EXTRACTION OF POLYPHENOL FROM PHYLLANTHUS NIRURI

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ABSTRACT

Phyllanthus niruri which is also known as Dukung Anak is a plant which contain miscellaneous bioactive components which provide the medical effects such as antioxidant, anti-cancer and treating Hepatitis-B. Extraction is a common method to obtain bioactive component from plant materials therefore this research is to elucidate the effect of solvents types and investigate the performance of different type extraction method on *P.Niruri.* Ultrasonic assisted extraction and microwave assisted extraction methods were studied because there is no previous work found in literature. Analysis such as Total Phenolic Content, Total Flavonoids Content, Ultra-Performance Liquid Chromatography were carried out throughout the research. From the works, it is found that highest yield of phyllanthin with 4.56mg Phy/g DW was obtained with 20% aqueous Isopropanol whereas the quercetin with the highest yield, 10.14mg Que/g DW in 20% aqueous ethanol and the highest yield 15.44mg GAE/g DW of gallic acid was obtained by using water. Central Composite Design analysis shown that microware assisted extraction with 250W power, 3.62 minutes and 52.58% ethanol concentration obtain the optimum yield of polyphenol extraction of 83.70%. In conclusion, the yield of bioactive component is highly dependent on solvent polarity used in extraction and microwave extraction method provides a fast extraction without significantly compromising the extraction yield compare to ultrasonic assisted extraction.

ABSTRAK

Dukung anak juga dikenali sebagai Dukung Anak merupakan tumbuhan yang mengandungi pelbagai komponen bioaktif yang dapat memberi kesan perubatan seperti antioksidan, anti-kanser dan juga merawat Hepatitis-B. Pengekstrakan adalah kaedah common untuk mendapatkan komponen bioaktif dari bahan tumbuhan dan kajian ini adalah untuk menjelaskan kesan jenis pelarut yang berlainan dan menyiasat prestasi yang berbeza kaedah pengekstrakan pada P.Niruri. Pengekstrakan ultrasonik dan pengekstrakan gelombang mikro dikaji dalam kajian ini kerana tidak ada kerja dijumpai di dalam kesusasteraan sebelum ini. Analisis seperti jumlah kandungan fenolik, jumlah kandungan flavonoids, Ultra-Performance Liquid Chromatography dijalankan sepanjang kajian. Kajian mendapati bahawa hasil tertinggi phyllanthin dengan 4.56mg Phy / g DW telah diperolehi dengan 20% akueus Isopropanol manakala quercetin dengan hasil tertinggi, 10.14mg Que / g DW dengan 20% etanol berair dan hasil tertinggi 15.44mg GAE / g DW asid Gallic telah diperolehi dengan menggunakan air. Analisis pusat Komposit Design menunjukkan bahawa pengekstrakan gelombong mikro dengan kuasa 250W, masa 3.62 minit dan kepekatan etanol 52.58% telah mendapatkan hasil optimum pengekstrakan polifenol iaitu 83.70%. Kesimpulannya, hasil daripada komponen bioaktif adalah sangat bergantung kepada kekutuban pelarut yang digunakan dalam kaedah pengekstrakan dan pengekstrakan gelombong mikro menyediakan pengekstrakan yang cepat tanpa menjejaskan hasil pengekstrakan dengan ketara bandingkan dengan pengekstrakan ultrasonic.

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LIST OF SYMBOLS

F	number of points in the factorial portion
Hr	Hour
N	dilution
V	extract volume
W	quantity of *P. Niruri* dry powder
Y	sample fluid concentration of total phenolic
cm	Centimeter
cP	viscosity
f(x)	vector function of *p* elements
g	gram
kHz	KiloHertz
kg	Kilogram
ml	Mililiter
mm	Milimeter
tan ∂	dissipator factor
wt	Weight
d	*degree model*
µm	Micrometer
˚C	Degree Celsius
β	vector of *p* unknown constant coefficients
ϵ	random experimental error
%	Percentage
λ	Wavelength
2^k	first-order
Ɛ’	dielectric constant
n_0	design center

LIST OF ABBREVIATIONS

AA	antioxidant
ANOVA	Analysis if variance
BHA	butylated hydroxyanisole
CCD	central composite design"
DE	Dextrose equivalent
DoE	design of experiments
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
EtOH	Ethanol
Eup	Eupatorin
FDA	food and drug administration
GA	Gallic acid
H20	Water
HPLC	High performances liquid chromatography
MAE	microwave assisted extraction
MoH	ministry of health of Malaysia
P. *niruri*	*phyllanthus niruri*
PHY	Phyllanthin
PWE	pressurized water extraction
QUE	Quercetin
RP	reverse phase
RSM	response surface methodology
SFE	supercritical liquid extraction
TFC	total flavonoid content
TPC	total phenolic content
UAE	ultrasonic assisted extraction
UPLC	ultra-performance liquid chromatography
WPI	Why protein isolat

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Phyllanthus niruri, locally termed as Dukung anak (Family: Phyllanthaceae) is an annual herb that grows up to measures of 50cm tall and having a smooth bark on the ascending branches with small flowers and tiny fruits that filled with seed. It is growing mainly in tropical areas and thrives in wet rainforest conditions and spreads rapidly throughout the tropical and subtropical countries including Malaysia and India. In India, *P.niruri* is a common herbs used to heal problems such as stomach, genitourinary system, liver, kidney and spleen. Traditionally, *P. niruri* act as home remedy in many countries due to well-known of its curative properties. Historically it can increase the appetite, relieve inflammations and fever. In terms of health, it helps in restricting the growth of hepatitis B virus found in blood stream, having antifungal, anti-viral and hypoglycaemic action and useful in the treatment of liver disease such as jaundice and liver cirrhosis. It also helps to remedy fatty liver and liver damage. *P. niruri* is diuretic and hence is used in urinary tract infections and bacterial infections like cystitis and prostatitis. 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.

From the analysis done by ministry of health of Malaysia (MoH), 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.*

1.2 Motivation and Problem Statement

The most important factor that affects the yield and recovery of the bioactive components from plant materials is the extraction method. Previous method of extraction performed by Tripathi et al (2011) was maceration that consumes 10 hours for the extraction process. The 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). 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 yield from the plant materials involving solid-liquid extraction where mass transfer process involving the solvent (liquid) transport to the inner part of the plant materials (solid), the solubility of the solute and release of solutes from the solid matrix to the external bulk phase of the plant. With the aid of UAE and MAE, reduction on the limitation of the mass transfers for both internal and external transport can be minimized. 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 done. Therefore, UAE and MAE methods were selected for this research study by RSM.

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. Hence, ethanol, isopropyl alcohol and water were used as the solvent study in this research work on the combined effect of various type of solvents and method of extraction on *P. Niruri*. 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* had been develop.

1.3 Objectives

This study boards on the following objectives:

1) To elucidate the combined effect of various type of solvents and method of extraction on *P. Niruri*.
2) To investigate the performance of ultrasonic assisted extraction and microwave assisted extraction in extraction polyphenol from *P. Niruri*.

1.4 Scopes of Research

The following are the scope of this research:

1) Extraction study of bioactive compound by using various solvents (water, ethanol and isopropyl alcohol), extraction methods (both UAE and MAE) and process condition (ethanol concentration, time, power and amplitude) by response surface methodology.
2) Performing a proximate analysis (total phenolic content and total flavonoids content) of the plant leaves extracts.
3) Performing UPLC analysis to quantify polyphenolic compounds (pyhllanthin, gallic acid and quercetin) of the plant leaves extracts.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

This chapter describes the review on the previous work or study related to the method of extraction (UAE & MAE), literature study that related on the extraction (factors) and the analytical method as well as the bioactive components from *P. Niruri*.

2.2 Introduction

There was 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; Oii, et al., 2014). 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.

2.3 Extraction Method for Flavonoids and Phenolic Content

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, contains 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 2 007).

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 where 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 have occurred during these conventional extraction methods as well.

In order to reduce the thermal degradation of bioactive components, other better polyphenol extraction method was developed and studied. In most recent years, ultrasonic assisted extraction (UAE), microwave assisted extraction (MAE), supercritical liquid extraction (SFE) 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.

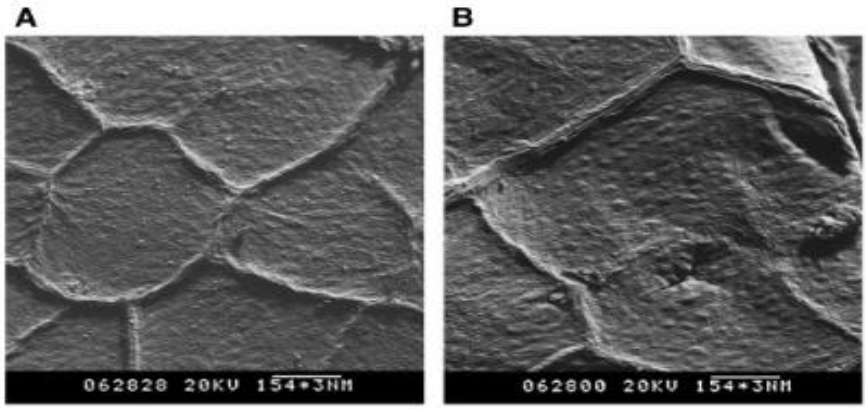


Figure 2-1: Scanning electron micrographs (100X) of Epimedium leaf sample. (A) Untreated leaf; (B) After ultrasonic assisted extraction by Zhang et al. (2009).

It is 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 affect the recovery rate of the cell content. Other than that, there are several researchers (Mediani et al., 2015; Murugaiyah and Chan., 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/cm^2, 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 undergoing 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 leaf which also enhance the mass transfer of solvent into the chloroplast as well.

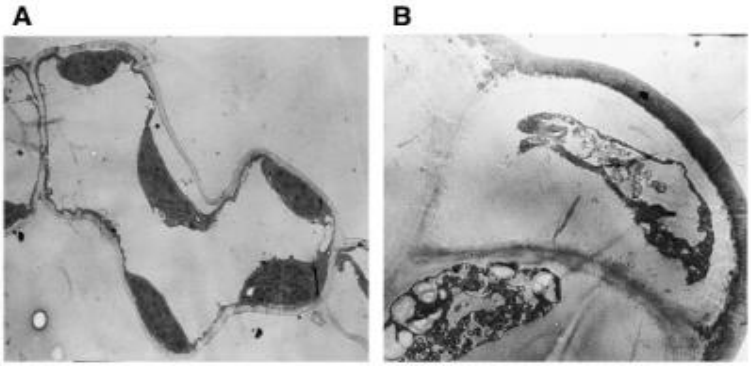


Figure 2-2 Transmission electron micrographs (3000×) of Epimedium leaf samples from (A), untreated leaf; (B), after ultrasonic-assisted extraction by Zhang et al., (2009)

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 outside (heating medium) to the interior of the sample (cell content). In MAE, heat is dissipated volumetrically inside the irradiated medium. In microwave heating, energy transfer by two mechanism, dipole rotation and ionic conduction through reversals of dipoles and displacement of charged ions present in the solute and the solvent (Routray 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). Figure 2.3 below describes energy transfer toward the materials during the MAE process are delivered directly to the interior (cell content) through the molecular interaction with electromagnetic fields through conversion of electromagnetic energy into heat energy (Thostenson and Chou, 1999).

There are several factors affecting the yield of MAE. The efficiency of the extraction refracted to the operating condition selected. The major contribution 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 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 2-1: 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 favorable 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 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 (Mandal 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.

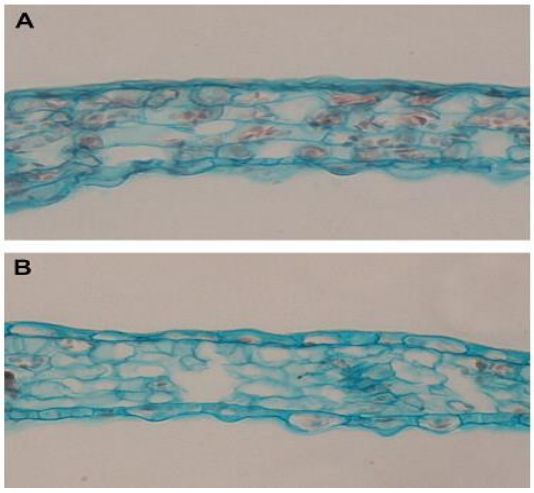


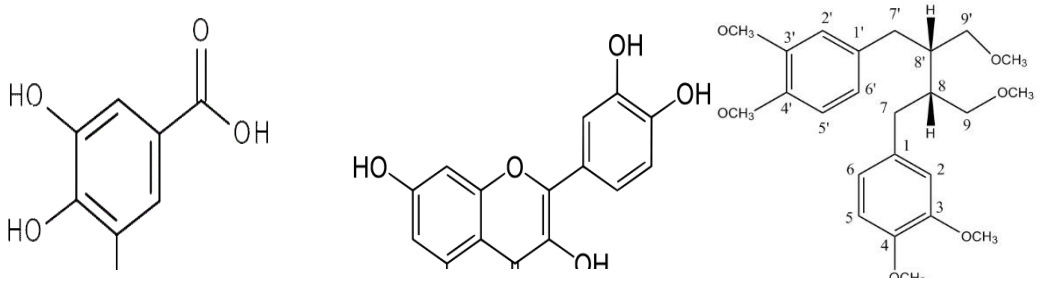
Figure 2-3 Light micrographs of Epimedium leaf samples: (A) untreated leaf sample; (B) leaf sample after microwave irradiation by Zhang et al., (2011).

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. 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 use in traditional medicine as 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

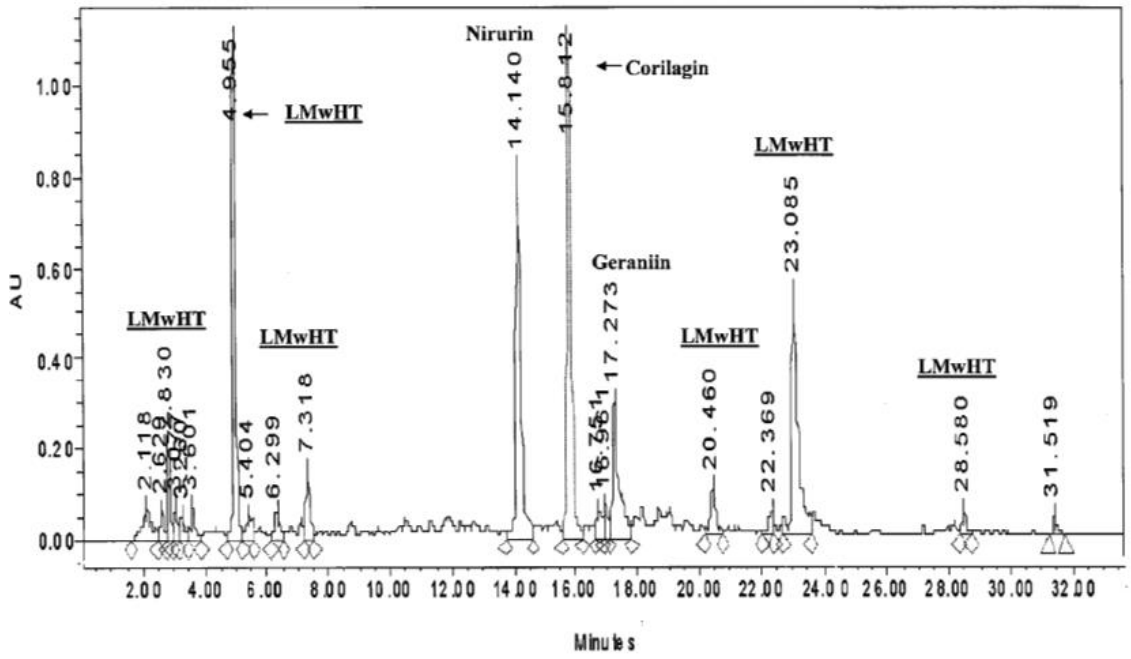
Figure 2-4: Active components in Phyllanthus Niruri extract

2.5 Ultra Performance Liquid Chromatography

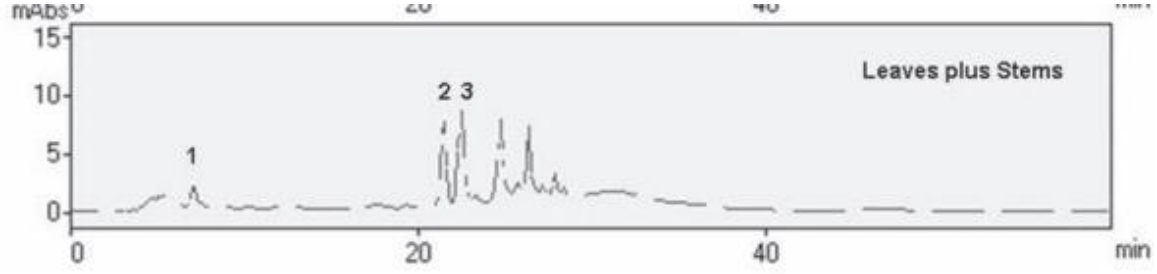
P. Niruri consists of variety of active components with its medical effect. Analytical method is required for the accurate and precise quantification and qualification of these active components. Mainly the work done before this regarding the analysis of active components was done by HPLC analysis. Summary of HPLC analysis methods are presented in Table 2.2. From the literature studies, it can be seen that LC analysis is the best method to analyses 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 and operate at higher pressure to increase speed, efficiency, and resolution by comparison of traditional HPLC method (Swartz, 2005). UPLC column with high packed pressure can provide the sharper and faster separation than HPLC therefore it is chosen and will be used for the analysis of active components. Refer 2 on the review of HPLC analysis for *P. Niruri* by different researcher.

Table 2-2: Review of HPLC analysis for Phyllanthus Niruri

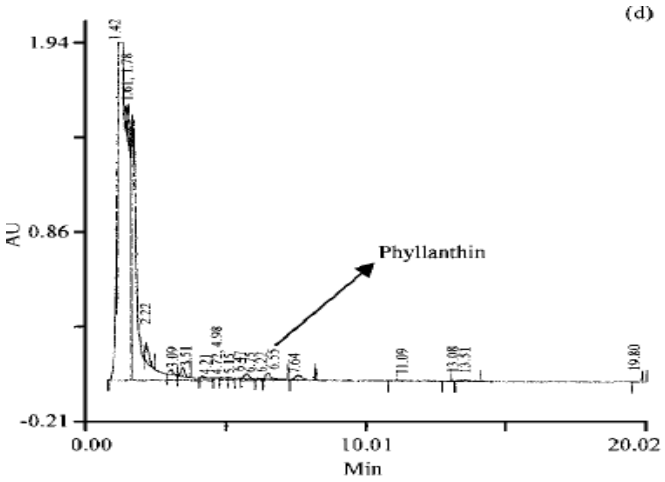
Authors	Column	Mobile Phase	Mode of column separation
Ghosal et al. (2012)	RP C18 250mm1.X 4mm id., 5um particles d	A: 0.1%phosporic acid, B: Acetronitrile	Reverse phase
Cuoto et al. (2013)	RP-18 Li Chrosher 250X4mm id, 5um particle diameter	A: 1% phosphoric acid, B: CAN: phosphoric acid 1% (w/w) (50: 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



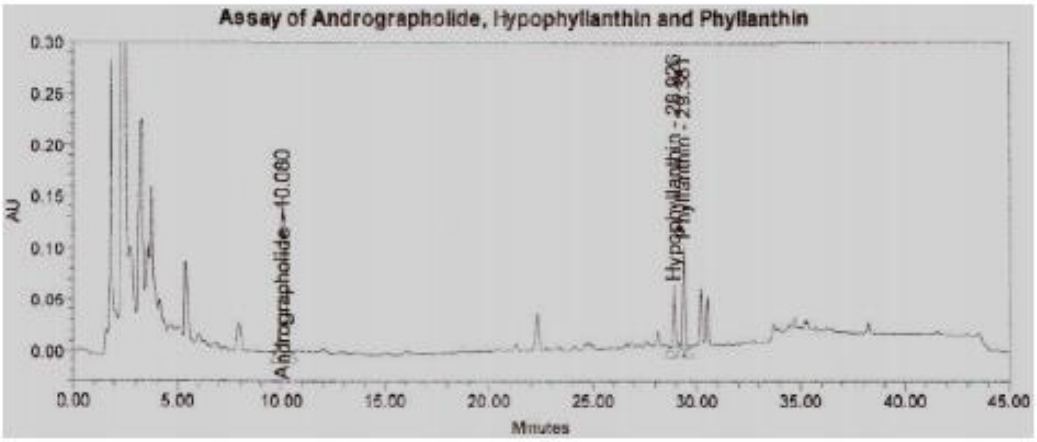
(A) Ghosal et al. (2012)



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



(C) Bhope et al. (2013)



(D) Annamalai and Lakshmi. (2009)

Figure 2-5: HPLC chromatogram of Phyllanthus Niruri extract from various authors.

2.6 Response Surface Methodology

Response surface methodology (RSM) is method of optimizing a response via a collection of mathematical and statistical techniques for model building. From the design of experiments, the respondent was influenced by independent variables which referring the input variables. An experiment is a series of tests (runs), in which changes are made in the input variables (factors) denoted by $x_1, x_2, \ldots, x_k$ in order to identify the reasons for changes in the output response, y. In overall, a relationship is unknown but can be approximated by a low-degree polynomial model of the form

$$y = f'(x)\beta + \epsilon \qquad \text{Eq. (2.1)}$$

Where $x = (x_1, x_2, \ldots, x_k)$'

$f(x)$ is a vector function of p elements that consists of powers and cross-products of power of x1, x2, …, xk up to a certain degree denoted by (d≥1).

β is a vector of p unknown constant coefficients referred to as parameters

ϵ is a random experimental error assumed to have a zero mean

It is believed that model above able to provide an adequate representation of the output response. For this model, the quantity of $f'(x)\beta$ is depends on the mean response, which is expected by the value of y, and denoted by $\mu(x)$. In RSM, the two important models included first-degree model (d=1) and second-degree model (d=2). The first-degree model and second-degree model are shown in the Eq.(2.2) and Eq.(2.3) below.

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \epsilon \qquad \text{Eq. (2.2)}$$

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i<j} \beta_{ij} x_i x_j + \sum_{i=j}^{k} \beta_{ij} x_i^2 + \epsilon \qquad \text{Eq. (2.3)}$$

According to Khuri and Mukhopadhyay (2010), three objectives of considering of the above model are due to the following factors.

1. To establish a relationship, albeit approximate, between y and $x_1, x_2, \ldots, x_k$ that can be used to predict response values for given settings of the control variables.
2. To determine, through hypothesis testing, significance of the factors whose levels are represented by $x_1, x_2, \ldots, x_k$.
3. To determine the optimum setting of $x_1, x_2, \ldots x_k$ that results in the maximum or minimum response over a certain region of interest.

RSM was first developed to model experimental response and then was migrated into modelling of numerical experiments. The difference is in the type of error generated by the response. In physical experiments, inaccuracy can be due, for example, to

measurement errors while, in computer experiments, numerical noise is a result of incomplete convergence of iterative processes, round-off errors or the discrete representation of continuous physical phenomena. In RSM, the errors are assumed to be random. With the implementation of the RSM in the optimization study, it reduces the number of run which able to reduce the cost of the analysis, chemicals and time.

2.6.1 Design of experiments.

Design of experiments (DoE) is a very important aspect in RSM. The strategies were first developed for the model fitting of the physical experiments that is also applied to the numerical experiments. DoE is developed for the selection of the points where the output response should be evaluated. The criteria of the optimal design of the experiments are depended with the mathematical model of the process. At the earlier stage, these mathematical models are polynomials with unknown structure where the corresponding experiments are designed for particular factors. Development of the DoE can be having the large influence of the accuracy of the approximation and the cost of response surface construction.

From the traditional way of DoE, screening experiments are performed as the initial stage for the eliminating of minor or no effect on the response, as there are many design variables were considered from the beginning. The reason of performing the screening experiments is to identify the factors that has the major effects for the further investigation. Nowadays, there is a genetic programming had been developed and shows good screening properties by suggesting the both selection of the relevant design variables and at the same time, identification of the model can be carried out (Gilbert et al., 1998).

