to minimal difference in the feed solution viscosity and a similar solid content employed in

this work. The size of the particle affects the powder flowability. According to Liu et al. (2008), the powder flowability increased when the particle size increased. Since the particle size is one of the main considerations in powder handling, a larger particle size is the most suitable form as the flowability is a vital parameter for an efficient and effective transportation, storage, handling, processing and tableting.

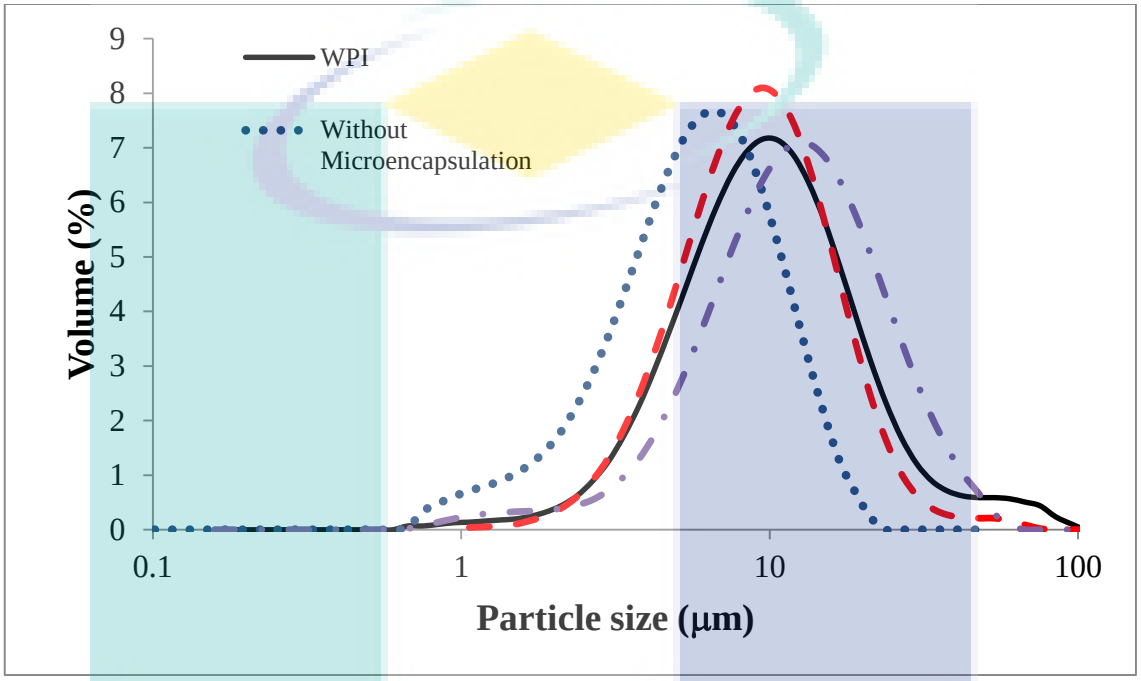


Figure 5.1 Particle size distribution at different formulation.

5.4 Particle morphology

The morphology of the spray-dried particle was examined using a scanning electron microscope. Figure 5.2 shows the morphology of spray-dried powder prepared from *P. niruri* extracts without encapsulation and with encapsulation by WPI, MD and its combination. The micrograph shows that different types of wall material affect the surface morphology of the microcapsule. Results from this study showed that highly deformed, with extensive wrinkle and dented surface were found in spray-dried particle without encapsulating agent as well as spray-dried powder encapsulated with WPI. On the other hand, compared to MD encapsulated spray-dried powder at identical solid concentration, it was found that the surface of the particle is more spherical and well developed. Figure 5.2 also illustrates that the spray-dried powder with an encapsulating agent of mixtures, WPI and MD at the ratio of 1:9 was able to produce spherical shape

consistently. The shape and morphology of the particles upon spray drying are possibly depending on the factor of droplet evaporation rate and the formulation composition (Chegini & Taheri, 2013). Researcher Rosenberget, et al., (1985) reported that the shrinkage of the particle during spray drying might be the factor in the dented surface formation of spray-dried particles. In addition, Wang & Langrish, (2010) suggested that the formation of the surface roughness may be decreased significantly with the increase of the contact adhesion between two particles with the spray dryer surface.

WPI encapsulated spray-dried powder tends to have more wrinkle surface than MD which may be due to the viscosity factor. Referring to Table 5.1, with WPI encapsulated contains higher viscosity (6.19 mPas) than MD encapsulated which indicates that higher viscosity tends to inhibit the flexibility of the droplet during the spray drying process. Therefore, the flowability of the feed solution in the spray-dryer may not be consistent in producing spherical and well developed droplet resulting in the dented and wrinkle surface of the particle. On the other hand, lowest viscosity (5.18 mPas) was recorded in the mixture of WPI and MD. Furthermore, the particle morphology for this spray powder are evenly in spherical shape and much more developed.

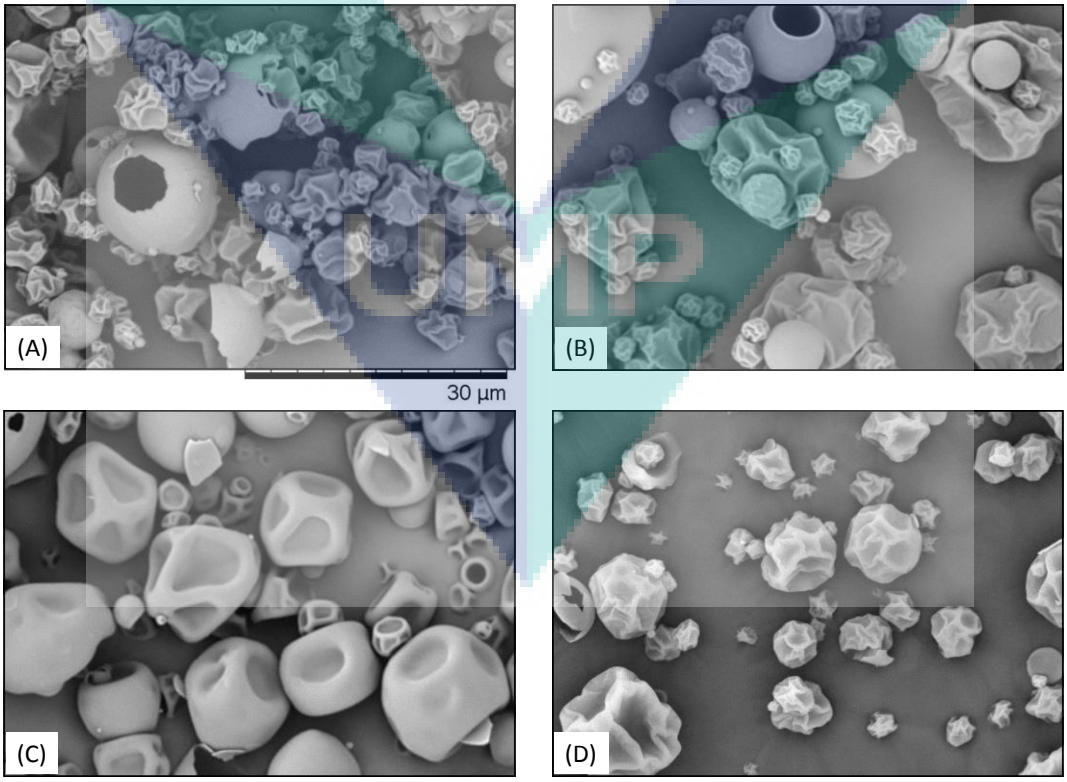


Figure 5.2 Surface morphology of the non-encapsulated and encapsulated spray dried powder. (A)-Without encapsulating agent; (B)- 10% MD; (C) 10% WPI; (D)- 10% WPI (1): MD (9).