2.6.2 Central Composite Design

Central composite design (CCD) is most popular analysis for second order model design. CCD are first-order (2^k) designs augmented by additional center and axial points to allow estimation of the tuning parameters of a second-order model.

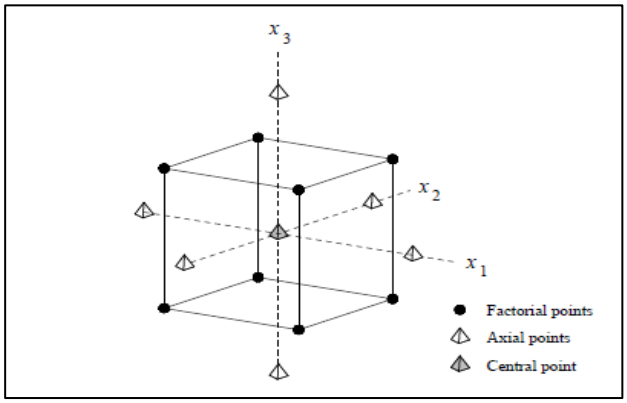


Figure 2-6: Central composite design for 3 design variables at 2 levels.

In Figure 2.6 demonstrates design involves 2^k factorial points, 2*K* axial points and 1 central point. CCD presents an alternative to 3*K* designs in the construction of second-order models because the number of experiments is reduced as compared to a full factorial design (15 in the case of CCD compared to 27 for a full-factorial design). In the case of problems with many designs variables, the experiments may be time-consuming even with the use of CCD.

The design of CCD consists of a complete for a fraction of 2^k factorial design whose factor level are coded as -1, 0 and 1(factorial portion). CCD also is an axial portion that consisting of 2*k* points arranged so that two points are chosen on the axis of each control variable at α distance from the design center, $n_{0.}$ CCD is obtained by augmenting a first-order design, 2^k factorial with additional experimental runs, replication of 2^k axial points and the n_0 center-point. Moreover, this design is developed in a consistent manner with the sequential nature of a response surface investigation by starting with a first-order design, to fit a first-degree model, followed by the addition of design points to fit the larger second-degree model. (Khuri and Mukhopadhyay, 2010). The first-order design serves as a preliminary phase to obtain initial information about the response system and to assess the importance of the factors in each experiment. The additional experimental runs are chosen for getting more information that can lead to the determination of optimum operating conditions on the control variables using the second-degree model.

The replication values of α, the axial parameter and n_0, center-point chosen so that the CCD can acquire certain desirable properties. For example, choosing $\alpha = F^{1/4}$, where F denotes the number of points in the factorial portion, causes the CCD to be rotatable. The value of n_0 can then be chosen so that the CCD can achieve either the orthogonality property or the uniform precision property.

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 ultra-performance liquid chromatography (UPLC). Details on experimental setup, condition and procedures and the source of chemicals and plant materials clearly presented in this chapter. Figure 3.1 below shows the flow chart of this research methodology.

Figure 3-1: Flow chart of research methodology.

3.2 Chemicals

Ethanol, isopropanol, sodium nitrate, sodium hydroxide, Folin & Ciocalteu reagent, butylated hydroxyanisole (BHA) and HPLC gradea cetonitrile were obtained from Merck (Darmstadt, Germany). Trifluroacetic acid, gallic acid and quercetin were obtained from Fisher Scienctific (Pittsburgh, PA). HPLC grade dimethyl sulfoxide, aluminium hexachloride, 2,2-diphenyl-1-picrylhydrazyl were obtained from sigma Aldrich (St/ Louis, MO).

3.3 Plant Material

Dried aerial plant of *P. Niruri* that been voucher specimen deposited at the Herbarium of Rimba Ilmu, Institute of Science Biology, University of Malaya, Kuala Lumpur (voucher number KLU46618) was obtained from Malaysia Herbal Shop, Selangor. Dried plant was grinded and sieved. Every particle size sieved was analysed to obtained accurate comparison.

Figure 3-2: Phyllanthus Niruri Plant.

3.4 Extraction Methods

Ultrasonic assisted extraction (UAE) and microwave assisted extraction (MAE) had been chosen as the extraction method in the research study. Solvent study and powder size study will be done by UAE and then the selection of the solvent and powder size for MAE will be follow the finding from UAE.

3.4.1 Ultrasonic Assisted Extraction (UAE)

Weight (2 wt. %) powdered *P. Niruri* then mix with solvent in 250mL sealed Erlenmeyer flask. Carry the UAE by using the sonicator Q700 with a microtip probe of 13mm diameter and employed in the response surface methodology in study the optimization of the extraction time, temperature and solvent concentration. All the parameters set are based on the literature and limitation of the equipment. According to the 2 level factorial studies, with three independent variables, ethanol concentration (X1), time (X2) and amplitude (X3). There are six dependents variables: phenolic content (Y1), flavonoid content (Y2), antioxidant (Y3), Phyllanthin (Y4), Gallic acid (Y5) and Quercetin (Y6). Total 8 experiments points will be carried out. After the screening of 2 level factorial, optimization of the independent variable will be done according to central composite design.

3.4.2 Microwave Assisted Extraction (MAE)

Powdered *P. Niruri to solvent ratio (2 wt. %)* is weighted and placed in test tube. 5 ml of plant material to solvent ratio (2g/100mL) was placed in a test tube for MAE using CEM microwave reactor (Matthews.Nc, Explorer SP 48, USA). The parameters were determined based on literature and equipment limitation. According to the 2 level factorial studies, with three independent variables, ethanol concentration (X1), time (X2)

and power (X3). There are six dependents variables: phenolic content (Y1), flavonoid content (Y2), antioxidant (Y3), Phyllanthin (Y4), Gallic acid (Y5) and Quercetin (Y6). Total 20 experiments points will be carried out. After the screening of 2 level factorial, optimization of the independent variable will be done according to central composite design.

3.5 ANALYSIS

3.5.1 Total Phenolic Content

By referring Trabelsi et al. (2010), total phenolic content (TPC) is assessed using the Folin–Ciocalteu reagent, adopted method from Singleton's method. Firstly, add the sample aliquot of 0.125ml to a centrifuge tube containing 0.5 ml of ultrapure water and 0.125 ml of the Folin–Ciocalteu reagent. Add 1.25ml of 7% Na2CO3 solution after 3 minutes, and then make up the final volume to 3 ml with ultrapure water. Mix the solution well and incubated for 60 min in the dark. Measure the absorbance against the prepared blank reagent at λ = 760 nm using a calibrated ultraviolet–visible spectroscopy (Hitachi U-1800, Japan). Express TPC of the leaves as mg gallic acid equivalents per gram dry weight (mg GAE/g DW) by comparing with the calibration curve for gallic acid in Figure 3.3 by using the Eq. 3.1(Pang et al., 2012).

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

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

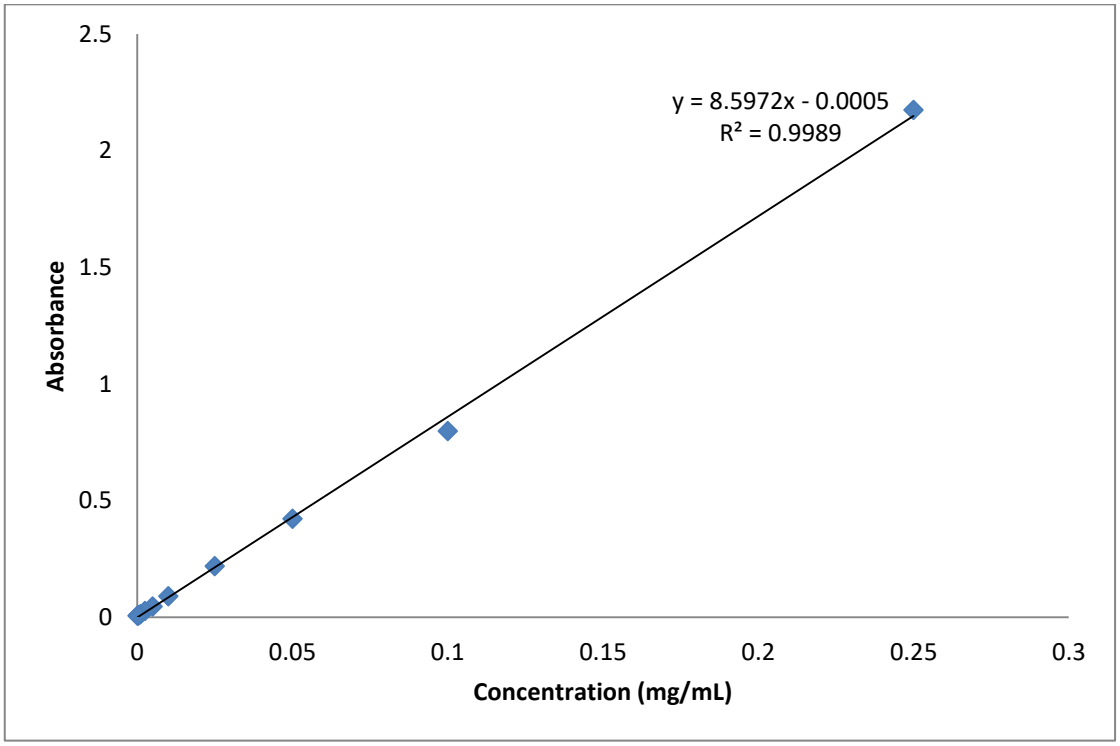


Figure 3.3: Standard Calibration Curve for Gallic Acid

3.5.2 Total Flavonoids Content

Measure the total flavonoids content by aluminium chloride colorimetric assay. (Abouzid and Elsherbeiny, 2008). Add 0.2mL of crude extract or standards solution of quercetin to a centrifuge tube containing 4.8ml ultrapure water. Then, add 0.3ml of 5% $NaNO_2$ into it and mix well. After 5 minutes, add 0.3ml of 10% $AlCl_3$ and mix well. At 6^{th} minutes, add 2ml 1M NaOH solution and make the final volume of 10 ml with ultrapure water. Measure the absorbance against prepared reagent blank at λ=414nm using a calibrated UV-Vis. (Chang et al., 2002). Express the total flavonoid content as mg quercetin in Figure 3.4 equivalents per gram dry weight (mg QE/g DW) by comparing the calibration curve for quercetin using equation Eq. 3.2 (Pan et al., 2012)

$$Total\ Flavonoid\ Content\left(\frac{mg}{g}\right) = \frac{Y \times N \times V}{W} \qquad Eq.(3.2)$$

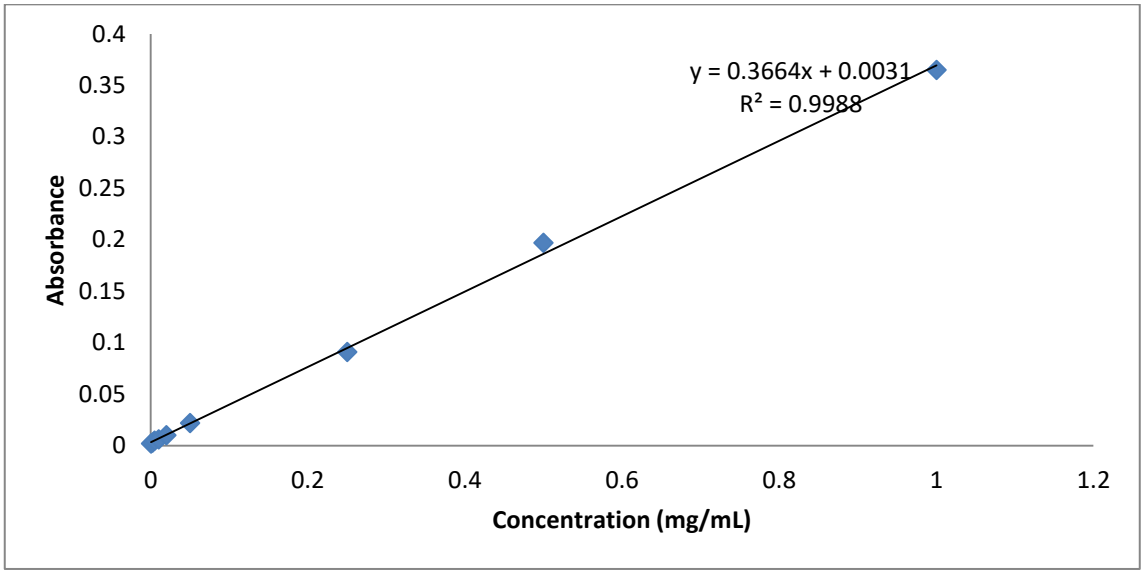


Figure 3.4: Calibration Curve for Quercetin

3.5.3 Ultra-performance liquid chromatography

Major constituents from the P. Niruri extract(Phyllanthin, Gallic acid and Quercetin) are determine and quantify by Waters Acquity UPLC H-Class (Milford, MA) fitted with Acquity UPLC HSS T3 Column (2.1x75mm, 1.8µm) and Acquity UPLC HSS T3 VanGuard column guard (2.1x5 mm, 1.8µm). The UPLC system is equipped with photodiode array detector and connected to a computer running Water Empower 2 software. Employ an eluent system combination of A (0.1% formic acid in H2O) and B (0.1% formic acid in acetonitrile) at a flow rate of 0.3ml/min. The gradient elution: 0-5minutes, 30% B; 5-10minutes, 30-40% B; 10-15minutes, 40-50% B; 15-35minutes, 50-95% B; 35-45minutes, 95-5% B. Maintained the temperature at room temperature. Inject the injection volume of 2µl for each sample. Filter the sample by using the 0.2µm PES membrane filter before the injection to UPLC system. Detect the peak at 350nm.

3.6 Statistical Analysis

Repeat each test with a new batch of P. Niruri in triplicates. Perform the analysis of variance (ANOVA) by using the data analysis tools in Microsoft Excel 2010, and test a least significant difference (LSD) to compare the means with a confidence interval of 95%.

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, 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 be 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 number of bioactive components extracted. Extraction study on the review of the previous extraction method was studied in section 2. 3 where most of the previous work done on the phyllanthus niruri extraction method was soxhlet extraction. (Murugaiyah & Chan, 2007; Markom et al., 2007). Soxhlet extraction required high temperature and longer time to obtain higher yield of bioactive components. These traditional methods often time consuming together encounter with higher risk of thermal degradation of the polyphenols extraction due to exposure under high temperature for several hours. In order to overcome the such problems, ultrasonic assisted extraction (UAE) and microwave assisted extraction (MAE) method were introduced (Mediani et al., 2015; Zhang et al., 2011). Extraction referring to a mass transfer mechanism which required solvent

transport to the solid phase for dissolution process to yield solutes and then released the solutes from the solid phase to the external transport. With the UAE and MAE method of extraction able to minimise the limitation of traditional extraction method on inner and outer mass transfer therefore it able to increase the yield of extraction. Moreover, from section 2.3.1 and 2.3.2 discussed the ability of UAE and MAE in breaking the cell membrane and cell wall of the plant, hence it reduces the control of the inner mass transport during extraction. As a result, from the studies, UAE and MAE had been chosen in this research study.

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 various respect to its phenolic and flavonoid by various solvent. Hence, the combined effect of different extraction method and solvent was one of the aim of this research study. TPC and TFC analytical method were outlined in chapter 3 with the details of components constituent.

4.3 UPLC Quantification of Polyphenol

The most extensive method to analyse active components in Phyllanthus Niruri was HPLC (Murugaiyah & Chan, 2007; Cuto et al., 2013; Markom et al., 2007). From reported analysis obtained by previous researcher, it showed the retention time of active components separation required 32 to 45 minutes. Further literature study regarding on HPLC application and quantification of polyphenol from Phyllanthus Niruri was studied in section 2.5. Quick quantification method always desired the better option in method selection, most of the researcher look into fast liquid chromatography method in reducing the time consuming in the analysis. Ultra-performance liquid chromatography (UPLC) also another approach that solving the deficiency of HPLC method. In addition, there is no previous work on UPLC application in Phyllanthus Niruri was done in the literature.

Acquity UPLC RP-18 endcapped column was used for the separation of active components. The comprehensive separation method was outlined in section 3.5.3. From Figure 4-2 the chromatogram obtained, it showed a good separation for both methoxylated and hydroxylated compounds. Active components were identified by mean

of retention time as shown in Figure 4-1) and UV spectra from the standard. From the results obtained, it showed similar match on the extract to the standard (Figure 4-3, Figure 4-4, and Figure 4.5). Quantitative of active components was measured by the peak areas from the extract and compared with the calibrated results series from the standard obtained by Sigma Aldrich. Studied phenolic compound calibration curves showed good linearity (r2=0.998) in of 0.005-0.5g/mL concentration. The analysis time for this UPLC method in qualitative and quantitative analysis spent 20 minutes. Which is 1/3 time faster than other reported methods such as Ghosal et al. (2012) 32 minutes; Cuoto et al. (2013) 40 minutes and Annamalai and Lakshmi. (2009) 45 minutes.

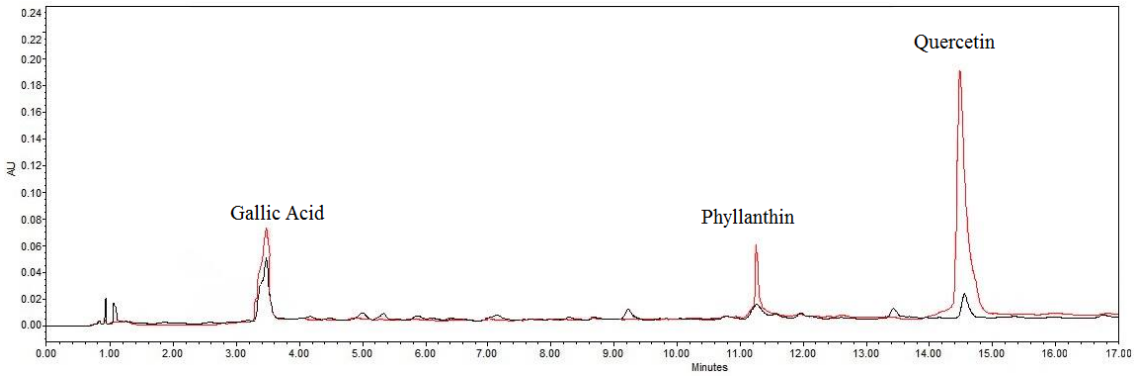


Figure 4-1: Identification of active compound by comparing retention time.

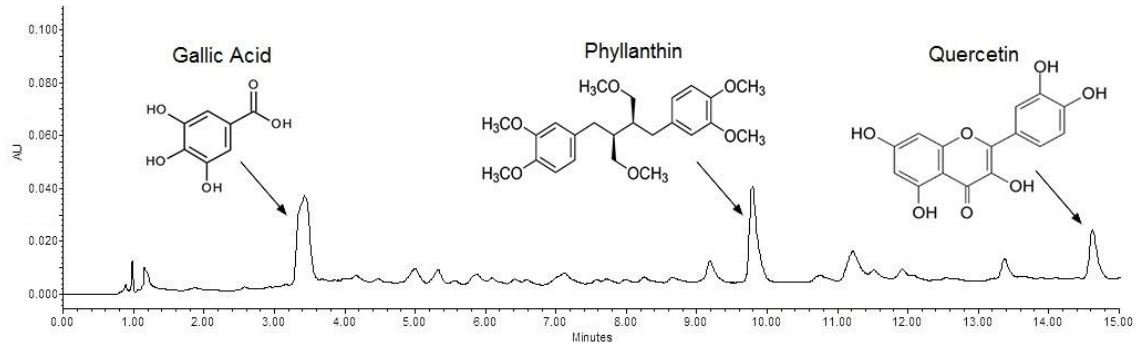


Figure 4-2: UPLC chromatogram of *P. Niruri* extract and chemical structures of the markers.

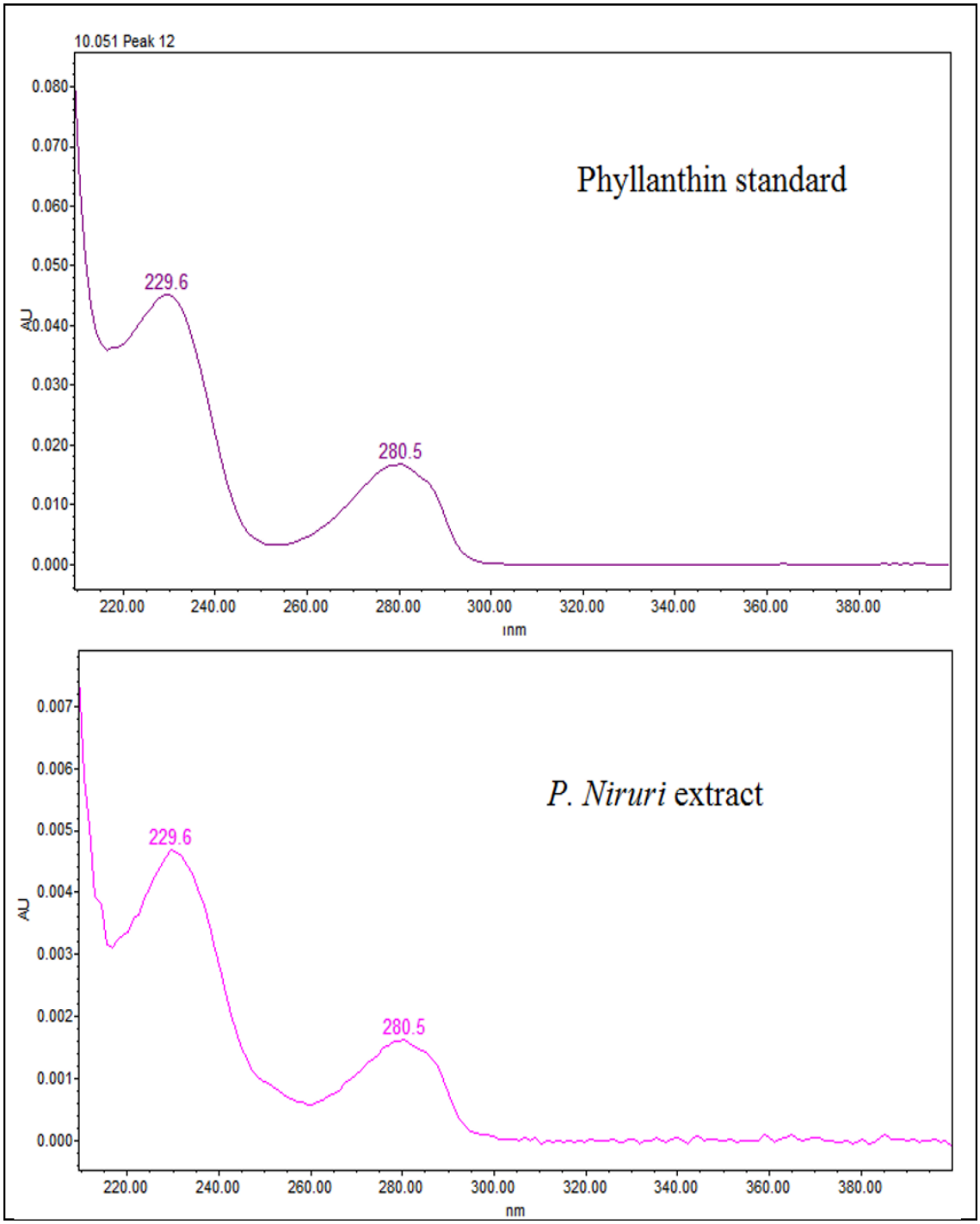


Figure 4-3: Identification of *phyllanthin* by matching UV spectra of sample to standard in Empower software library.

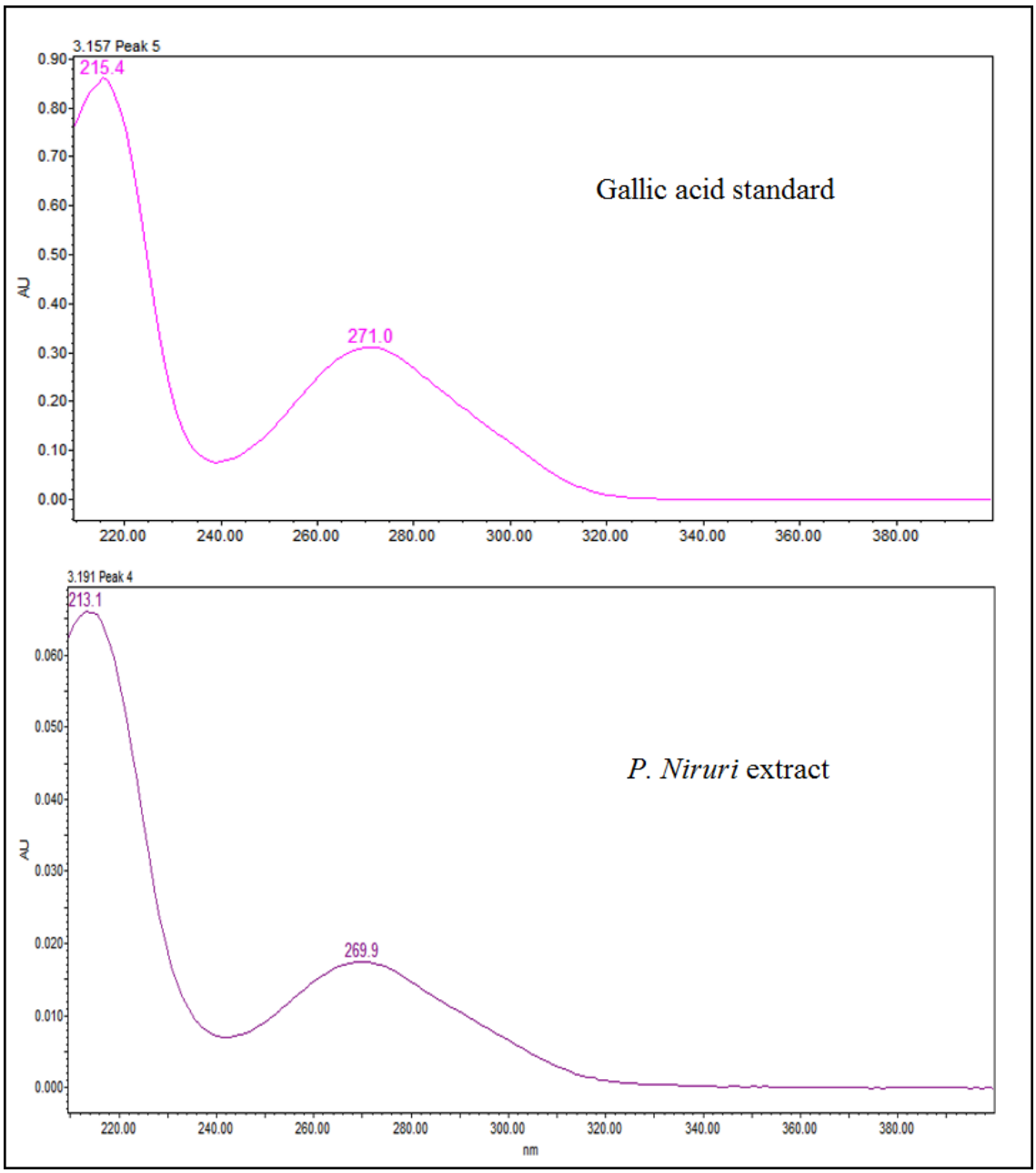


Figure 4-4: Identification of gallic acid by matching UV spectra of sample to standard in Empower software library.

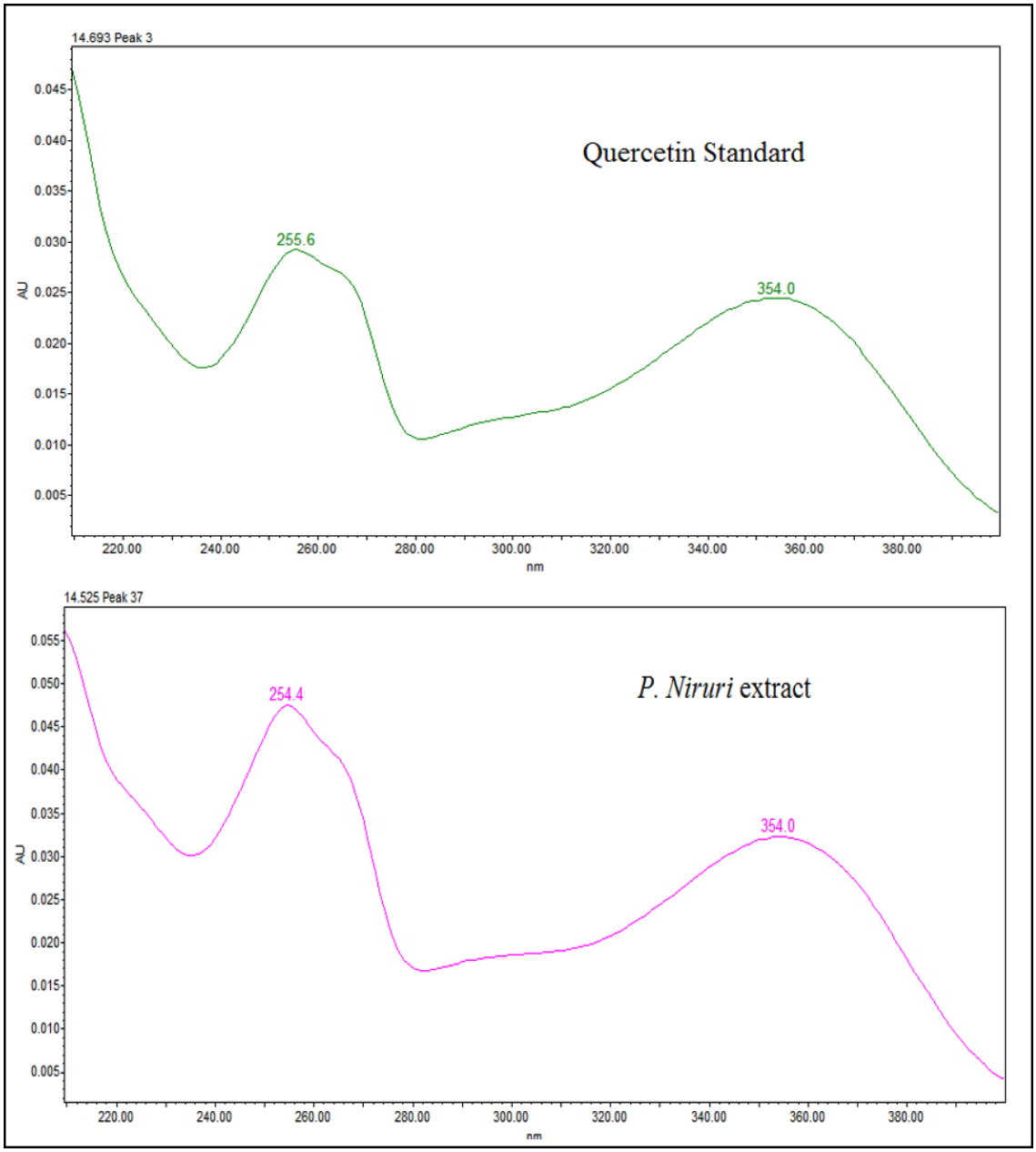


Figure 4-5: Identification of quercetin by matching UV spectra of sample to standard in Empower software library.

4.4 Influence of solvent type to the polyphenols extraction

Solvent type is the one of the factor affecting the extraction rate of the polyphenol. The rate of polyphenol extraction was determined by using the different polarities solvent (water, 20% ethanol, 40% ethanol, 60% ethanol, 80% ethanol, 100% ethanol, 20% isopropanol, 40% isopropanol, 60% isopropanol, 80% isopropanol and 100%

isopropanol) for UAE method. The details of the setup of the experiment was outlined in chapter 3 where variable to study the on the optimization of the solvent type and its polarities was examined at the end of this subchapter. In order to get a fair comparison, all extractions was carried out at equivalent solid to solvent ratio. Identical component of the bioactive compound was examined via UPLC and total phenolic content, total flavonoid content and antioxidant activity were determined spectrophotometrically.

Solvent the might yield different extraction performance toward the bioactive component extracted. Its structural characteristic enhances the ability of bioactive component to soluble in different solvent. Franco et al, (2008) and Metrouh-Amir et al. (2015) also found that the extraction yield is subjective toward the distinct polarities of extraction solvents together with its solubility of the compounds in the extraction solvent. From the finding of this chapter, higher methoxylated compounds; for example, phyllanthin and quercetin, the lipophilic compound which shows good stability towards lower polarity solvent. Similarly, Akowuah et al. (2005) 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, chloroform extract. These results also supported by find from Pang, S. F. (2013) where the at lower polarity solvent, isopropanol able to yield the highest methoxylated compounds. On the other hand, when dealing with highly hydroxylated compounds, gallic acid, it is more hydrophilic which yields higher solubility in ethanol. Thus, it is found highest extraction yield in water compared to phyllanthin and quercetin.

From the result obtained in Table 4-1, it suggests that alcoholic solvent with the range from 40 to 60% of both ethanol and isopropanol has a higher extracting capacity toward total phenolic content and flavonoid content compared to pure solvent such as 100% ethanol, 100% isopropanol and pure water. These finding is similar with what is reported by Zhi-feng et al. (2016) whereas the higher total phenolic content was found in the ethanol range of 50 to 70%. Polarity index of water, ethanol and isopropanol is 9.0, 4.2 and 3.9 respectively. From Table 4-1, 40% of isopropanol yields the highest phenolic (44.55mg GAE/g DW), flavonoid content (61.99 mg QE/g DW) and antioxidant activity (145.41 mg/g). 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. For instance, solvent with a lower polarity

index such as the isopropyl alcohol has a better efficiency in the extraction wider range of phenolic content. This finding is supported by the researcher Masturah et al. (2006) and 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, these finding had been confirmed by Tian et al. (2009).

Total phenolic content (TPC) and total flavonoid content (TFC) representing the total amount of polyphenols but not specific bioactive compound. TPC and TFC found least when using isopropanol as the extracting solvent which resulting 5.74 mg GAE/g DW and 12.30 mg QE/g DW respectively. On the other hand, bioactive compound yield from UPLC result showing the phyllanthin and quercetin having high yield at 4.31 mg Phy/g DW and 8.55 mg Que/g DW respectively when extracted by isopropanol. Thus, TPC and TFC is consider the result from the proximate analysis where it shouldn’t be taken in measuring the exact extraction yield.

Table 4-1: Effect of solvent on polyphenols extraction from *Phyllanthus Niruri.*

Solvent Type	**Polyphenol (mg GAE/g DW)**	**Flavonoid (mg QE/g DW)**	**Bioactive component**		
			Phyllanthin (mg Phy/g DW)	**Gallic Acid (mg GAE/g DW)**	**Quercetin (mg Que/g DW)**
Ethanol	23.48±0.078	32.17±0.369	4.43±0.146	0.99±0.088	7.34±0.730
Isopropanol	5.74±0.589	12.30±0.056	4.31±0.043	1.26±0.011	8.55±0.843
Water	33.43±1.082	38.39±1.742	0.55±0.290	15.44±2.436	3.19±0.539
20% Ethanol	40.26±0.461	49.57±1.041	4.41±0.038	13.19±0.368	10.14±4.519
40% Ethanol	42.54±0.943	60.74±0.214	4.41±0.132	9.86±0.125	5.48±0.314
60% Ethanol	40.63±0.215	53.30±1.094	4.45±0.050	9.20±0.102	6.12±0.206
80% Ethanol	37.51±0.739	48.21±0.942	4.51±0.196	4.42±0.221	6.74±1.148
20% Isopropanol	42.96±0.128	60.74±3.159	4.56±0.388	3.06±0.336	9.10±2.545
40% Isopropanol	44.55±0.078	61.99±1.609	4.34±0.013	2.67±0.323	8.50±1.437
60% Isopropanol	41.38±1.797	57.02±1.004	4.33±0.014	2.34±1.190	9.63±1.128
80% Isopropanol	33.12±0.63	49.57±0.672	4.30±0.083	2.21±0.139	8.45±0.455

Note: Means (three or more replicates) follows by at least one same letter are not significantly different (P > 0.0

4.5 Factorial Analysis on UAE

2^{5-1} factorial design with four 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 model fitting, interpreting the model graphically, finding the optimum point, and model validation.

4.5.1 Effect of Solvent purity, Time and Amplitude on UAE

The effect of solvent purity, time and amplitude in characterizing the extraction yield were summarized in Table 4-2. Variable on the solvent purity 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	Amplitude	Total Phenolic Content	Total Flavonoid content	Phyllanthin	Gallic Acid	Quercetin
		%	Minutes	%	mg GAE/g DW	mg QE/g DW	mg Phy/g DW	mg GAE/g DW	mg Que/g DW
8	1	80	9	90	32.074	41.496	80.842	4.009	97.825
4	2	80	9	20	22.420	27.850	54.790	2.803	84.179
5	3	20	3	90	30.271	31.944	80.063	3.784	88.273
1	4	20	3	20	21.373	22.391	42.704	2.672	78.720
3	5	20	9	20	28.992	37.402	80.211	3.624	93.731
7	6	20	9	90	36.960	45.590	92.638	4.620	101.919
2	7	80	3	20	13.638	12.839	30.724	1.705	69.168
6	8	80	3	90	25.502	27.850	67.953	3.188	84.179

4.5.1.1 Model Fitting and Effect Estimation for UAE

Simulation and analysis of experimental data by a completed 8 fractional factorial design was conducted using Design Expert 8.0.6 (Stat-Ease, USA), to calculate effect estimates using Yates algorithms systematically. From the researcher Anderson et al., (2009), the percent of contribution of the model comes from the consideration of total sum of square, then each sum of squares of the term was dividing by the total to yield a percentage. Table 4-3, Table 4-4, Table 4-5, Table 4-6 and Table 4-7 and illustrates the

effect of estimate and percent contributions calculated for phyllanthin, gallic acid, quercetin, total phenolic content and total flavonoid content. Low p-value (<0.05) for the factor indicates the statistical significant at 95% confidence level. From the tables below, it showed that the p-value for all the main factors and interactive factors are lesser than 0.05. Thus, this had confirmed that all the factors are statistically significant. Apparently, factor A, B and C played the major contribution in the polyphenol extraction which contributes more than 90% compared to interactive factors. The fitted model for the factorial analysis in coded form for phyllanthin, gallic acid, quercetin, total phenolic content, total flavonoid content and antioxidant activity was shown in Equation (4.1), Equation (4.2), Equation (4.3), Equation (4.4) and Equation (4.5) respectively.

$$Phyllanthin = 3.29 - 0.37 * A + 0.46 * B + 0.59 * C + 0.017 * \mathrm{A} * \mathrm{B} + 0.073 * A * C - 0.055 * B * C \quad (0.1)$$

$$Gallic\ acid = 4.18 - 1.18 * A + 0.95 * B + 0.92 * C - 0.12 * A * B - 0.24 * A * C + 0.028 * B * C \quad (4.2)$$

$$Quercetin = 66.24 - 7.66 * A + 10.88 * B + 14.13 * C - 1.64 * A * B + 1.69 * \mathrm{A} * C - 4.51 * B * C \quad (4.3)$$

$$Total\ Phenolic\ Content = 26.41 - 3.04 * A + 3.66 * B + 4.78 * C + 0.14 * A * B + 0.62 * A * C - 0.36 * B * C \quad (4.4)$$

$$Total\ Flavonoid\ Content = 30.92 - 3.41 * A + 7.16 * B + 5.80 * C + 1.36 * A * C - 0.34 * B * C \quad (4.5)$$

Table 4-3: Sum of squares and the percent contribution for each term for Phyllanthin.

Term	Effect Estimate	Sum of Squares	% Contribution
A-EtOH Purity	-0.74794	1.118818	19.666
B-Time	0.915347	1.67572	29.454
C-Amplitude	1.187965	2.82252	49.612
AB	0.033568	0.002254	0.040
AC	0.14625	0.042778	0.752
BC	-0.1097	0.024066	0.423

Table 4-4: Sum of squares and the percent contribution for each term for Gallic acid.

Term	Effect Estimate	Sum of Squares	% Contribution
A-EtOH Purity	-2.360	11.140	43.440
B-Time	1.890	7.160	27.830
C-Amplitude	1.840	6.840	26.590
AB	-0.240	0.110	0.440
AC	-0.480	0.460	1.780
BC	0.056	0.006	0.024

Table 4-5: Sum of squares and the percent contribution for each term for Quercetin.

Term	Effect Estimate	Sum of Squares	% Contribution
A-EtOH Purity	-15.33	469.82	14.48
B-Time	21.76	946.94	29.18
C-Amplitude	28.27	1597.99	49.23
AB	-3.28	21.54	0.66
AC	3.37	22.77	0.70
BC	-9.03	162.99	5.02

Table 4-6: Sum of squares and the percent contribution for each term for Total Phenolic Content.

Term	Effect Estimate	Sum of Squares	% Contribution
A-EtOH Purity	-6.07732	73.8677	20.059
B-Time	7.317505	107.0918	29.080
C-Amplitude	9.559948	182.7852	49.635
AB	0.281713	0.158725	0.043
AC	1.244669	3.098404	0.841
BC	-0.71434	1.020561	0.277

Table 4-7: Sum of squares and the percent contribution for each term for Total Flavonoid Content.

Term	Effect Estimate	Sum of Squares	% Contribution
A-EtOH Purity	-6.82	93.11	11.81
B-Time	14.33	410.62	52.07
C-Amplitude	11.60	269.09	34.12
AB	0	0	0
AC	2.73	14.90	1.89
BC	-0.68	0.93	0.12

The relative effects were visually demonstrated by Pareto chart in Figure 4-6, Figure 4-7, Figure 4-8, Figure 4-9 and Figure 4-10 where the bar length is proportional to the absolute value of estimated effect. For the main effect, positive effect to be said when there's an increase to its high level result an increase in the response. On the other hand, negative effect is defined when an increase in its high level will yield in a decrease in response. For interactions, when both factors were a chance to the same level (either low or high) and the response will increase, that represent the positive effect. However, negative effect results both factors were change to the opposite level such as one at its low and another at its high, the response will increase. According to Martendal et al., (2007), positive effect (colored in orange) and negative effect (colored in blue) shown in the Pareto chart. Effect of t-value limit (black colored line) is considered as statistically significant at 95% confidence level. For the effect below t-value limit, are not likely to be statistically significant. Mee, (2009) stated that model with a small global p-value, Bonferroni's corrected t-test were performed based on the individual terms in the model in order to justify individual terms in forward selection of models. Anderson et al., (2009) found that any effect above Bonferroni's corrected t-value limit, colored red line in the Pareto chart is almost certainly significant. A quick analysis was performed on the selected effects using Pareto chart to statistically check for significance of the selected effects at 95% confidence level. All the selected effects (A, B and C) shown to be significant at t-value limit except for total flavonoid content while Interaction factor (AC) shown to be significant at t-value limit for gallic acid and quercetin.

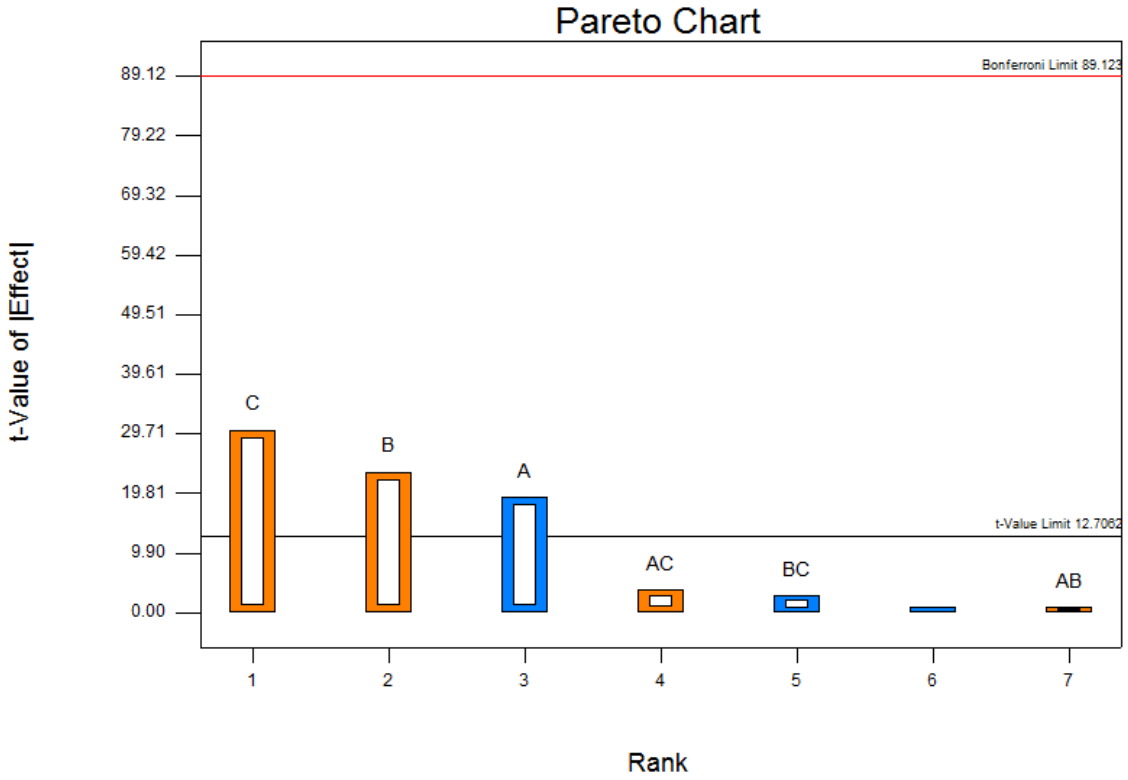


Figure 4-6: Pareto chart of effects of interfacial polymerization factors on Phyllanthin.

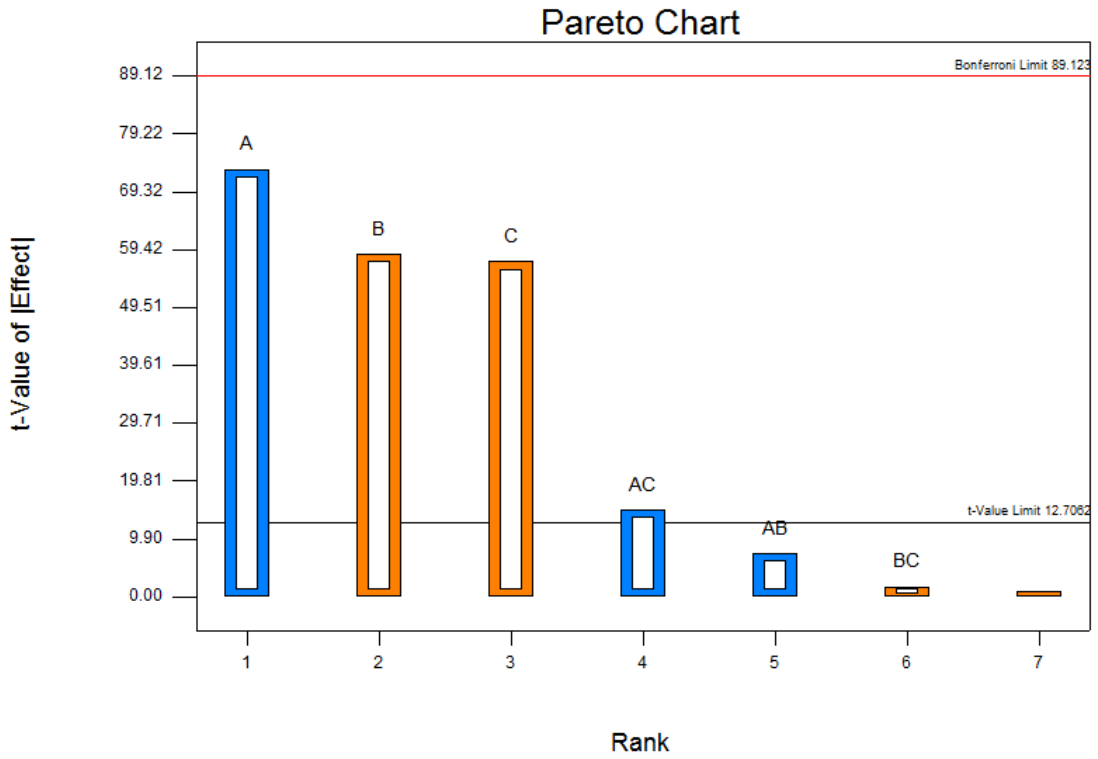


Figure 4-7: Pareto chart of effects of interfacial polymerization factors on Gallic Acid.