5.5 Microencapsulation of polyphenolic compounds from *P. niruri*

Comparison of the polyphenols retention of spray powder without encapsulating agent, WPI encapsulated and MD encapsulated powder with initial feed solution were conducted in this study to determine the level of polyphenols preservation by microencapsulation method. Phyllanthin, gallic acid and quercetin content were analyzed in both initial feed solution and the spray-dried powders by UPLC. Retention of the polyphenols for all the formulations prepared showed a good retention result that ranges from 71.98% to 91.53% as depicted in Figure 5.3. The sample without microencapsulation is named as 'control' in Figure 5.3. The retentions of individual polyphenol without microencapsulation for phyllanthin, gallic acid and quercetin are 73.37%, 74.22% and 70.51%, respectively. For instance, quercetin showed higher degradation than phyllanthin which may be possibly due to the presence of hydroxyl groups in their molecular structure. Bucher et al. (2006) reported that polyphenol with more hydroxyl groups were found to be more feasible to thermal degradation. Another possible reason that may lead to thermal degradation of the polyphenol is the benzene ring substitution pattern. According to Makris & Rossiter (2000), the position of the benzene ring substitution plays an important role in thermal degradation. The molecular structure of phyllanthin that contains no hydroxyl group illustrated better thermal stability during spray drying compared to quercetin. Based on UPLC analysis results, it can be concluded that the sample with mixtures of WPI and MD at the ratio of 1:9 encapsulated provides a better retention on all three targeted compounds; phyllanthin (84.33%), gallic acid (88.92%) and quercetin (88.39%), as shown in Figure 5.3. By comparing WPI and MD encapsulate, MD encapsulate spray powder resulting in a better retention of polyphenol than WPI encapsulate spray powder. Similar finding was reported by Pang et al. (2014) where 10.67% of MD encapsulate spray powder were able to preserve polyphenol better compared to 10.67% WPI encapsulated spray powder. The summary of this study proposed that microencapsulation using mixture of WPI and MD provides the best preservation of polyphenol during spray drying as opposed to single protein encapsulate of WPI or MD. In addition, spray powder with protein encapsulated

associated to a smooth and spherical particle surface that improve its particle flowability and may enhance the particle collection and particle handling.

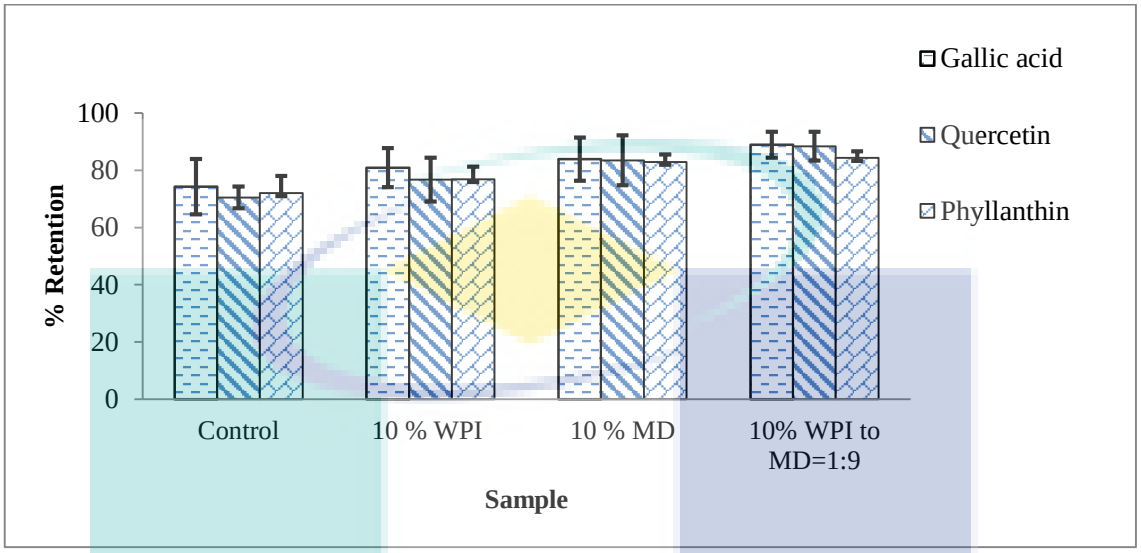


Figure 5.3 Retention of phyllanthin, gallic acid and quercetin using different encapsulation strategies.