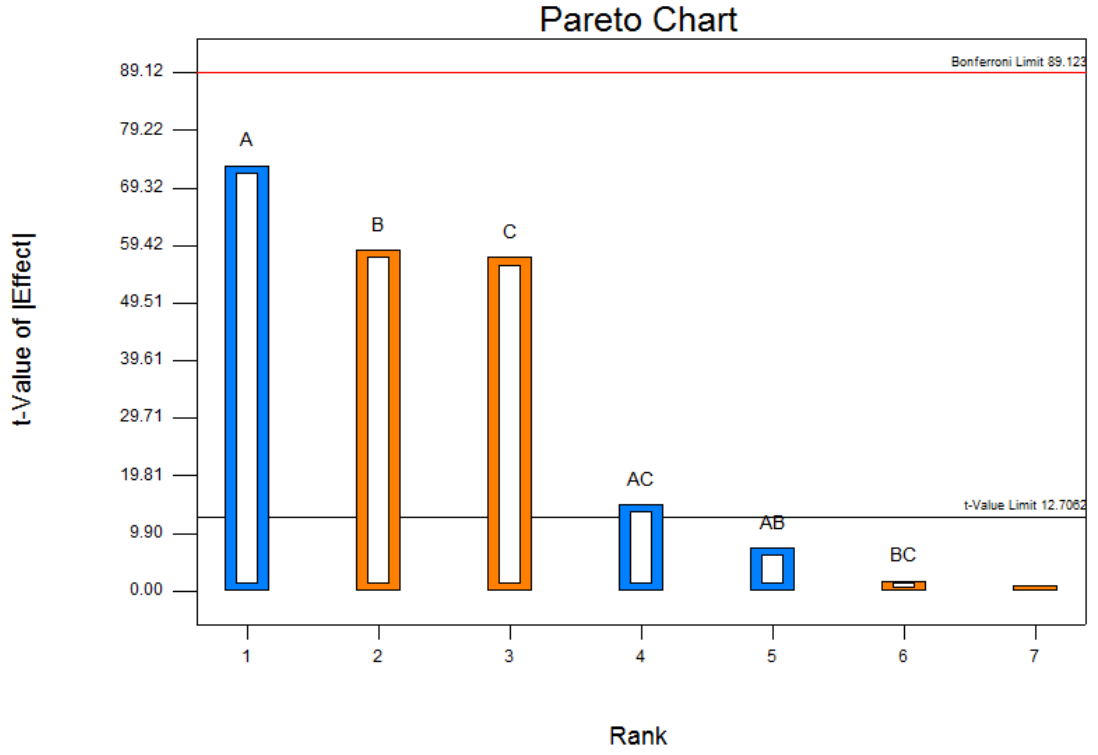


Figure 4-8: Pareto chart of effects of interfacial polymerization factors on Quercetin.

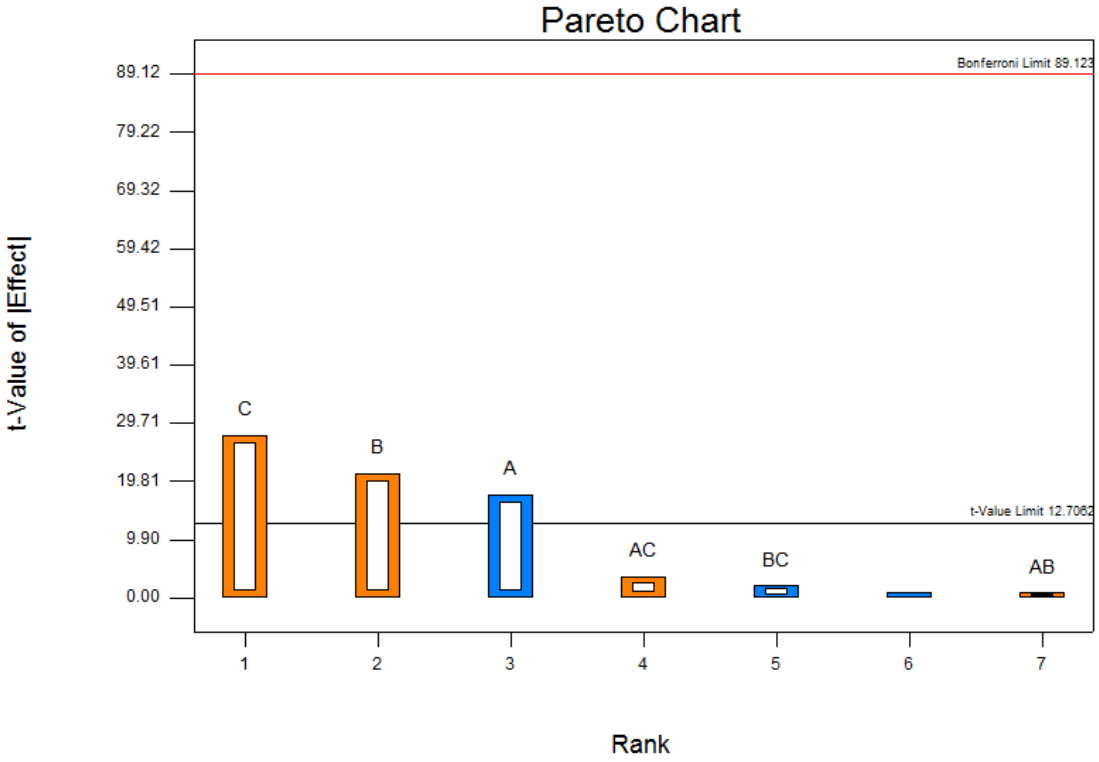


Figure 4-9: Pareto chart of effects of interfacial polymerization factors on Total Phenolic Content.

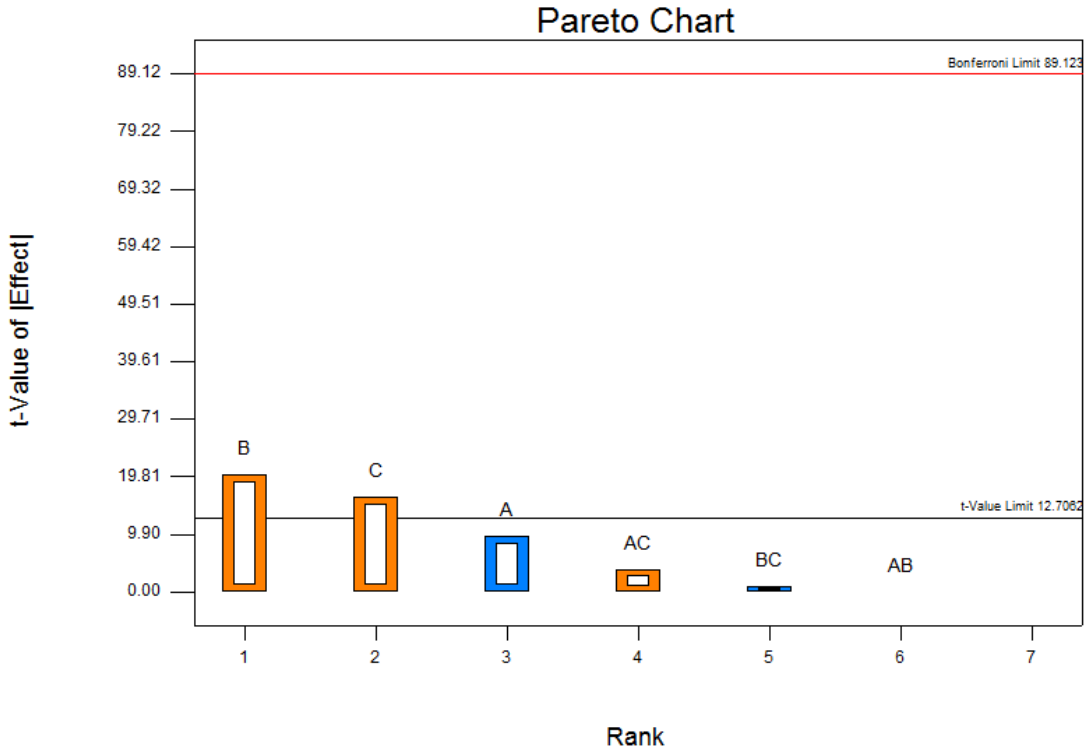


Figure 4-10: Pareto chart of effects of interfacial polymerization factors on Total Flavonoid Content.

4.5.1.2 ANOVA

All the models from section 4.5.1.1 with the selected effects were analyzed using analysis of variance (ANOVA) method and found significant for phyllanthin, gallic acid, quercetin, total phenolic content and total flavonoid content as presented in Table 4-8, Table 4-9, Table 4-10, Table 4-11 and Table 4-12 respectively. R^2, the coefficient of determination representing the proportion of variation in the response attributed to the model. High correlation ($R^2 \geq 0.9961$) between the experimental data and model data was obtained for all the responses. From this study, the regression coefficient for all the selected model terms is lower than the interception, which indicated the existent of the design plateau. Thus, this plateau showed that the design had an optimum point, where further optimization experiment can be performed (Box et al., 1978). The best experimental condition for factors in polyphenol extraction was shown in Table 4-13.

Table 4-8: ANOVA analysis for the factorial model for Phyllanthin.

Source	**SS[a]**	**Df[b]**	**MS[c]**	**F-Value**	**p-value**	
Model	5.686156	6	0.947693	309.4664	0.0435	significant
A-Extraction time	1.118818	1	1.118818	365.3469	0.0333	
B-Power	1.67572	1	1.67572	547.2015	0.0272	
C-Ethanol	2.82252	1	2.82252	921.686	0.0210	
AB	0.002254	1	0.002254	0.735909	0.5486	
AC	0.042778	1	0.042778	13.96906	0.1664	
BC	0.024066	1	0.024066	7.858847	0.2181	
Residual	0.003062	1	0.003062			
Cor Total	5.689219	7				
C.V. =1.68; R^2=0.9995; Adjusted R^2=0.9962; Adeq. Precision=55.08.						

[a]Sum of squares.
[b]Degree of freedom.
[c]Mean Square

Table 4-9: ANOVA analysis for the factorial model for Gallic Acid.

Source	**SS[a]**	**Df[b]**	**MS[c]**	**F-Value**	**p-value**	

Model	25.71014	6	4.285024	2058.347	0.0169	**significant**
A-Ethanol Purity	11.14118	1	11.14118	5351.761	0.0087	
B-Time	7.155794	1	7.155794	3437.346	0.0109	
C-Amplitude	6.836402	1	6.836402	3283.923	0.0111	
AB	0.112875	1	0.112875	54.22032	0.0859	
AC	0.45767	1	0.45767	219.8457	0.0429	
BC	0.006216	1	0.006216	2.986122	0.3340	
Residual	2.08E-03	1	2.08E-03			
Cor Total	25.71222	7				
C.V. =1.09%; R^2=0.9999; Adjusted R^2=0.9994; Adeq. Precision=142.94.						

[a]Sum of squares.
[b]Degree of freedom.
[c]Mean Square

Table 4-10: ANOVA analysis for the factorial model for Quercetin.

Source	**SS[a]**	**Df[b]**	**MS[c]**	**F-Value**	**p-value**	
Model	6581.796	6	1096.966	2058.347	0.0169	**significant**
A-Ethanol Purity	2852.143	1	2852.143	5351.761	0.0087	
B-Time	1831.883	1	1831.883	3437.346	0.0109	
C-Amplitude	1750.119	1	1750.119	3283.923	0.0111	
AB	28.89593	1	28.89593	54.22032	0.0859	
AC	117.1636	1	117.1636	219.8457	0.0429	
BC	1.59141	1	1.59141	2.986122	0.3340	
Residual	5.33E-01	1	5.33E-01			
Cor Total	6582.329	7				
C.V. =1.07%; R^2=0.9999; Adjusted R^2=0.9994; Adeq. Precision=140.82						

[a]Sum of squares.
[b]Degree of freedom.
[c]Mean Square

Table 4-11: ANOVA analysis for the factorial model for Total Phenolic Content.

Source	**SS[a]**	**Df[b]**	**MS[c]**	**F-Value**	**p-value**	
Model	368.0223	6	61.33706	257.1104	0.0477	significant
A-Extraction time	73.8677	1	73.8677	309.6359	0.0361	
B-Power	107.0918	1	107.0918	448.9033	0.0300	
C-Ethanol	182.7852	1	182.7852	766.1924	0.0230	
AB	0.158725	1	0.158725	0.665336	0.5644	
AC	3.098404	1	3.098404	12.98778	0.1723	
BC	1.020561	1	1.020561	4.277952	0.2867	
Residual	0.238563	1	0.238563			
Cor Total	368.2609	7				
C.V. =1.85%; R^2=0.9994; Adjusted R^2=0.7968; Adeq. Precision=7.198.						

[a]Sum of squares.
[b]Degree of freedom.
[c]Mean Square

Table 4-12: ANOVA analysis for the factorial model for Total Flavonoid Content.

Source	**SS[a]**	**Df[b]**	**MS[c]**	**F-Value**	**p-value**	
Model	788.6467	6	131.4411	63660000	< 0.0001	**significant**
A-Ethanol Purity	93.11059	1	93.11059	63660000	< 0.0001	
B-Time	410.6177	1	410.6177	63660000	< 0.0001	
C-Amplitude	269.0896	1	269.0896	63660000	< 0.0001	
AB	0	1	0			
AC	14.89769	1	14.89769	63660000	< 0.0001	
BC	0.931106	1	0.931106	63660000	< 0.0001	
Residual	0.00E+00	1	0.00E+00			
Cor Total	788.6467	7				
C.V. =0.00%; R^2=1.0000; Adjusted R^2=1.0000.						

[a]Sum of squares.
[b]Degree of freedom.
[c]Mean Square

Table 4-13: 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

4.5.1.3 Effect of main factors on all the responses

All the main factors studied were statistically significant at 95 % confidence level toward phtyllanthin, gallic acid, quercetin, total phenolic content, total flavonoid content and antioxidant activity was presented in Pareto chart in section 4.5.1.2. Factor A, B and C was found to have positive effect. The main effects on polyphenol extraction were factor A, B and C. Factor B and C was described in past study to be the main factor in determining the extraction yield, by higher the factor B and C will increased the polyphenol extraction. But polyphenol tense to degrade if extract when further increase the factor A, B and C. (Sousa et al., 2016). Factor A, B and C highly affected quercetin extraction. This might be due to the quercetin component contain hydroxyl group which is prone to higher solubility when contact to lower polarity of factor A (Akowuah et al., 2005). Interaction of AC showed positive effect towards gallic acid and quercetin. From the previous researcher, Hashemi et al., (2016) and Quy et al., (2014), factor A higher C at the constant factor B and at lower A with the constant B tense to yield higher phenolic and flavonoid content. This finding supported AC interaction yield the positive effect in 2 level factorial analysis.

4.5.1.4 Validation of Model

The validation experiments were conducted based on one suggested best condition in from Design Expert 8.0.4 in triplicate. The experiments were performed according to the suggested best condition in Table 4-13 and the result is presented in Table 4.14. The validation experiments were conducted at the suggested best conditions and the error from these runs were not more than 10%. Based on the predicted and experimental results presented, the experimental values were in good agreement with the

predicted values proposed by the model with an error less than 10 % and proved to be an adequate model.

Table 4-14: Comparison between predicted and experimental value for best condition.

Response		Predicted Value	Experimental Value	**Error**
Phyllanthin	**Run 1**	5.225	5.245	0.381
	Run 2	5.225	4.620	7.028
	Run 3	5.225	4.366	2.628
Gallic Acid	**Run 1**	8.231	8.215	0.195
	Run 2	8.231	7.596	8.360
	Run 3	8.231	7.574	8.674
Quercetin	**Run 1**	90.926	91.085	0.175
	Run 2	90.926	89.448	1.652
	Run 3	90.926	92.891	2.115
Total Phenolic Content	**Run 1**	38.800	38.960	0.411
	Run 2	38.800	36.960	4.978
	Run 3	38.800	37.460	3.577
Total Flavonoid Content	**Run 1**	47.734	47.733	0.002
	Run 2	47.734	45.590	4.703
	Run 3	47.734	48.990	2.564
Antioxidant Activity	**Run 1**	89.289	94.213	5.226
	Run 2	89.289	92.638	3.615
	Run 3	89.289	92.733	3.714

4.5.2 Optimization on the polyphenol extraction.

CCD with a total of 20 experiments which including 7 runs for factorial design, 7 runs for axial points and 6 runs of repetitions at the central point were performed and analysed at the end of this chapter. The CCD experimental design and tabulated results were shown in Table4-15. All the responses were also analysed from the fitting a model, and interpreting the model graphically, then find the optimum point and validate the model

Table 4-15: 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)
16	1	48.52	11.00	82.50	4.446	8.207	14.996	44.462	64.585
20	2	15.00	15.00	75.00	4.231	7.214	14.569	42.310	62.433
11	3	27.50	11.00	95.11	3.841	6.114	14.043	38.414	58.537
15	4	27.50	11.00	82.50	3.859	7.524	19.092	38.588	58.711
4	5	27.50	17.73	82.50	4.696	10.254	16.139	46.963	67.086
1	6	40.00	7.00	75.00	4.266	8.125	14.954	42.659	62.782
7	7	27.50	11.00	82.50	4.690	7.414	16.994	46.905	67.028
2	8	27.50	11.00	82.50	4.115	8.820	18.645	41.147	61.270
19	9	27.50	11.00	69.89	4.458	6.365	14.741	44.578	64.701
14	10	15.00	15.00	90.00	4.440	8.781	14.269	44.404	64.527
13	11	27.50	4.27	82.50	3.789	5.786	15.053	37.890	58.013
5	12	27.50	11.00	82.50	4.103	8.082	19.616	41.031	61.154
9	13	27.50	11.00	82.50	4.283	7.937	17.875	42.834	62.957
8	14	40.00	7.00	90.00	4.208	7.948	18.433	42.078	62.201
10	15	15.00	7.00	75.00	3.370	6.044	14.635	33.703	53.826
17	16	6.48	11.00	82.50	3.551	7.598	18.422	35.506	55.629
6	17	27.50	11.00	82.50	4.225	6.898	18.388	42.252	62.375
12	18	15.00	7.00	90.00	3.638	7.465	12.931	36.378	56.501
18	19	40.00	15.00	75.00	4.708	9.737	18.113	47.079	67.202
3	20	40.00	15.00	90.00	5.214	11.500	15.711	52.139	72.262

4.5.2.1 Model Fitting

The experimental data shown in Table 4-15 were used to estimate the appropriate model for the response using Design Expert software. Fit Summary is a part of Design Expert, providing statistical tables that can be used to identify which model to choose for in depth study (Anderson et al., 2009). The statistical tables are sequential model sum of squares in Table 4-16, Table 4-17, Table 4-18, Table 4-19 and Table 4-20. For lack of fit test in was tabulated in Table 4-21, Table 4-22, Table 4-23, Table 4-24 and Table 4-25.

Montgomery and Runger, (2010) defined the reduction in the error sum of squares when one or more predictor variables are added to the regression model as sequential sum of squares. It is performed by starting with the mean and adding terms such as linear, two-factor interaction, quadratic, and cubic. The F-statistic is calculated for each type of model, and the highest order model with significant terms would be chosen for the statistic. Significance of the model is judged by the probability of the F-statistic calculated from the data exceeds a theoretical value. The probability decreases as the value of the F-statistic increases. According to Simon, (2003), if the probability is less than 0.05 the terms are significant and their inclusion improves the model. Thus, the model with p-value less than 0.05 in sequential model sum of square for all the response can be considered to be chosen to fit the response. From quadratic model fits the criteria to be chosen to fit the response.

Table 4-16: Sequential model sum of squares for Phyllanthin.

Source	Sum of Squares	df	Mean Square	F-Value	p-value
Mean vs Total	366.263	1	366.263		
Linear vs Mean	3.0159	3	1.0053	15.4221	< 0.0001
2FI vs Linear	0.07559	3	0.0252	0.33858	0.7978
Quadratic vs 2FI	0.07128	3	0.02376	0.26514	0.8490
Cubic vs Quadratic	0.45485	4	0.11371	1.54623	0.3012
Residual	0.44125	6	0.07354		
Total	370.322	20	18.5161		

Table 4-17: Sequential model sum of squares for Gallic Acid.

Source	Sum of Squares	df	Mean Square	F-Value	p-value
Mean vs Total	1245.22	1	1245.22		
Linear vs Mean	23.8102	3	7.93672	8.21372	0.0016
2FI vs Linear	1.68524	3	0.56175	0.53014	0.6695
Quadratic vs 2FI	4.14249	3	1.38083	1.43349	0.2905
Cubic vs Quadratic	4.33849	4	1.08462	1.22922	0.3903
Residual	5.29418	6	0.88236		
Total	1284.49	20	64.2247		

Table 4-18: Sequential model sum of squares for Quercetin.

Source	Sum of Squares	df	Mean Square	F-Value	p-value
Mean vs Total	3461.13	1	3461.13		
Linear vs Mean	16.0267	3	5.34224	9.78384	0.0007
2FI vs Linear	0.4956	3	0.1652	0.26061	0.8525
Quadratic vs 2FI	1.34904	3	0.44968	0.65249	0.5993
Cubic vs Quadratic	6.45041	4	1.6126	21.9213	0.0010
Residual	0.44138	6	0.07356		
Total	3485.9	20	174.295		

Table 4-19: Sequential model sum of squares for Total Phenolic Content.

Source	Sum of Squares	df	Mean Square	F-Value	p-value
Mean vs Total	35391	1	35391		
Linear vs Mean	288.097	3	96.0323	16.247	< 0.0001
2FI vs Linear	3.78956	3	1.26319	0.18089	0.9075
Quadratic vs 2FI	6.08173	3	2.02724	0.23934	0.8670
Cubic vs Quadratic	40.9251	4	10.2313	1.40231	0.3382
Residual	43.7761	6	7.29601		
Total	35773.7	20	1788.68		

Table 4-20: Sequential model sum of squares for Total Flavonoid Content.

Source	Sum of Squares	df	Mean Square	F-Value	p-value
Mean vs Total	94919	1	94919		
Linear vs Mean	354.333	3	118.111	18.6444	< 0.0001
2FI vs Linear	4.63074	3	1.54358	0.20745	0.8894
Quadratic vs 2FI	7.88384	3	2.62795	0.29579	0.8277
Cubic vs Quadratic	50.7195	4	12.6799	1.99554	0.2143
Residual	38.1246	6	6.3541		
Total	95374.7	20	4768.74		

First, the type of model such as linear, sequential sum of squares for the two-factor interaction, quadratic, and cubic need to be selected, then perform a lack of fit test using ANOVA to compare the residual error to the pure error from replication. If residual error significantly exceeds pure error, the model will show significant lack of fit, and another model may be more appropriate. Thus, the desired result in a lack of fit test is to test the model selected is insignificant in lack of fit (p-value > 0.1) (Anderson et al., 2009; Simon, 2003). The lack of fit test in Table 4-21, Table 4-22, Table 4-23, Table 4-24, and Table 4-25 shows both quadratic and cubic model is insignificant in lack of fit.

Table 4-21: Lack of fit test for Phyllanthin.

Source	Sum of Squares	df	Mean Square	F-Value	p-value
Linear	0.63784	11	0.05799	0.71564	0.7017
2FI	0.56225	8	0.07028	0.86739	0.5924
Quadratic	0.49098	5	0.0982	1.2119	0.4191
Cubic	0.03612	1	0.03612	0.44582	0.5339
Pure Error	0.40513	5	0.08103		

Table 4-22: Lack of fit test for Gallic Acid.

Source	Sum of Squares	df	Mean Square	F-Value	p-value
Linear	13.2844	11	1.20767	2.77492	0.1349
2FI	11.5991	8	1.44989	3.33148	0.1004
Quadratic	7.45663	5	1.49133	3.42669	0.1013
Cubic	3.11814	1	3.11814	7.16469	0.0440
Pure Error	2.17604	5	0.43521		

Table 4-23: Lack of fit test for Quercetin.

Source	Sum of Squares	df	Mean Square	F-Value	p-value
Linear	8.40348	11	0.76395	11.4723	0.0073
2FI	7.90787	8	0.98848	14.8441	0.0043
Quadratic	6.55883	5	1.31177	19.6989	0.0026
Cubic	0.10843	1	0.10843	1.62823	0.2580
Pure Error	0.33295	5	0.06659		

Table 4-24: Lack of fit test for Total Phenolic Content.

Source	Sum of Squares	df	Mean Square	F-Value	p-value
Linear	56.5446	11	5.14041	0.67588	0.7273
2FI	52.755	8	6.59437	0.86705	0.5926
Quadratic	46.6733	5	9.33465	1.22734	0.4138
Cubic	5.74821	1	5.74821	0.75579	0.4244
Pure Error	38.0278	5	7.60557		

Table 4-25: Lack of fit test for Total Flavonoid Content.

Source	Sum of Squares	df	Mean Square	F-Value	p-value
Linear	70.4676	11	6.40614	1.03689	0.5204
2FI	65.8368	8	8.22961	1.33203	0.3918
Quadratic	57.953	5	11.5906	1.87604	0.2533
Cubic	7.23347	1	7.23347	1.1708	0.3286
Pure Error	30.8911	5	6.17823		

From the observation of sequential model sum of squares and lack of fit table, it's summarize quadratic model is the most suitable model to be use in fitting the all responses. The results were fitted with a second-order polynomial equation. The values of regression coefficients were calculated, the response variable and the test variables are related by the second-order polynomial equation in Equation (4.6), Equation (4.7), Equation (4.8), Equation (4.9) and Equation (4.10). These equations are in coded form.

$$Phyllanthin = 4.25 + 0.33 * A + 0.33 * B + 0.00760 * C - 0.00023 * A * B - 0.031 * A * C + 0.092 * B * C - 0.033 * A^2 + 0.055 * B^2 + 0.021 * C^2 \quad (4.6)$$

$$Gallic\ Acid = 7.75 + 0.65 * A + +1.11 * B + 0.30 * C + 0.33 * A * B - 0.18 * A * C + 0.26 * B * C + 0.25 * A^2 + 0.29 * B^2 - 0.34 * C^2 \quad (4.7)$$

$$Quercetin = 13.59 + 1.60 * \mathrm{A} + 0.44 * \mathrm{B} - 0.25 * \mathrm{C} + 0.43 * A * B - 0.53 * A * C - 0.67 * B * C - 0.095 * -0.25 * B^2 + 0.056C^2 \quad (4.8)$$

$$Total\ Phenolic\ Content = 42.08 + 3.09 * A + 3.40 * B - 0.082 * C - 0.27 * A * B - 0.036 * A * C + 0.63 * B * C - 0.47 * A^2 + 0.39 * B^2 + 0.06 * C^2 \quad (4.9)$$

$$Total\ Flavonoid\ Content = 69.24 + 3.44 * A + 3.75 * B - 0.11 * C - 0.30 * A * B - 0.040 * A * C + 0.70 * B * C - 0.65 * A^2 + 0.27 * B^2 - 0.12 * C^2 \quad (4.10)$$

4.5.2.2 ANOVA

Table 4-26 summarizes the ANOVA results by considering a model is significant if the p-value is lower than 0.05. The p-value lower than 0.05 indicate that only 5% chance that a 'Model F-value' could occur due to noise. According to Tan et al., (2011) it is also used as indicator to evaluate the significance of the effects of each linear, quadratic and interaction term on the response. The p-value for the fitted model for all responses was less than 0.05, the fitted model equation adequately describes the response. In addition, the p-values for each model term suggest that A, B and C are the model terms that have significant effects on phyllanthin, gallic acid, total phenolic content and total flavonoid content. For quercetin and antioxidant activity response, only factor A is significant. Linear factor B and C is not significant as the p-value greater than 0.1. Although these factor B and C were not significant, these factors could not be excluded from the model in order to retain model hierarchy.

Table 4-26: ANOVA analysis for the optimization model for Phyllanthin.

Source	**SS[a]**	**Df[b]**	**MS[c]**	**F-Value**	**p-value**	
Model	3.162760987	9	0.351417887	3.9216	0.0221	**significant**
A-Ethanol Purity	1.51E+00	1	1.51E+00	16.8681	0.0021	
B-Time	1.50E+00	1	1.50E+00	16.7786	0.0022	
C-Amplitude	7.89E-04	1	7.89E-04	0.0088	0.9271	
AB	4.12E-07	1	4.12E-07	4.6E-06	0.9983	
AC	7.71E-03	1	7.71E-03	0.08606	0.7752	
BC	0.067872906	1	0.067872906	0.75742	0.4045	
A^2	0.016005984	1	0.016005984	0.17862	0.6815	
B^2	0.043805228	1	0.043805228	0.48884	0.5004	
C^2	0.006620866	1	0.006620866	0.07388	0.7913	
Residual	0.896107358	10	8.96E-02			
Lack of Fit	0.490976375	5	9.82E-02	1.2119	0.4191	**not significant**
Pure Error	0.405130983	5	8.10E-02			
Cor Total	4.058868344	19				
C.V. =7.00%; R^2=0.78; Adjusted R2=0.58; Adeq. Precision=6.86.						

[a]Sum of squares.
[b]Degree of freedom.
[c]Mean Square

Table 4-27: ANOVA analysis for the optimization model for Gallic Acid.

Source	**SS[a]**	**Df[b]**	**MS[c]**	**F-Value**	**p-value**	
Model	29.63788427	9	3.293098253	3.41868	0.0344	**significant**
A-Ethanol Purity	5.71E+00	1	5.71E+00	5.92743	0.0352	
B-Time	1.68E+01	1	1.68E+01	17.481	0.0019	
C-Amplitude	1.26E+00	1	1.26E+00	1.30973	0.2791	
AB	8.96E-01	1	8.96E-01	0.93055	0.3575	
AC	2.45E-01	1	2.45E-01	0.2548	0.6247	
BC	0.543431396	1	0.543431396	0.56415	0.4699	
A^2	0.916648025	1	0.916648025	0.9516	0.3523	
B^2	1.24260443	1	1.24260443	1.28999	0.2825	
C^2	1.624604273	1	1.624604273	1.68656	0.2232	
Residual	9.632672223	10	9.63E-01			
Lack of Fit	7.456627531	5	1.49E+00	3.42669	0.1013	**not significant**
Pure Error	2.176044692	5	4.35E-01			
Cor Total	39.2705565	19				
C.V. =12.44%; R^2=0.75; Adjusted R2=0.53; Adeq. Precision=5.99.						

[a]Sum of squares.
[b]Degree of freedom.
[c]Mean Square

Table 4-28: ANOVA analysis for the optimization model for Quercetin.

Source	**SS[a]**	**Df[b]**	**MS[c]**	**F-Value**	**p-value**	
Model	46.79881371	9	5.19986819	4.06631	0.0196	**significant**
A-Ethanol Purity	3.49E+01	1	3.49E+01	27.2858	0.0004	
B-Time	2.59E+00	1	2.59E+00	2.0216	0.1855	
C-Amplitude	8.85E-01	1	8.85E-01	0.69205	0.4249	
AB	1.50E+00	1	1.50E+00	1.16996	0.3048	
AC	2.21E+00	1	2.21E+00	1.72822	0.2180	
BC	3.612257907	1	3.612257907	2.82479	0.1237	
A^2	0.129754846	1	0.129754846	0.10147	0.7566	
B^2	0.935909641	1	0.935909641	0.73188	0.4123	
C^2	0.044637566	1	0.044637566	0.03491	0.8555	

Residual	12.78768395	10	1.28E+00			
Lack of Fit	12.28485028	5	2.46E+00	7.4312	0.3016	**significant**
Pure Error	0.502833666	5	1.01E-01			
Cor Total	59.58649766	19				
C.V. =8.45%; R^2=0.79 Adjusted R2=0.59; Adeq. Precision=8.23.						

[a]Sum of squares.
[b]Degree of freedom.
[c]Mean Square

Table 4-29: ANOVA analysis for the optimization model for Total Phenolic Content.

Source	**SS[a]**	**Df[b]**	**MS[c]**	**F-Value**	**p-value**	
Model	297.9682591	9	33.10758435	3.90875	0.0224	**significant**
A-Ethanol Purity	1.31E+02	1	1.31E+02	15.4118	0.0028	
B-Time	1.57E+02	1	1.57E+02	18.5907	0.0015	
C-Amplitude	9.20E-02	1	9.20E-02	0.01086	0.9191	
AB	5.79E-01	1	5.79E-01	0.06834	0.7991	
AC	1.06E-02	1	1.06E-02	0.00125	0.9725	
BC	3.200180443	1	3.200180443	0.37782	0.5525	
A^2	3.241501334	1	3.241501334	0.3827	0.5500	
B^2	2.184584809	1	2.184584809	0.25792	0.6226	
C^2	0.052486972	1	0.052486972	0.0062	0.9388	
Residual	84.70110753	10	8.47E+00			
Lack of Fit	46.67326198	5	9.33E+00	1.22734	0.4138	**not significant**
Pure Error	38.02784554	5	7.61E+00			
Cor Total	382.6693666	19				
C.V. =6.92%; R^2=0.78; Adjusted R2=0.58; Adeq. Precision=6.935.						

[a]Sum of squares.
[b]Degree of freedom.
[c]Mean Square

Table 4-30: ANOVA analysis for the optimization model for Total Flavonoid Content.

Source	**SS[a]**	**Df[b]**	**MS[c]**	**F-Value**	**p-value**	
Model	366.8476642	9	40.76085158	4.5879	0.0130	**significant**
A-Ethanol Purity	1.62E+02	1	1.62E+02	18.2062	0.0016	
B-Time	1.92E+02	1	1.92E+02	21.6579	0.0009	
C-Amplitude	1.64E-01	1	1.64E-01	0.01848	0.8946	
AB	7.07E-01	1	7.07E-01	0.07961	0.7836	
AC	1.29E-02	1	1.29E-02	0.00145	0.9703	
BC	3.910527899	1	3.910527899	0.44016	0.5220	
A^2	6.170824082	1	6.170824082	0.69457	0.4241	
B^2	1.020001497	1	1.020001497	0.11481	0.7417	
C^2	0.224572472	1	0.224572472	0.02528	0.8768	
Residual	88.84415595	10	8.88E+00			
Lack of Fit	57.95300958	5	1.16E+01	1.87604	0.2533	**not significant**
Pure Error	30.89114638	5	6.18E+00			
Cor Total	455.6918202	19				
C.V. =4..33%; R^2=0.81; Adjusted R2=0.63; Adeq. Precision=7.515.						

[a]Sum of squares.
[b]Degree of freedom.
[c]Mean Square

The R^2 for the model was ranging from 0.75 until 0.81, implying a good correlation between the observed and predicted values, as shown in Figure 4-11, Figure 4-12, Figure 4-13, Figure 4-14 and Figure 4-15. Based on the R^2 value, it indicates that not more than 25 % of the total variability was not explained by the model terms in the model. The good R^2 value represent the model obtained will be able to give a convincingly good estimate of response of the system within the range studied. The lack of fit test, which was not significant for the model, shows that the model satisfactorily fits the data. From all of these statistical tests, we can summarize that the developed model was suitable to represent the data. Furthermore, these data able to provide a good description on the relationship between the process variables and response.

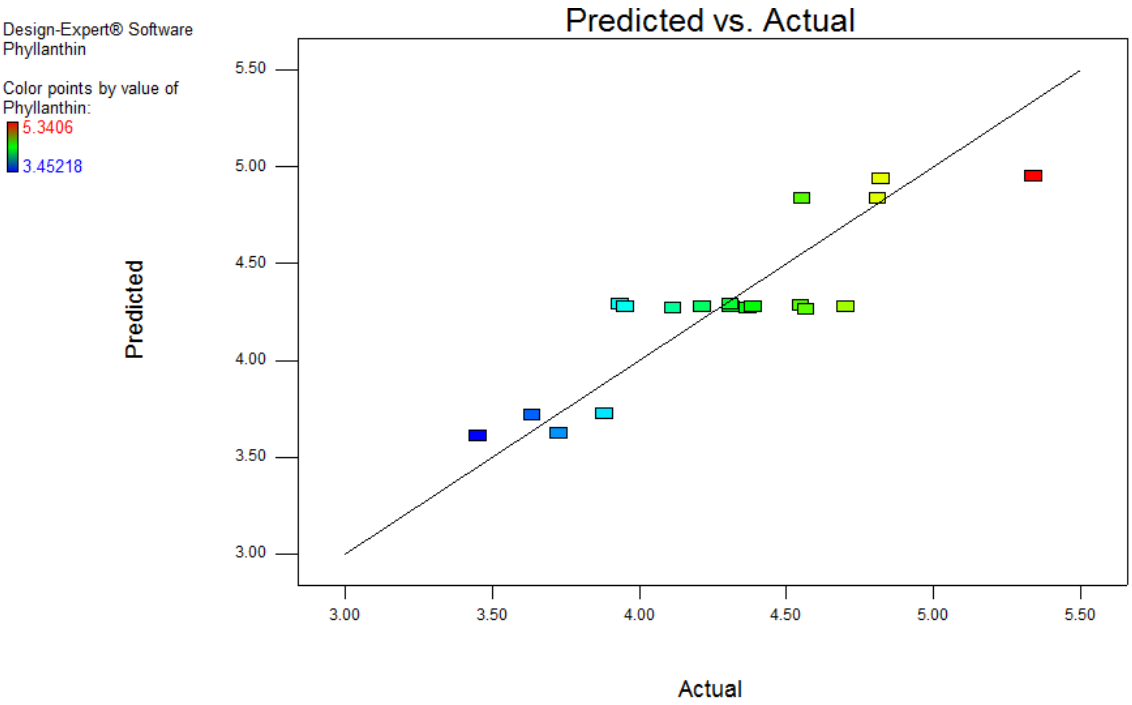


Figure 4-11: Predicted vs. actual phyllanthin colored by standard order.

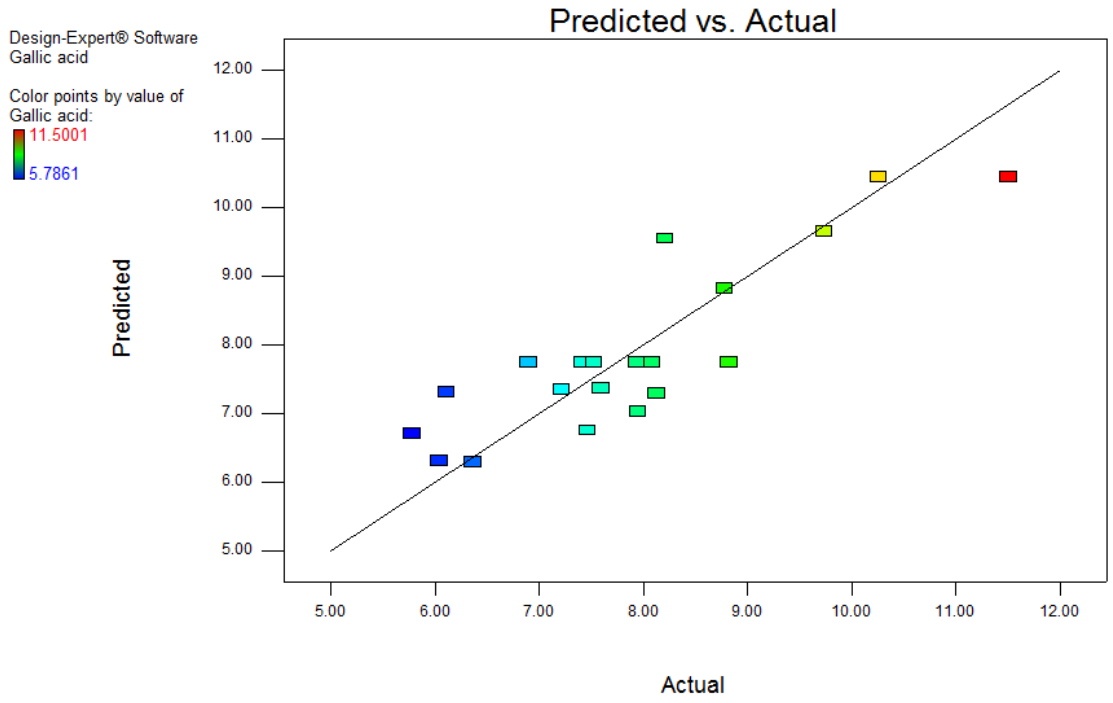


Figure 4-12: Predicted vs. actual gallic acid colored by standard order.

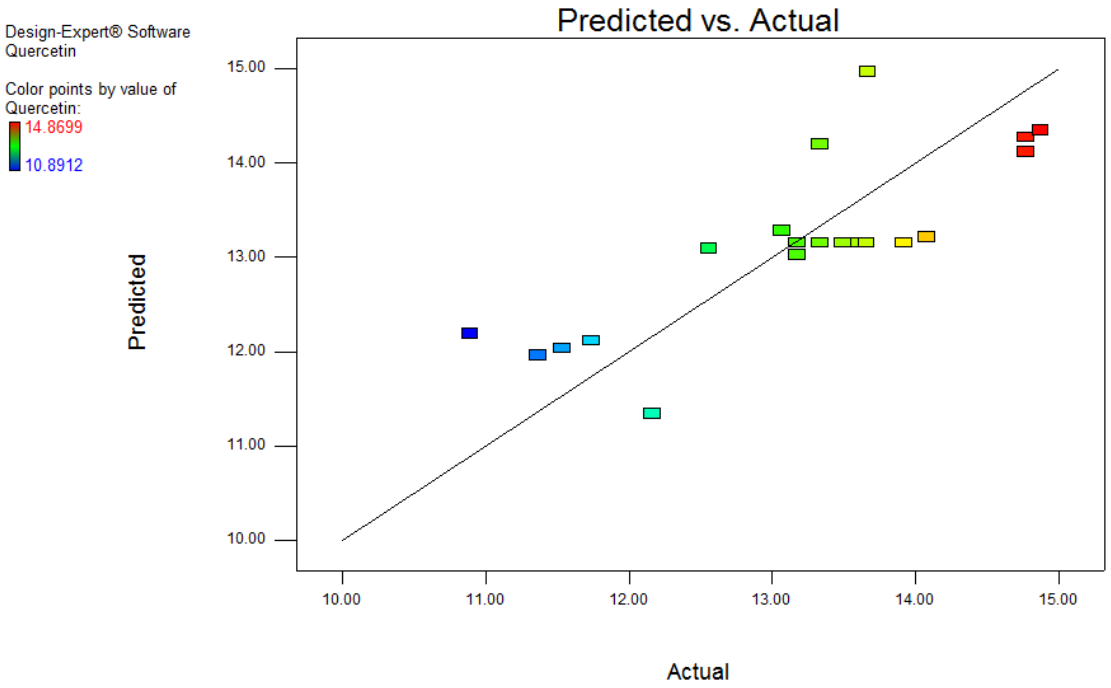


Figure 4-13: Predicted vs. actual quercetin colored by standard order.

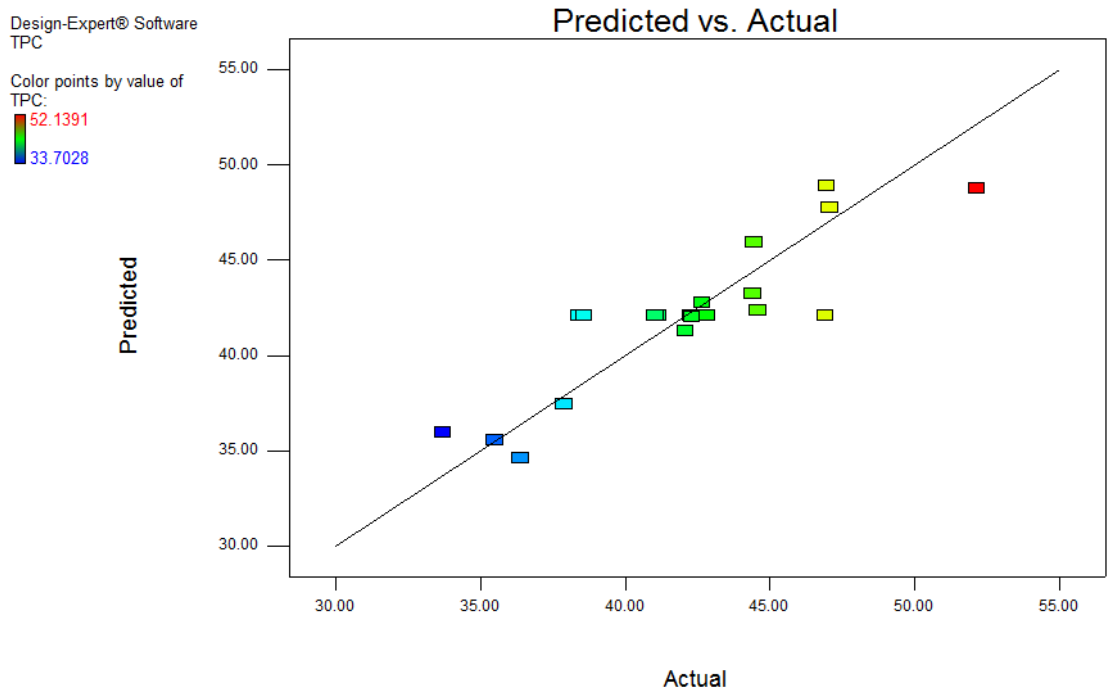


Figure 4-14: Predicted vs. actual total phenolic content colored by standard order.

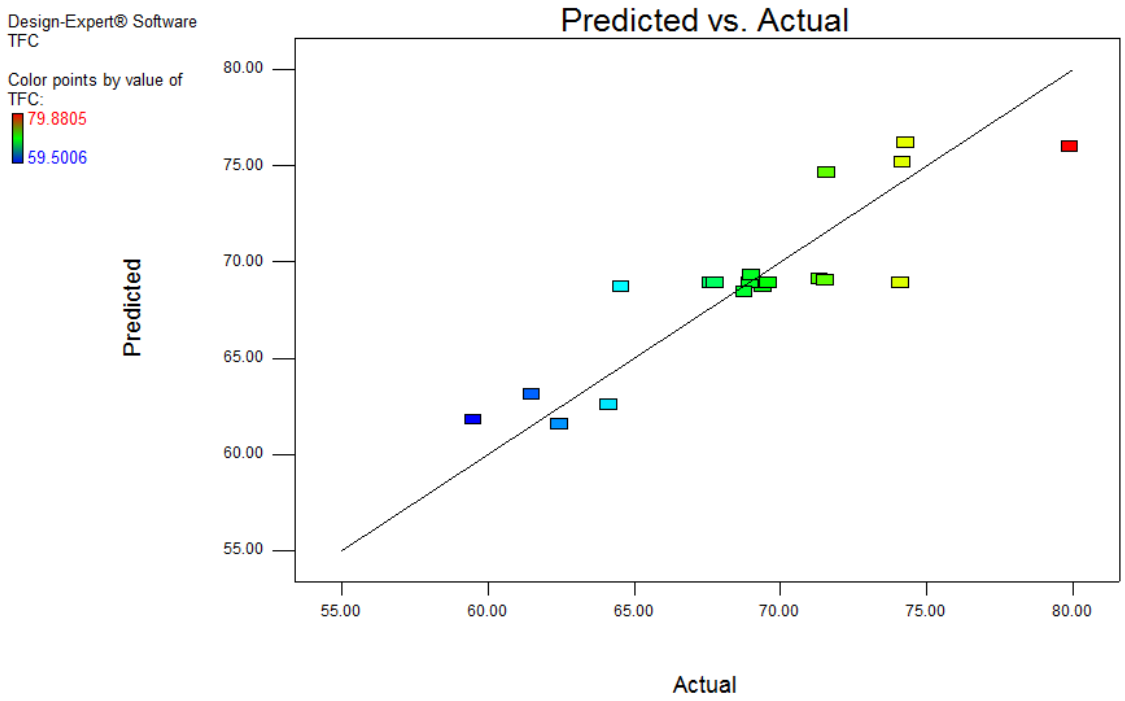


Figure 4-15: Predicted vs. actual total flavonoid content colored by standard order.