5.6 Summary

Microencapsulation by spray drying using WPI and MD yielded a good polyphenol retention from *P. niruri* extracts. Microencapsulation is a compromising technique to reduce polyphenol degradation during the spray drying process. This study demonstrated that low viscosity of the feed solution reflects low moisture content and lower solid content that leads to a larger particle size, which allowed the lesser deformed particle surface yields during the spray drying process which may improve the flowability of the particle.

Microencapsulation using a mixture of WPI and MD at the ratio of 1:9 yielded the highest retention of phyllanthin (84.33%), gallic acid (88.93%) and quercetin (88.39%) followed by MD and WPI. Microencapsulation using a mixture of WPI and MD at ratio 1:9 is recommended as it provides a better preservation of polyphenol during spray drying as opposed to single protein encapsulate of WPI and MD. The results analysis shows that gallic acid and quercetin are more susceptible to thermal degradation than phyllanthin during spray drying.

CHAPTER 6

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

From these research finding, ethanol is the best solvent for the bioactive components extraction which is aligned to FDA regulatory and at 40% ethanol, it yielded the total phenolic content at 42.54mgGAE/g, 60.74mg QE/g of total flavonoid content, 127.85mg BHA/g antioxidant activity, 4.41mg Phy/g of phyllanthin, 9.86mg GA/g gallic acid, and 5.48mg Que/g of quercetin. From the optimization study of UAE, it is found that the best ethanol purity is 40% of ethanol, best extraction time is 15 minutes and the amplitude is ranging from 75 to 90 %. For MAE, it is found that ethanol purity ranging from 36.58% to 76.31 % subjective to which response is required. The best power is suggested at 250W except for antioxidant activity extraction, the power is suggested to be at 30W. The extraction time for MAE is suggested to be ranging from 2.47 minutes to 5.72 minutes. By comparing both UAE and MAE, MAE is suggested to be used or more preferable method for polyphenol extraction as it required lesser than and able to obtain higher yield compared to UAE method. Apart from that, from the MAE analysis, it's shown that the optimization result is more convincing based of the three dimensional response surface plot based on the factors and its responses. Based on the result in section 4.6.2.1, some optimum points of the factors are able to be identified at the factors ranged in the table 4.23.

From both UAE and MAE extraction, the identified bioactive components in *P. niruri* are phyllanthin, gallic acid and quercetin. These three bioactive components are separated based on the UPLC method developed in this research study and there were positively identified by comparing their retention time and UV spectra with its standard. The analysis time for this UPLC method in qualitative and quantitative analysis spent 20

minutes which also developed for the first time for *P. Niruri*. MAE extract obtained slightly higher polyphenol content (Phyllanthin: 5.513mg Phy/g DW; Gallic Acid: 15.714 mg GAE/g; Quercetin: 77.656mg Que/g DW) than UAE extract (Phyllanthin: 5.026mg Phy/g DW; Gallic Acid: 10.498 mg GAE/g; Quercetin: 17.212mg Que/g DW).

WPI and MD encapsulation spray powder had generated a good polyphenol retention from *P. niruri*. Microencapsulation is a compromising technique to reduce polyphenol degradation during spray drying process. This study demonstrated that low viscosity of the feed solution reflects low moisture content and lower solid content that leads to larger particle size which allowed the lesser deformed particle surface yields during spray drying process could be resulting uniform flowability of the particle through the process. Microencapsulation using mixture of WPI and MD at the ratio of 1:9 delivered the highest retention of phyllanthin (84.33%), gallic acid (88.93%) and quercetin (88.39%) followed by MD and WPI encapsulation. Microencapsulation using mixture of WPI and MD at ratio 1:9 is recommended as it provides a better preservation of polyphenol during spray drying as opposed to single protein encapsulate of WPI and MD. The results analysis illustrate that gallic acid and quercetin are more susceptible to thermal degradation than phyllanthin during spray drying.