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

The effect of the three polymerization conditions, ethanol purity, extraction time and amplitude on the polyphenol extraction were analysed using RSM. Three-dimensional response surface and contour plot were generated to study the interactive effect of the variables on the response. The variables were studied by setting one constant at central level. Thus, a three-dimensional plat can give a clearer geometrical representation of the nature and the extent of the interaction between the variables and response within the experimental range studied.

The effect of non-interaction factors ethanol purity (A), time (B) and amplitude (C) on polyphenol extraction is depicted in Figure 4-16. Figure 4-17, Figure 4-18, Figure 4-19, Figure 4-20, Figure 4-21, Figure 4-22, Figure 4-23, Figure 4-24, Figure 4-25, Figure 4-26, Figure 4-27, Figure 4-28 and Figure 4-29 for all the response. Interaction effects has p-value higher than 0.100 indicating both interaction was non-significant to the response. This was also demonstrated in previously discussed factorial analysis. The non-existent of interaction can be explained in a simple manner. Interactions cannot be seen because the factors were not affecting the other factors in a same process but two different processes.

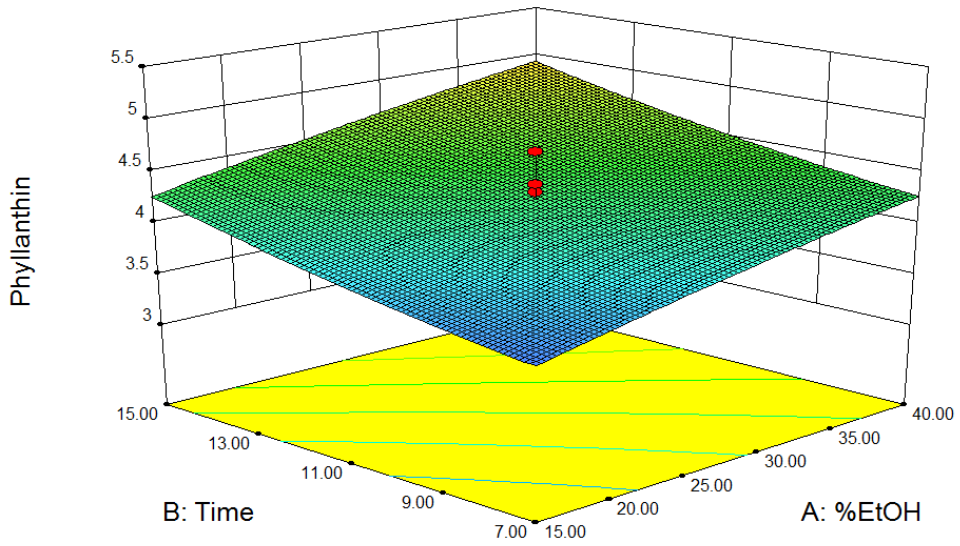


Figure 4-16: Three-dimensional response surface plot the effects of ethanol purity (A) and time (B) on phyllanthin at a constant amplitude at 82.50%.

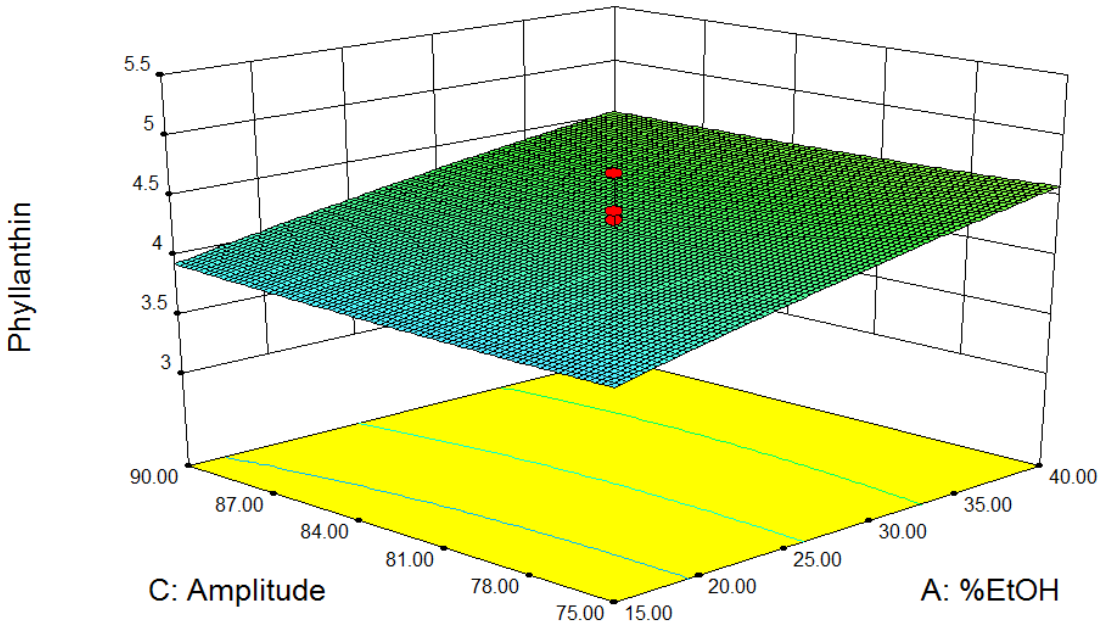


Figure 4-17: Three-dimensional response surface plot for the effects of ethanol purity (A) and amplitude (C) on phyllanthin at a constant time at 11 minute.

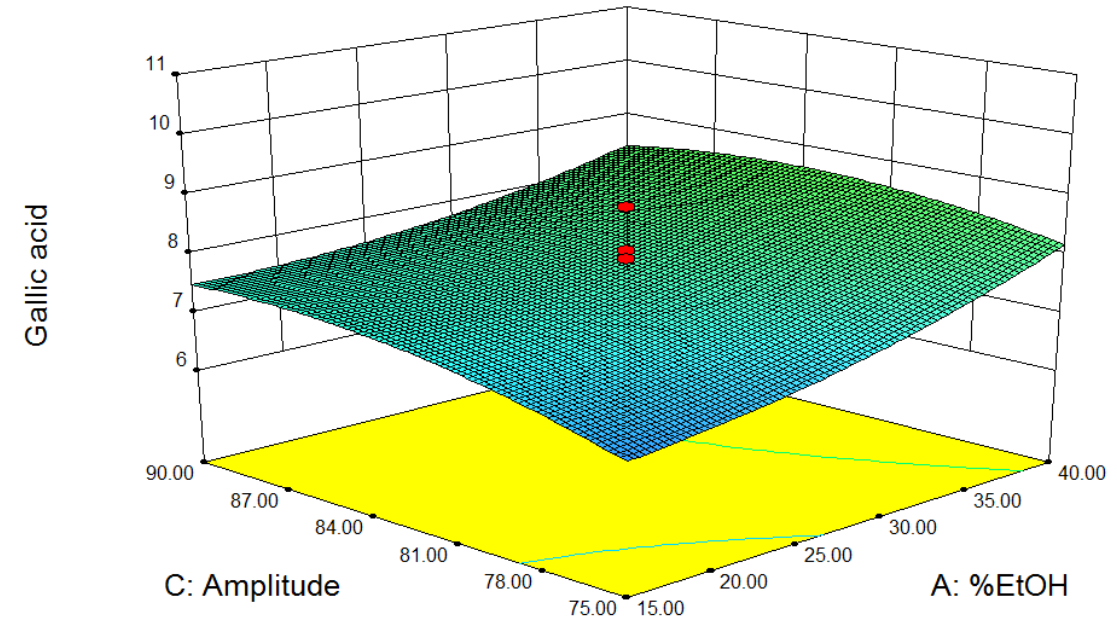


Figure 4-18: Three-dimensional response surface plot for the effects of ethanol purity (A) and amplitude (C) on gallic acid at a constant time at 11 minute.

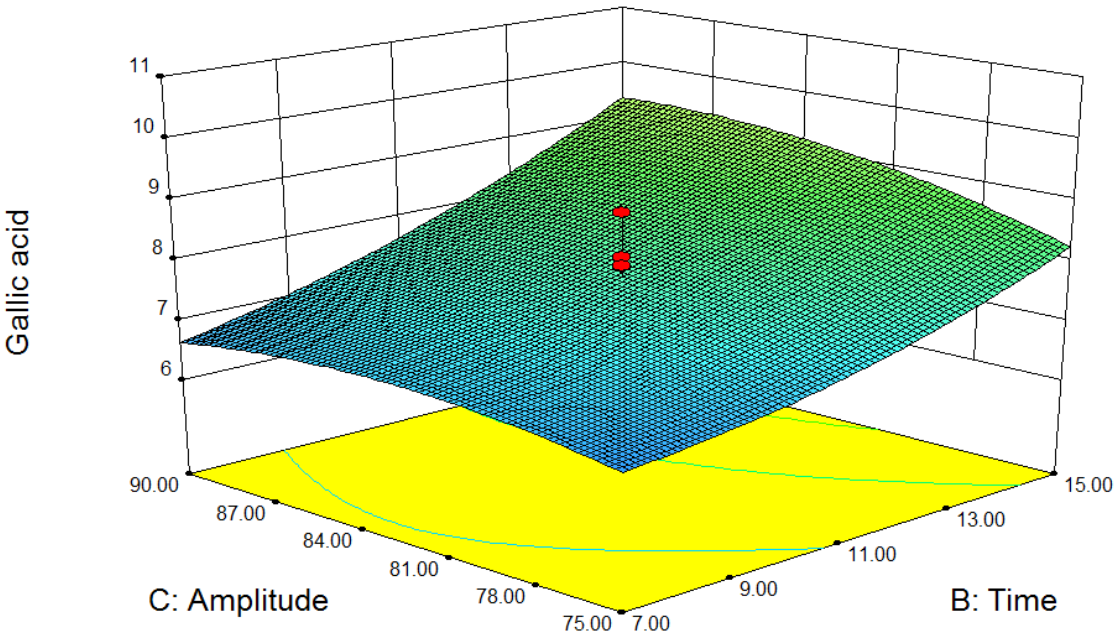


Figure 4-19: Three-dimensional response surface plot for the effects of time (B) and amplitude (C) on gallic acid at a constant ethanol purity at 27.50%.

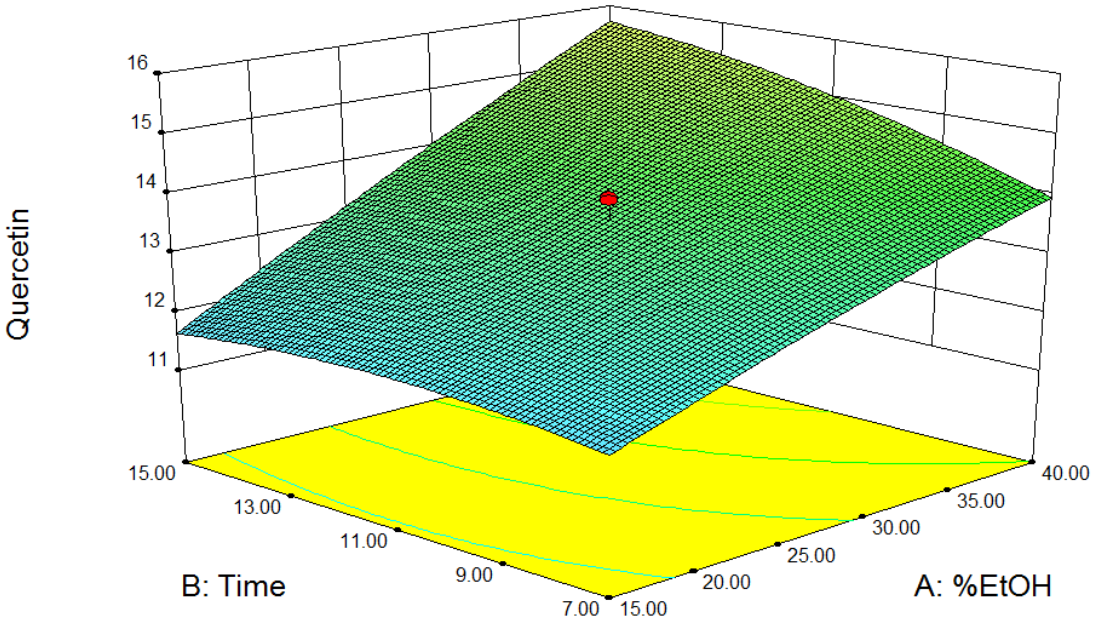


Figure 4-20: Three-dimensional response surface plot for the effects of ethanol purity (A) and time (B) on quercetin at a constant amplitude at 82.50%.

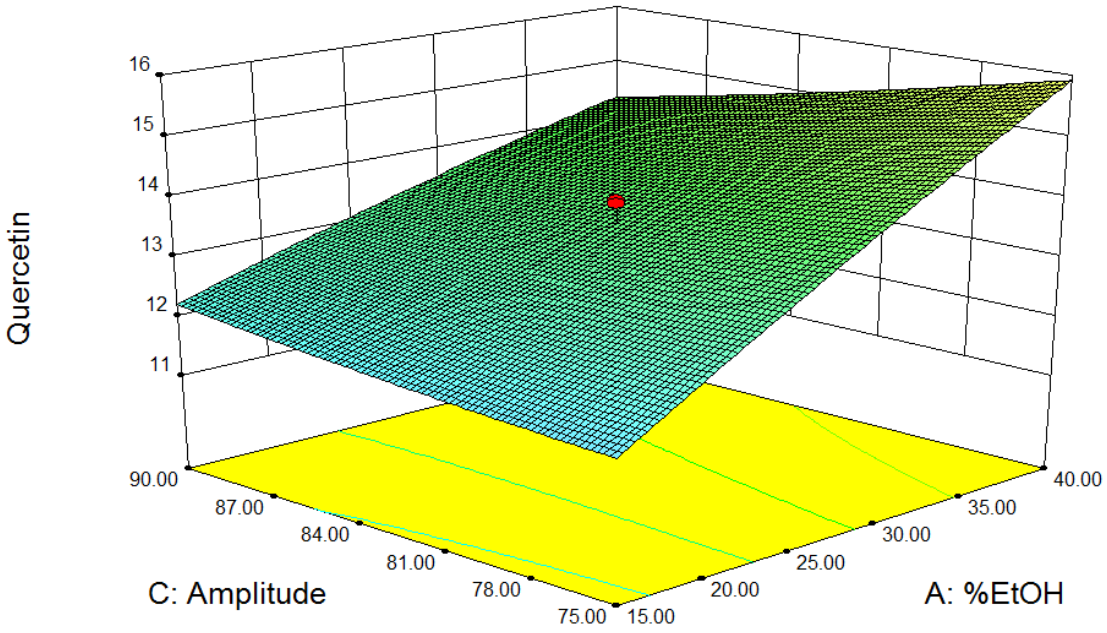


Figure 4-21: Three-dimensional response surface plot for the effects of ethanol purity (A) and amplitude (C) on quercetin at a constant time at 11 minute.

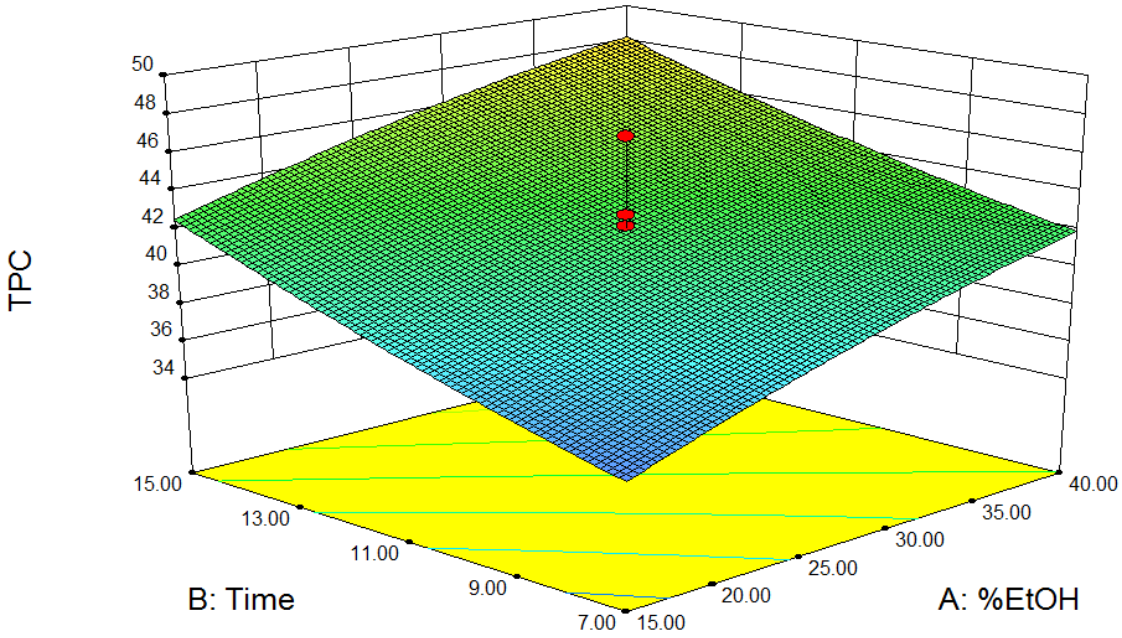
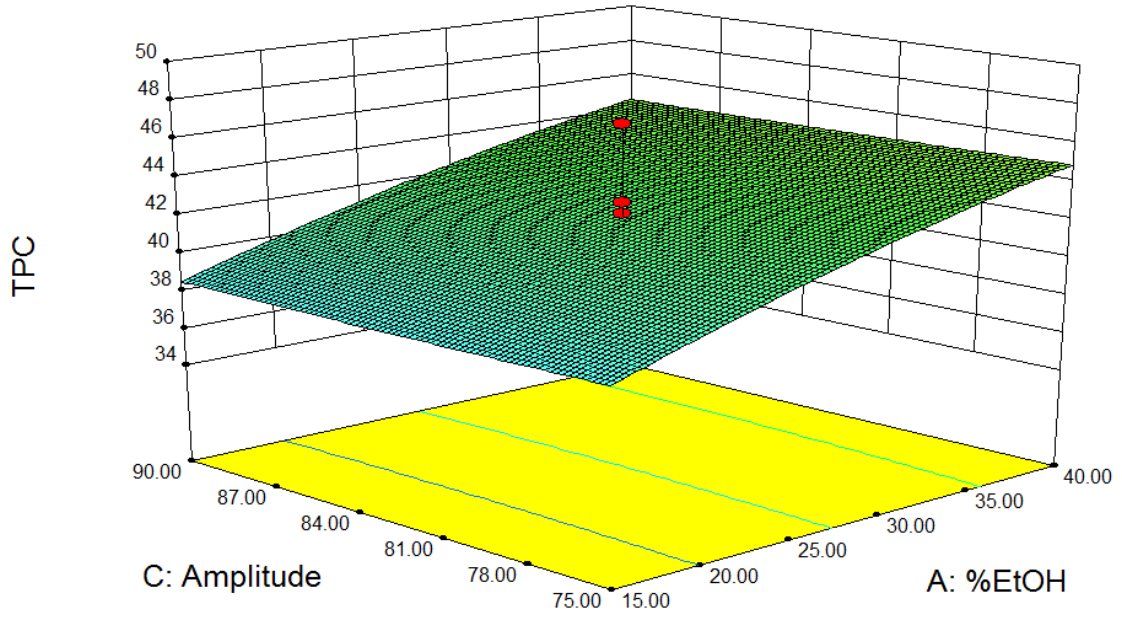


Figure 4-22: Three-dimensional response surface plot for the effects of ethanol purity (A) and time (B) on total phenolic content



at a constant amplitude at 82.50%.

Figure 4-23: Three-dimensional response surface plot for the effects of ethanol purity (A) and amplitude (C) on total phenolic content at a constant time at 11 minute.

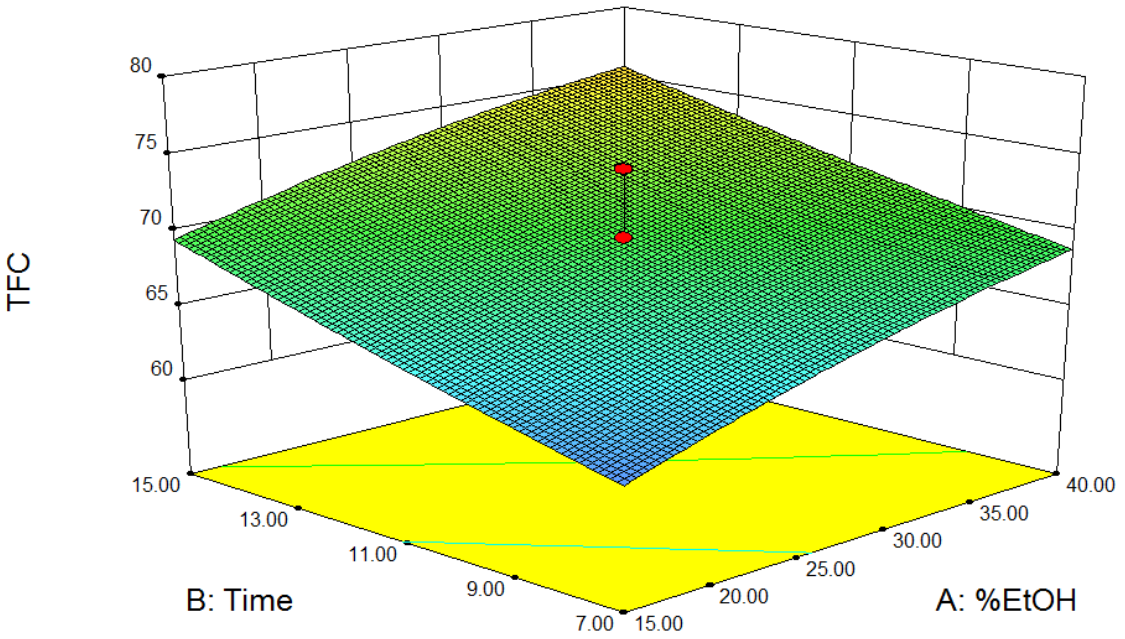


Figure 4-24: Three-dimensional response surface plot for the effects of ethanol purity (A) and time (B) on total flavonoid content at a constant amplitude at 82.50%.