6.2 Recommendation

The proposed solution in this research study might require in depth investigation before take in consideration as a guideline for the production of functional foods. Another recommendation on this research study is to study the pharmacological test on the toxicity of the extract. Toxicity analysis of *P. niruri* extract is suggested to undergo animal test in ACCU accredited lab before release for human consumption. Once all these unknown had been clarified, it is good to develop a standard for the extraction as well as the quality control for the production of functional foods. Error analysis on the solvent concentration preparation should be taken into consideration to reflect more precise solvent concentration.

Even though microencapsulation is one of the preservation method for polyphenols content from thermal degradation during spray drying, it is still a gap on terminology of the biological activity of the spray dried powder compared to its initial extract. Although various tests such as UPLC and colorimetric assay resulting the spray

dried powder still retained the most of the bioactive compound as compared to its initial extract, the best method to ensure or confirm the uncertainty is by conducting the animal testing or other relevant test. Functionality and properties of the polyphenols may change in spray dried powder form. Hence, an appropriate test on the functionality and properties of the polyphenols after spray dried is needed to confirm the uncertainty.

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APPENDIX A

RELEVANT PUBLICATIONS, THESIS, SEMINARS/CONFERENCES PRESENTATION

Journal

1) S. L. Nguang et al., 2017. Ultrasonic assisted extraction on phenolic and flavonoid content from *Phyllanthus Niruri* plant. *Indian Journal of Science and Technology*. 10(2), 1-5. ISI Indexed, Zool. Record.
2) S. L. Nguang et al., 2018. Optimisation of Gallic Acid and Quercetin Extraction from Phyllanthus Niruri. *International Journal of Engineering & Technology*. 7(1.13), 90-94. Scopus indexed.
3) J. Gimbun et al., 2018. Assessment of phenolic compounds stability and retention during spray drying of *Phyllanthus niruri's* extracts. *Industrial & Engineering Chemistry Research*. Q1 ranked ISI indexed SCIE.

Conference

1) Nguang, S. L., Yeong, Y. L., Pang, S. F., & Gimbun J. (2017). Ultrasonic assisted extraction on phenolic and flavonoid content from *Phyllanthus Niruri* plant. 2nd International Conference on Fluid and Chemical Engineering (FluidsChE), Sabah, Malaysia. April 4-6, 2017.
2) Nguang, S. L., Yeong, Y. L., Pang, S. F., & Gimbun J. (2018). Optimisation of Gallic Acid and Quercetin Extraction from *Phyllanthus niruri*. 5th International Conference on Computer Science, Engineering and Technologies (ICCSET), Bangkok, Thailand. June 18-19, 2018.

Awards

1) **Gold medal**, Functional Food from *Phyllanthus niruri*, 7-8 March 2016, CITREX 2016, UMP
2) **Gold prize**, Functional Food from *Phyllanthus niruri*, 8-10 April 2016, I-ENVEX 2016, UNIMAP
3) **ENVEX Best Award Classed C (Food, Health and Biotechnology)**, Functional Food from *Phyllanthus niruri*, 8-10 April 2016, I-ENVEX 2016, UNIMAP
4) **Gold medal**, Polyphenol-Rich Microcapsule from *Phyllanthus niruri*, 9-11 December 2016, KIDE 2016, Kaohsiung, Taiwan.
5) **International Best Invention Award (Hong Kong)**, Polyphenol-Rich Microcapsule from *Phyllanthus niruri*, 9-11 December 2016, KIDE 2016, Kaohsiung, Taiwan.
6) **Bronze medal,** Polyphenol-Rick Microcapsule from *Phyllanthus niruri*, 15-16 March 2017, CITREX 2017, UMP