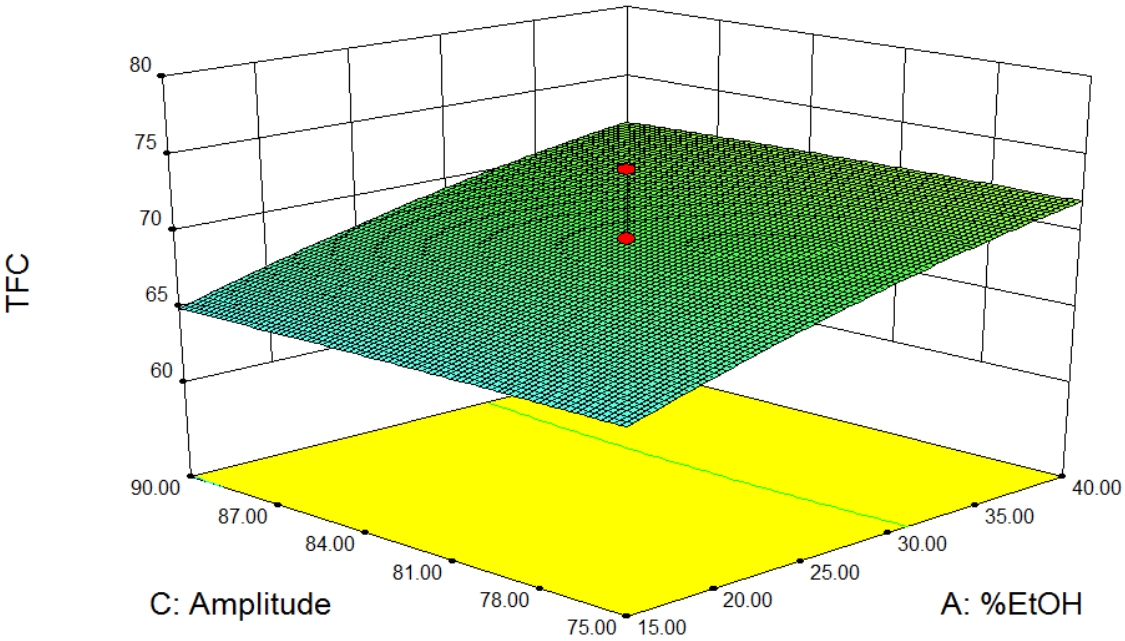


Figure 4-25: Three-dimensional response surface plot for the effects of ethanol purity (A) and amplitude (C) on total flavonoid content at a constant time at 11 minute.

4.5.2.4 Validation Model

Optimization can be performed by using mathematical (numerical) or graphical (contour plot) approaches. Graphical optimization is limited to cases due to few responses. Simon, (2003) explained that numerical optimization requires defining an objective function (called a desirability or score function) that reflects the levels of each response in terms of minimum (zero) to maximum (one) desirability. Numerical optimization was performed with the goal to maximize the response and gave the following best solution as shown in Table 4-31 with desirability of 0.889.

Table 4-31: Condition for factors optimizing polyphenol extraction.

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

To determine the suitability of the model equation, prediction on the optimum response value was tested under the optimum conditions as described in Table 4-31. The experiments were performed based on the suggested best condition in Table 4-31 and the result is presented in Table 4-32. The validation experiments were conducted at the suggested best conditions and the error from these runs were range from 0.138% to 4.742%. Referring on the predicted and experimental results presented, the experimental values were in good agreement with the predicted values proposed by the model with an error less than 5 % and proved to be an adequate model.

Table 4-32: 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

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

2^{4-1} factorial design with total of 16 experiments were performed for MAE. The fractional factorial experimental design and the resulted response is shown in Table 4-33. All the responses were analysed through examining model fitting, interpreting the model graphically, finding the best point, and then validate the model. The effect of time, power, solvent purity and solid liquid ratio in characterizing the extraction yield were summarized in Table 4-33. Variable on the time ranged from 1 min to 6 min, power ranged from 30W to 250W, solvent purity ranged from 30% to 80% and solid liquid ratio ranged from 0.025g/ml to 0.2g/ml were studied in the factorial design.

Table 4-33: 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	Phyllanthin	Gallic Acid	Quercetin
		minutes	W	%	g/ml	mg GAE/g DW	mg QE/g DW	mg Phy/g DW	mg GAE/g DW	mg Que/g DW
4	1	6	250	30	0.025	16.197	17.877	4.049	3.239	7.989
5	2	1	30	80	0.025	6.485	5.595	1.621	1.297	3.198
11	3	1	250	30	0.200	79.299	155.704	19.825	15.860	39.112
10	4	6	30	30	0.200	75.461	118.859	18.435	15.092	37.219
15	5	1	250	80	0.200	86.511	146.152	21.628	17.302	42.669
16	6	6	250	80	0.200	67.726	110.671	16.952	13.545	33.403
6	7	6	30	80	0.025	11.661	13.783	2.915	2.332	5.751
2	8	6	30	30	0.025	8.753	8.324	2.188	1.751	4.317
13	9	1	30	80	0.200	29.748	45.169	7.437	5.950	14.672
9	10	1	30	30	0.200	64.585	91.567	16.146	12.917	31.854
7	11	1	250	80	0.025	17.186	24.700	4.296	3.437	8.476
1	12	1	30	30	0.025	16.255	16.512	4.064	3.251	8.017
14	13	6	30	80	0.200	92.327	143.422	23.082	18.465	45.537
12	14	6	250	30	0.200	81.335	120.224	20.334	16.267	40.116
3	15	1	250	30	0.025	12.824	16.512	3.206	2.565	6.325
8	16	6	250	80	0.025	18.582	26.064	4.645	3.716	9.165

4.6.1 Model Fitting and Effect Estimation

Simulation and analysis of experimental data by a completed 16 fractional factorial design was conducted using Design Expert 8.0.6 (Stat-Ease, USA), to calculate effect estimates using Yates algorithms systematically. From the researcher Anderson et al., (2009), the percent of contribution of the model comes from the consideration of total sum of square, then each sum of squares of the term was dividing by the total to yield a percentage. Table 4-34, Table4-35, Table 4-36, Table 4-37, Table 4-38 and Table-39 illustrates the effect of estimate and percent contributions calculated for phyllanthin, gallic acid, quercetin, total phenolic content and total flavonoid content. Low p-value (<0.05) for the factor indicates the statistical significant at 95% confidence level. From the tables below, it showed that the p-value for all the main factors and interactive factors are lesser than 0.05. Thus, this had confirmed that all the factors are statistically significant. Apparently, factor D played the major contribution in the polyphenol extraction which contributes more than 80% compared to linear factor and interactive factors. The fitted model for the factorial analysis in coded form for phyllanthin, gallic acid, quercetin, total phenolic content, total flavonoid content and antioxidant activity was shown in Equation (4.11), Equation (4.12), Equation (4.13), Equation (4.14) and Equation (4.15) respectively.

$$Phyllanthin = 10.68 + 0.90 * A + 1.19 * B - 0.35 * C + 7.30 * D - 1.27 * A * B + 0.68 * A * C + 0.82 * A * D + 0.37 * B * C + 0.51 * B * D - 0.35 * C * D \quad (4.11)$$

$$Gallic\ Acid = 8.56 + 0.74 * A + 0.93 * B - 0.31 * C + 5.86 * D - 1.04 * A * B + 0.52 * A * C + 0.68 * A * D + 0.31 * B * C + 0.39 * B * D - 0.30 * C * D \quad (4.12)$$

$$Quercetin = 21.11 + 1.82 * A + 2.29 * B - 0.75 * C + 14.46 * D - 2.56 * A * B + 1.28 * A * C + 1.67 * A * D + 0.78 * B * C + 0.96 * B * D - 0.75 * C * D \quad (4.13)$$

$$Total\ Phenolic\ Content = 42.81 + 3.70 * A + 4.65 * C - 1.53 * C + 29.32 * D - 5.19 * A * B + 2.60 * A * C + 3.39 * A * D + 1.57 * B * C + 1.94 * B * D - 1.52 * C * D \quad (4.14)$$

$$Total\ Flavonoid\ Content = 66.32 + 3.58 * A + 10.95 * B - 1.88 * C + 50.15 * D - 12.11 * A * B + 5.46 * A * V + 3.24 * A * D + 1.54 * B * C - 3.24 * C * D \quad (4.15)$$

Table 4-34: Sum of squares and the percent contribution for each term for phyllanthin.

Term	Effect Estimate	Sum of Squares	% Contribution
A-Extraction Time	1.797224	12.92005	1.259773
B-Power	2.380896	22.67467	2.210898
C-Ethanol Purity	-0.70877	2.009433	0.19593
D-Solid liquid ratio	14.60663	853.4142	83.21233
AB	-2.54076	25.82193	2.517773
AC	1.355853	7.353353	0.71699
AD	1.644558	10.81828	1.054839
BC	0.735832	2.165794	0.211176
BD	1.028712	4.232993	0.412739
CD	-0.7015	1.968423	0.191932

Table 4-35: Sum of squares and the percent contribution for each term for gallic acid.

Term	Effect Estimate	Sum of Squares	% Contribution
A-Extraction Time	0.776205	2.409979	0.544241
B-Power	1.441368	8.310171	1.876671
C-Ethanol Purity	-1.21602	5.91481	1.335731
D-Solid liquid ratio	9.773603	382.0932	86.28744
AB	-1.24584	6.20842	1.402037
AC	1.167056	5.448083	1.230331
AD	0.654073	1.711244	0.386447
BC	0.594314	1.412837	0.319059
BD	0.359621	0.517308	0.116823
CD	-1.2102	5.858368	1.322985

Table 4-36: Sum of squares and the percent contribution for each term for quercetin.

Term	Effect Estimate	Sum of Squares	% Contribution
A-Extraction Time	1.478679	8.745968	1.323965
B-Power	1.859617	13.8327	2.093996
C-Ethanol Purity	-0.61212	1.498753	0.226881
D-Solid liquid ratio	11.7262	550.0152	83.26136
AB	-2.07771	17.26754	2.613962
AC	1.039583	4.322928	0.654405
AD	1.356546	7.360872	1.114289
BC	0.629565	1.585411	0.24
BD	0.77787	2.420324	0.366389
CD	-0.6063	1.470409	0.222591

Table 4-37: Sum of squares and the percent contribution for each term for total phenolic content.

Term	Effect Estimate	Sum of Squares	% Contribution
A-Extraction Time	3.504759	49.13335	1.33361
B-Power	4.45008	79.21287	2.150049
C-Ethanol Purity	-1.31798	6.948258	0.188594
D-Solid liquid ratio	27.74574	3079.304	83.58053
AB	-5.00257	100.1029	2.717061
AC	2.909958	33.87141	0.919361
AD	3.205808	41.10881	1.115803
BC	1.392212	7.75302	0.210438
BD	1.802225	12.99206	0.352639
CD	-1.30374	6.798969	0.184542

Table 4-38: Sum of squares and the percent contribution for each term for total flavonoid content.

Term	Effect Estimate	Sum of Squares	% Contribution
A-Extraction Time	7.393396	218.6492	1.323965
B-Power	9.298085	345.8176	2.093996
C-Ethanol Purity	-3.06059	37.46883	0.226881
D-Solid liquid ratio	58.63101	13750.38	83.26136
AB	-10.3886	431.6884	2.613962
AC	5.197913	108.0732	0.654405
AD	6.782732	184.0218	1.114289
BC	3.147827	39.63526	0.24
BD	3.889348	60.5081	0.366389
CD	-3.03151	36.76022	0.222591

The relative effects were visually demonstrated by Pareto chart in Figure 4-26, Figure 4-27, Figure 4-28, Figure 4-29 and Figure 4-30 where the bar length is proportional to the absolute value of estimated effect. For the main effect, positive effect to be said when there's an increase to its high level result an increase in the response. On the other hand, negative effect is defined when an increase in its high level will yield in a decrease in response. For interactions, when both factors were a chance to the same level (either low or high) and the response will increase, that represent the positive effect.

However, negative effect results both factors were change to the opposite level such as one at its low and another at its high, the response will increase. According to Martendal et al., (2007), positive effect (colored in orange) and negative effect (colored in blue) shown in the Pareto chart. Effect of t-value limit (black colored line) is considered as statistically significant at 95% confidence level. For the effect below t-value limit, are not likely to be statistically significant. Mee, (2009) stated that model with a small global p-value, Bonferroni's corrected t-test were performed based on the individual terms in the model in order to justify individual terms in forward selection of models. Anderson et al., (2009) found that any effect above Bonferroni's corrected t-value limit, colored red line in the Pareto chart is almost certainly significant. A quick analysis was performed on the selected effects using Pareto chart to statistically check for significance of the selected effects at 95% confidence level. Only effect D shown to be significant at t-value limit except for total flavonoid content while Interaction factor (AC) shown to be significant at both t-value limit and Bonferroni's corrected t-value limit.

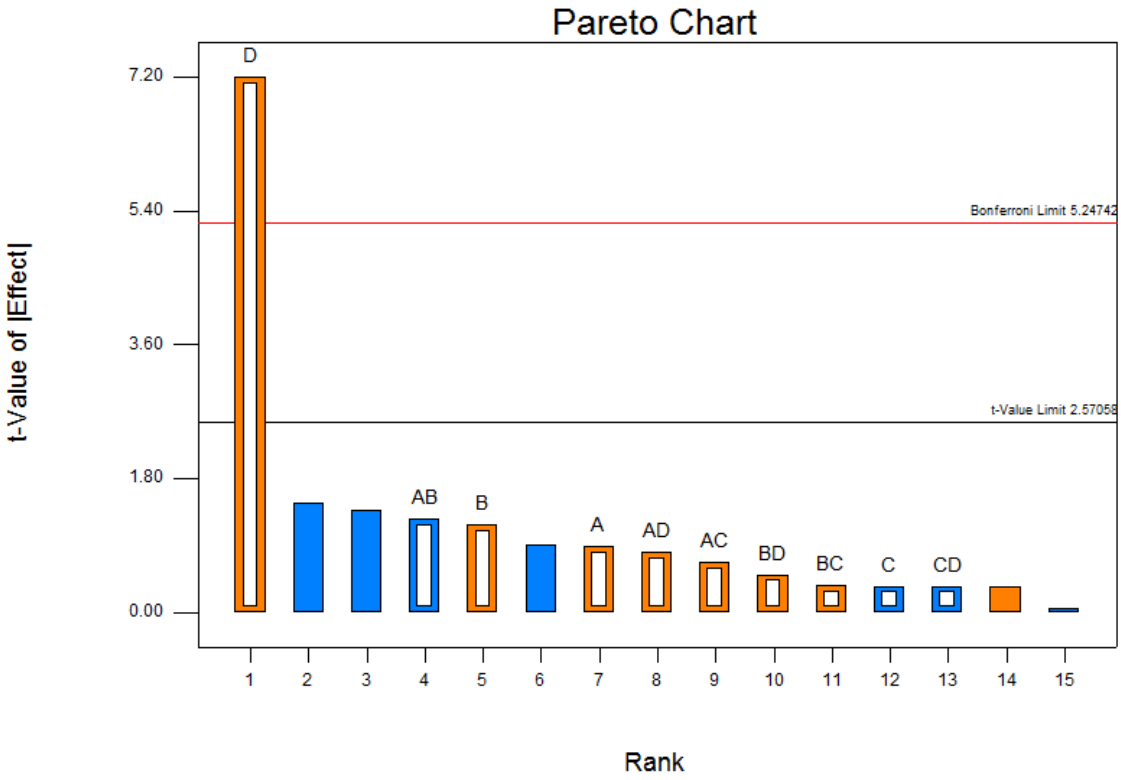


Figure 4-26: Pareto chart of effects of MAE factors on phyllanthin.

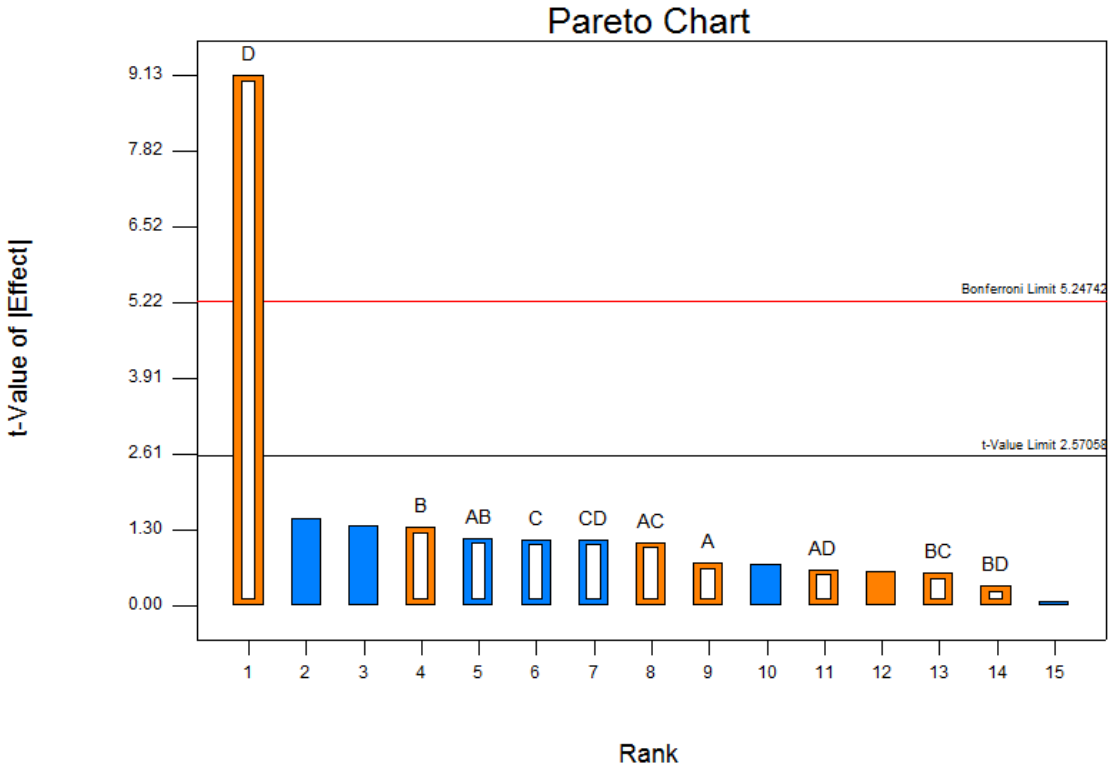


Figure 4-27: Pareto chart of effects of MAE factors on gallic acid.

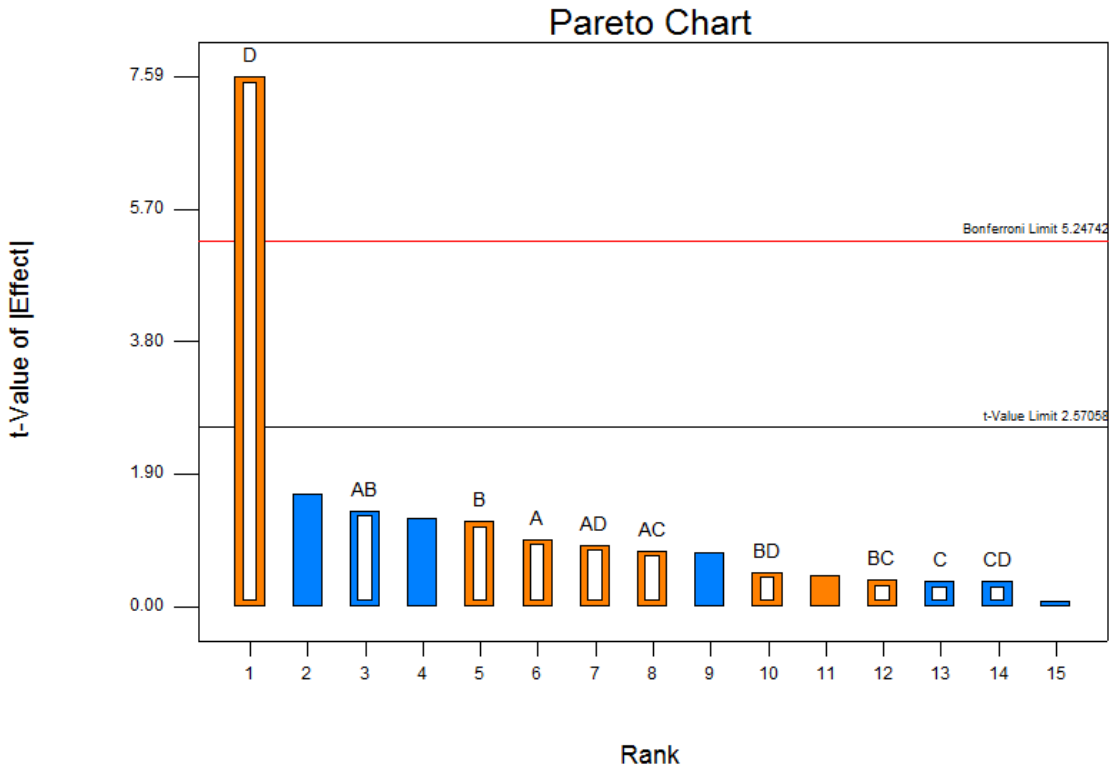


Figure 4-28: Pareto chart of effects of MAE factors on quercetin.

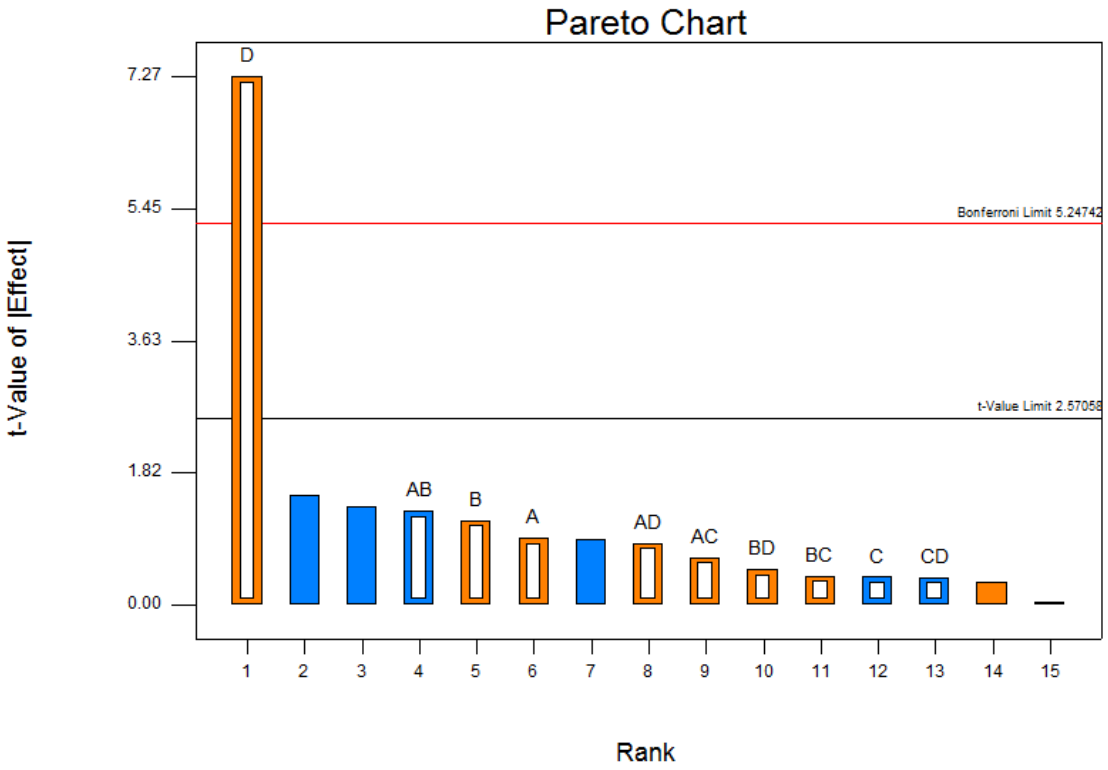


Figure 4-29: Pareto chart of effects of MAE factors on total phenolic content.

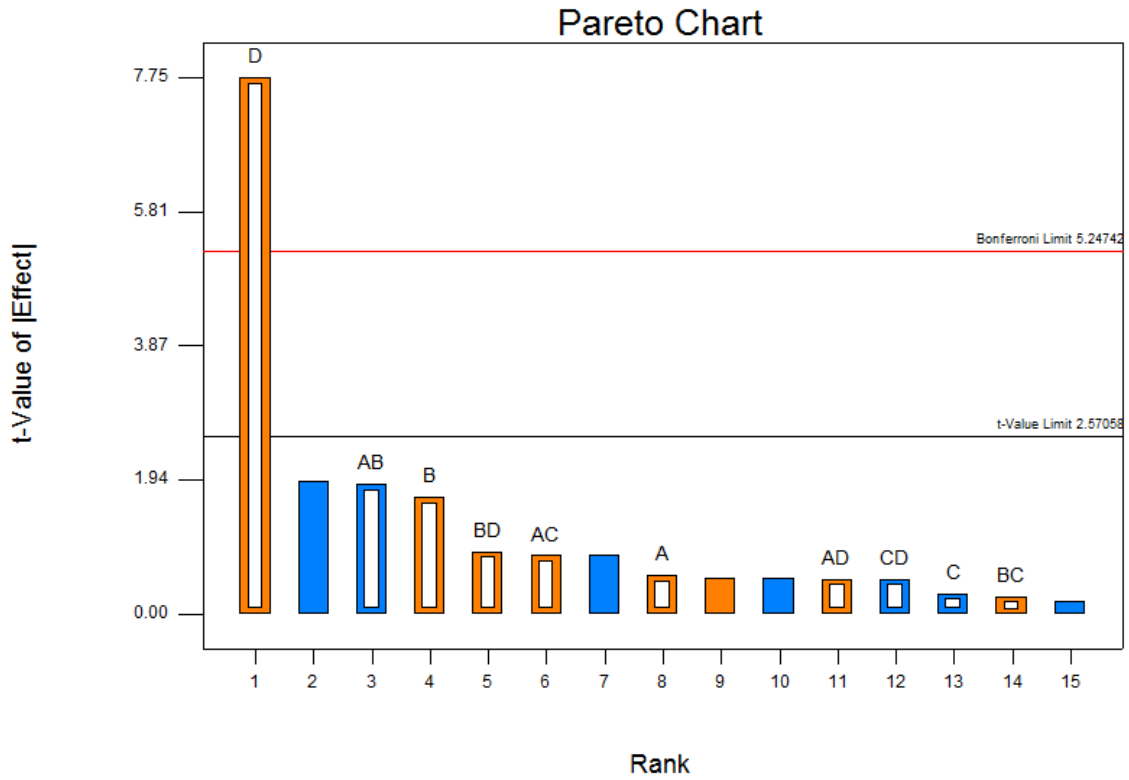


Figure 4-30: Pareto chart of effects of MAE factors on total flavonoid content.

4.6.1.1 ANOVA

All the models from section 4.6.1 with the selected effects were analyzed using analysis of variance (ANOVA) method and found significant for phyllanthin, gallic acid, quercetin, total phenolic content, total flavonoid content and antioxidant activity as presented in Table 4-39, Table 4-40, Table 4-41, Table 4-42 and Table 4-43 respectively. R^2, the coefficient of determination representing the proportion of variation in the response attributed to the model. High correlation ($R^2 \geq 0.9198$) between the experimental data and model data was obtained for all the responses. From this study, the regression coefficient for all the selected model terms is lower than the interception, which indicated the existent of the design plateau. Thus, this plateau showed that the design had an optimum point, where further optimization experiment can be performed (Box et al., 1978). The best experimental condition for factors in polyphenol extraction was shown in Table 4-44.

Table 4-39: ANOVA analysis for the factorial model for phyllanthin.

Source	**SS[a]**	**Df[b]**	**MS[c]**	**F-Value**	**p-value**	
Model	943.37914	10	94.33791	5.737818	0.0338	**significant**
A-Extraction time	12.920055	1	12.92005	0.785823	0.4160	
B-Power	22.67467	1	22.67467	1.379118	0.2931	
C-Ethanol	2.0094331	1	2.009433	0.122218	0.7409	
D-pH aqueous solution	853.4142	1	853.4142	51.90633	0.0008	
AB	25.821931	1	25.82193	1.570541	0.2655	
AC	7.3533529	1	7.353353	0.447245	0.5333	
AD	10.818283	1	10.81828	0.657989	0.4541	
BC	2.1657938	1	2.165794	0.131728	0.7315	
BD	4.2329929	1	4.232993	0.257459	0.6335	
CD	1.9684234	1	1.968423	0.119723	0.7434	
Residual	8.22E+01	5	1.64E+01			
Cor Total	1025.5863	15				
C.V. =37.98%; R^2=0.9198; Adjusted R^2=0.7595; Adeq. Precision=6.001.						

[a]Sum of squares.
[b]Degree of freedom.
[c]Mean Square

Table 4-40: ANOVA analysis for the factorial model for gallic acid.

Source	SS[a]	Df[b]	MS[c]	F-Value	p-value	
Model	419.88447	10	41.98845	9.155801	0.0123	**significant**
A-Extraction time	2.409979	1	2.409979	0.525509	0.5010	
B-Power	8.3101707	1	8.310171	1.812076	0.2361	
C-Ethanol	5.9148103	1	5.91481	1.289755	0.3076	
D-pH aqueous solution	382.09325	1	382.0932	83.31744	0.0003	
AB	6.2084204	1	6.20842	1.353779	0.2971	
AC	5.4480826	1	5.448083	1.187983	0.3255	
AD	1.7112437	1	1.711244	0.373146	0.5680	
BC	1.4128374	1	1.412837	0.308077	0.6028	
BD	0.5173084	1	0.517308	0.112802	0.7506	
CD	5.8583681	1	5.858368	1.277448	0.3097	
Residual	2.29E+01	5	4.59E+00			
Cor Total	442.81444	15				
C.V. =28.23%; R^2=0.9482; Adjusted R^2=0.8447; Adeq. Precision=7.991.						

[a]Sum of squares.
[b]Degree of freedom.
[c]Mean Square

Table 4-41: ANOVA analysis for the factorial model for quercetin.

Source	SS[a]	Df[b]	MS[c]	F-Value	p-value	
Model	3417.2254	10	341.7225	6.399047	0.0269	**significant**
A-Extraction time	49.133348	1	49.13335	0.920064	0.3815	
B-Power	79.212866	1	79.21287	1.483329	0.2776	
C-Ethanol	6.9482583	1	6.948258	0.130112	0.7331	
D-pH aqueous solution	3079.3037	1	3079.304	57.6626	0.0006	
AB	100.10292	1	100.1029	1.874513	0.2293	
AC	33.871411	1	33.87141	0.634271	0.4619	
AD	41.108813	1	41.10881	0.769798	0.4204	
BC	7.7530204	1	7.75302	0.145182	0.7188	
BD	12.992057	1	12.99206	0.243287	0.6427	
CD	6.7989694	1	6.798969	0.127317	0.7358	
Residual	2.67E+02	5	5.34E+01			
Cor Total	3684.2358	15				
C.V. =35.69%; R^2=0.9275; Adjusted R^2=0.7826; Adeq. Precision=6.387.						

[a]Sum of squares.
[b]Degree of freedom.
[c]Mean Square

Table 4-42: ANOVA analysis for the factorial model for total phenolic content.

Source	SS[a]	Df[b]	MS[c]	F-Value	p-value	
Model	15213.003	10	1521.3	5.843436	0.0325	**significant**
A-Extraction time	218.64919	1	218.6492	0.839849	0.4015	
B-Power	345.81757	1	345.8176	1.328313	0.3012	
C-Ethanol	37.468832	1	37.46883	0.143921	0.7200	
D-pH aqueous solution	13750.38	1	13750.38	52.81631	0.0008	
AB	431.68844	1	431.6884	1.65815	0.2542	
AC	108.07321	1	108.0732	0.415118	0.5478	
AD	184.02179	1	184.0218	0.706842	0.4388	
BC	39.635264	1	39.63526	0.152242	0.7125	
BD	60.508102	1	60.5081	0.232416	0.6501	
CD	36.760218	1	36.76022	0.141199	0.7225	
Residual	1.30E+03	5	2.60E+02			
Cor Total	16514.72	15				
C.V. =37.69%; R^2=0.9212; Adjusted R^2=0.7635; Adeq. Precision=6.027.						

[a]Sum of squares.
[b]Degree of freedom.
[c]Mean Square

Table 4-43: ANOVA analysis for the factorial model for total flavonoid content.

Source	SS[a]	Df[b]	MS[c]	F-Value	p-value	
Model	46144.678	10	4614.468	6.881283	0.0230	**significant**
A-Extraction time	205.30885	1	205.3089	0.306165	0.6039	
B-Power	1906.9049	1	1906.905	2.843655	0.1525	
C-Ethanol	56.331908	1	56.33191	0.084004	0.7836	
D-pH aqueous solution	40240.535	1	40240.54	60.00833	0.0006	
AB	2346.8524	1	2346.852	3.499722	0.1203	
AC	476.72623	1	476.7262	0.710914	0.4376	
AD	168.06462	1	168.0646	0.250625	0.6379	
BC	37.709789	1	37.70979	0.056234	0.8220	
BD	538.17922	1	538.1792	0.802555	0.4114	
CD	168.06462	1	168.0646	0.250625	0.6379	
Residual	3.35E+03	5	6.71E+02			
Cor Total	49497.59	15				
C.V. =39.05%; R^2=0.9323; Adjusted R^2=0.7968; Adeq. Precision=7.198.						

[a]Sum of squares.
[b]Degree of freedom.
[c]Mean Square

Table 4-44: Suggested best condition for factors in polyphenol extraction in maximizing polyphenol extraction by MAE.

Factors	**Phyllanthin**	**Gallic Acid**	**Quercetin**	**Total Phenolic Content**	**Total Flavonoid Content**
A-Extraction time (Min)	6	1	6	6	1
B-Power (W)	250	250	250	250	250
C-Ethanol Purity (%)	80	30	80	80	30
D-Solid liquid ratio (%)	0.2	0.2	0.2	0.2	0.2

4.6.1.2 Effect of main factors on polyphenol extraction by MAE

All the main factors studied were statistically significant at 95 % confidence level toward phtyllanthin, gallic acid, quercetin, total phenolic content and total flavonoid content was presented in Pareto chart in section 4.6.1. Only factor D was found to have positive effect. The main effect from this study on polyphenol extraction was factor D. Factor D was described in past study done by Sousa et al., (2016) as increasing factor D, it allowed to obtain higher yield of polyphenol extraction.

4.6.1.3 Validation of Model

The validation experiments were conducted based on one suggested best condition in from Design Expert 8.0.6 in triplicate. The experiments were performed according to the suggested best condition in Table 4-44 and the result is presented in Table 4-45. The validation experiments were conducted at the suggested best conditions and the error from these runs were ranging from 0.817% until 8.503%. Based on the predicted and experimental results presented, the experimental values were in good agreement with the predicted values proposed by the model with an error less than 10 % and proved to be an adequate model.

Table 4-45: Comparison between predicted and experimental value for best condition.

Response		Predicted Value	Experimental Value	**Error**
Phyllanthin	**Run 1**	20.476	19.214	6.568
	Run 2	20.476	20.041	2.171
	Run 3	20.476	19.630	4.310
Gallic Acid	**Run 1**	14.780	15.206	2.802
	Run 2	14.780	15.851	6.757
	Run 3	14.780	15.397	4.007
Quercetin	**Run 1**	39.172	38.634	1.393
	Run 2	39.172	41.041	4.554
	Run 3	39.172	38.128	2.738
Total Phenolic Content	**Run 1**	81.738	78.306	4.383
	Run 2	81.738	77.237	5.828
	Run 3	81.738	80.513	1.521
Total Flavonoid Content	**Run 1**	147.516	143.067	3.110
	Run 2	147.516	148.943	0.958
	Run 3	147.516	146.320	0.817

4.6.1.4 Effect of solid liquid ratio in polyphenol extraction

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).

4.6.2 Optimization on polyphenol extraction by MAE

CCD with total of 20 experiments, including 7 for factorial design, 7 for axial points and 6 repetitions at the central point, were performed. The CCD experimental design and the resulted response is shown in Table 4-46. Responses were analysed by examining fitting a model, then, interpreting the model graphically, finding the optimized point, and lastly validating the model.

Table 4-46: Experimental design and response for MAE optimization

Standard	Run	Ethanol Purity (%)	Time (Min)	Power (W)	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)
16	1	55	3.5	140	61.41006	80.5131	158.2963	10.155	5.145591
6	2	80	1	250	46.97618	114.0085	110.9406	2.956891	3.936167
15	3	55	3.5	140	67.01442	90.43767	140.4171	7.71028	5.615184
11	4	55	1	140	50.57143	73.06967	116.7393	1.853916	4.237415
7	5	30	6	250	56.91599	80.5131	126.4037	15.06662	4.76903
8	6	80	6	250	53.90233	96.64053	141.3836	6.581836	4.516513
3	7	30	6	30	51.68173	80.5131	146.699	5.345144	4.330448
10	8	80	3.5	140	47.61064	86.71596	130.7527	4.117301	3.989328
20	9	55	3.5	140	63.68352	100.3622	153.4641	8.603874	5.336087
9	10	30	3.5	140	52.15757	79.27253	137.5178	13.0576	4.370319
12	11	55	6	140	64.42372	95.39996	144.2829	9.502456	5.398108
4	12	80	6	30	43.8039	78.03196	102.2426	1.558093	3.670359
5	13	30	1	250	57.12748	79.27253	145.2494	7.571712	4.786751
19	14	55	3.5	140	66.32709	96.64053	170.8601	9.965286	5.557593
2	15	80	1	30	39.04548	69.34796	83.88022	1.012162	3.271648
13	16	55	3.5	30	52.31619	86.71596	160.2292	2.209649	4.383609
18	17	55	3.5	140	59.03085	92.91882	158.2963	8.702618	4.946235
14	18	55	3.5	250	67.33165	102.8434	139.4507	8.187193	5.641765
17	19	55	3.5	140	61.67441	101.6028	158.7796	7.100528	5.167742
1	20	30	1	30	45.60153	58.18281	135.1017	1.444053	3.820984

4.6.2.1 Model Fitting

The experimental data shown in Table 4-47, Table 4-48, Table 4-49, Table 4-50 and Table 4-51 were used to estimate the appropriate model for the all the responses using Design Expert software. Fit Summary is a part of Design Expert which providing statistical tables. These statistical tables used to identify best model to choose for in depth study (Anderson et al., 2009). The statistical tables are sequential model sum of squares in Table 4-47, Table 4-48, Table 4-49, Table 4-50 and Table 4-51 and lack of fit test in Table 4-52, Table 6-53, Table 6-54, Table 6-55 and Table 6-56.

Montgomery and Runger, (2010) defined the reduction in the error sum of squares when one or more predictor variables are added to the regression model as sequential sum of squares. It is performed by starting with the mean and adding terms such as linear, two-factor interaction, quadratic, and cubic. The F-statistic is calculated for each type of model, and the highest order model with significant terms would be chosen for the statistic. Significance of the model is judged by the probability of the F-statistic calculated from the data exceeds a theoretical value. The probability decreases as the value of the F-statistic increases. According to Simon, (2003), if the probability is less than 0.05 the terms are significant and their inclusion improves the model. Thus, the model with p-value less than 0.05 in sequential model sum of square for all the response can be considered to be chosen to fit the response. From quadratic model fits the criteria to be chosen to fit the response.

Table 4-47: Sequential model sum of squares of phyllanthin.

Source	Sum of Squares	df	Mean Square	F-Value	p-value
Mean vs Total	408.126	1	408.126		
Linear vs Mean	2.682	3	0.894	2.193	0.1286
2FI vs Linear	0.052	3	0.017	0.035	0.9909
Quadratic vs 2FI	4.619	3	1.540	8.303	**0.0046**
Cubic vs Quadratic	0.314	4	0.078	0.305	0.8645
Residual	1.540	6	0.257		
Total	417.333	20	20.867		

Table 4-48: Sequential model sum of squares of gallic acid.

Source	Sum of Squares	df	Mean Square	F-Value	p-value
Mean vs Total	880.494	1	880.494		
Linear vs Mean	205.764	3	68.5881	11.0397	0.0004
2FI vs Linear	21.9494	3	7.31647	1.22797	0.3391
Quadratic vs 2FI	51.6761	3	17.2254	6.68157	**0.0094**
Cubic vs Quadratic	14.2117	4	3.55294	1.8427	0.2398
Residual	11.5687	6	1.92811		
Total	1185.66	20	59.2832		

Table 4-49: Sequential model sum of squares of quercetin.

Source	Sum of Squares	df	Mean Square	F-Value	p-value
Mean vs Total	88560.725	1	88560.725		
Linear vs Mean	416.920	3	138.973	2.854	0.0700
2FI vs Linear	6.702	3	2.234	0.038	0.9898
Quadratic vs 2FI	649.236	3	216.412	17.589	**0.0003**
Cubic vs Quadratic	42.602	4	10.650	0.794	0.5697
Residual	80.439	6	13.406		
Total	89756.623	20	4487.831		

Table 4-50: Sequential model sum of squares of total phenolic content.

Source	Sum of Squares	df	Mean Square	F-Value	p-value
Mean vs Total	61450.4	1	61450.4		
Linear vs Mean	450.018	3	150.006	2.72858	0.0784
2FI vs Linear	6.55516	3	2.18505	0.03254	0.9917
Quadratic vs 2FI	730.684	3	243.561	17.107	**0.0003**
Cubic vs Quadratic	62.9536	4	15.7384	1.18898	0.4038
Residual	79.4215	6	13.2369		
Total	62780	20	3139		

Table 4-51: Sequential model sum of squares of total flavonoid content.

Source	Sum of Squares	df	Mean Square	F-Value	p-value
Mean vs Total	151903	1	151903		
Linear vs Mean	1597.04	3	532.346	4.89402	0.0134
2FI vs Linear	630.228	3	210.076	2.45997	0.1090
Quadratic vs 2FI	611.095	3	203.698	4.08151	**0.0393**
Cubic vs Quadratic	173.601	4	43.4003	0.80007	0.5668
Residual	325.474	6	54.2457		
Total	155240	20	7762.02		

From the observation of sequential model sum of squares and lack of fit table, it's summarize quadratic model is the most suitable model to be use in fitting the all responses. The results were fitted with a second-order polynomial equation. The values of regression coefficients were calculated, the response variable and the test variables are related by the second-order polynomial equation in Equation (4.16), Equation (4.17), Equation (4.18), Equation (4.19) and Equation (4.20). These equations are in coded form.

$$Phyllanthin = 5.03 - 0.21 * A + 0.31 * B + 0.35 * C + 0.004 * A * B + 0.077 * A * C + 0.022 * B * C - 0.82 * A^2 - 0.19 * B^2 - 0.029 * C^2 \quad (4.16)$$

$$Gallic\ Acid = 8.25 - 2.63 * A + 2.32 * B + 2.88 * C - 0.90 * A * B - 1.11 * A * C + 0.83 * B * C + 1.02 * A^2 - 1.89 * B^2 - 2.37 * C^2 \quad (4.17)$$

$$Quercetin = 72.76 - 3.26 * A + 3.03 * B + 4.67 * C + 0.79 * A * B + 0.096 * A * C - 0.45 * B * C - 9.30 * A^2 - 2.47 * B^2 - 0.65 * C^2 \quad (4.18)$$

$$Total\ Phenolic\ Content = 61.95 - 3.21 * A + 3.14 * B + 4.98 * C + 0.73 * A * B + 0.16 * A * C - 0.52 * B * C - 10.19 * A^2 - 2.58 * B^2 - 0.25 * C^2 \quad (4.19)$$

$$Total\ Flavonoid\ Content = 92.65 + 6.70 * A + 3.72 * B + 10.05 * C - 4.03 * A * B + 5.27 * A * C - 5.89 * B * C - 8.01 * A^2 - 6.77 * B^2 + 3.78C^2 \quad (4.20)$$

4.6.2.2 ANOVA

Table 4-52 summarizes the ANOVA results by considering a model is significant if the p-value is lower than 0.05. The p-value lower than 0.05 indicate that only 5% chance that a 'Model F-value' could occur due to noise. According to Tan et al., (2011) it is also used as indicator to evaluate the significance of the effects of each linear, quadratic and interaction term on the response. The p-value for the fitted model for all responses was less than 0.05, the fitted model equation adequately describes the response. In addition, the p-values for each model term suggest that A, B and C are the model terms that have significant effects on, gallic acid, quercetin and total phenolic content. For phyllanthin and total flavonoid content response, only factor A and C is significant.

Table 4-52: ANOVA analysis for the optimization model of phyllanthin.

Source	**SS[a]**	**Df[b]**	**MS[c]**	**F-Value**	**p-value**	
Model	7.353	9.000	0.817	4.406	0.0150	**significant**
A-Ethanol Purity	0.460	1.000	0.460	2.483	0.1462	
B-Extraction Time	0.969	1.000	0.969	5.228	0.0453	
C-Power	1.253	1.000	1.253	6.756	0.0265	
AB	0.000	1.000	0.000	0.001	0.9787	
AC	0.048	1.000	0.048	0.259	0.6220	
BC	0.004	1.000	0.004	0.020	0.8897	
A^2	1.839	1.000	1.839	9.916	0.0104	
B^2	0.097	1.000	0.097	0.522	0.4865	
C^2	0.002	1.000	0.002	0.012	0.9133	
Residual	1.854	10.000	0.185			
Lack of Fit	0.386	5.000	0.077	0.263	0.9157	**not significant**
Pure Error	1.469	5.000	0.294			
Cor Total	9.207	19.000				
C.V. =9.53%; R^2=0.80; Adjusted R2=0.62; Adeq. Precision=7.52.						

[a]Sum of squares.
[b]Degree of freedom.
[c]Mean Square

Table 4-53: ANOVA analysis for the optimization model of gallic acid.

Source	**SS[a]**	**Df[b]**	**MS[c]**	**F-Value**	**p-value**	
Model	279.390	9.000	31.043	12.041	0.0003	**significant**
A-Ethanol Purity	68.953	1.000	68.953	26.746	0.0004	
B-Extraction Time	53.896	1.000	53.896	20.906	0.0010	
C-Power	82.916	1.000	82.916	32.162	0.0002	
AB	6.525	1.000	6.525	2.531	0.1427	
AC	9.858	1.000	9.858	3.824	0.0790	
BC	5.566	1.000	5.566	2.159	0.1725	
A^2	2.879	1.000	2.879	1.117	0.3155	
B^2	9.782	1.000	9.782	3.795	0.0800	
C^2	15.392	1.000	15.392	5.970	0.0346	
Residual	25.780	10.000	2.578			
Lack of Fit	18.516	5.000	3.703	2.549	0.1638	**not significant**
Pure Error	7.265	5.000	1.453			
Cor Total	305.170	19.000				
C.V. =24.20%; R^2=0.92; Adjusted R2=0.84; Adeq. Precision=13.79.						

[a]Sum of squares.
[b]Degree of freedom.
[c]Mean Square

Table 4-54: ANOVA analysis for the optimization model of quercetin.

Source	**SS[a]**	**Df[b]**	**MS[c]**	**F-Value**	**p-value**	
Model	1072.858	9.000	119.206	9.688	0.0007	**significant**
A-Ethanol Purity	106.575	1.000	106.575	8.662	0.0147	
B-Extraction Time	92.025	1.000	92.025	7.479	0.0210	
C-Power	218.321	1.000	218.321	17.744	0.0018	
AB	4.986	1.000	4.986	0.405	0.5387	
AC	0.074	1.000	0.074	0.006	0.9398	
BC	1.642	1.000	1.642	0.133	0.7225	
A^2	237.903	1.000	237.903	19.335	0.0013	

B^2	16.813	1.000	16.813	1.366	0.2695	
C^2	1.166	1.000	1.166	0.095	0.7645	
Residual	123.041	10.000	12.304			
Lack of Fit	81.023	5.000	16.205	1.928	0.2442	**not significant**
Pure Error	42.018	5.000	8.404			
Cor Total	1195.898	19.000				
C.V. =5.27%; R^2=0.90; Adjusted R2=0.81; Adeq. Precision=11.59.						

[a]Sum of squares.
[b]Degree of freedom.
[c]Mean Square

Table 4-55: ANOVA analysis for the optimization model of total phenolic content.

Source	**SS[a]**	**Df[b]**	**MS[c]**	**F-Value**	**p-value**	
Model	1187.257	9.000	131.917	9.265	0.0009	**significant**
A-Ethanol Purity	103.335	1.000	103.335	7.258	0.0225	
B-Extraction Time	98.631	1.000	98.631	6.928	0.0251	
C-Power	248.052	1.000	248.052	17.422	0.0019	
AB	4.228	1.000	4.228	0.297	0.5977	
AC	0.201	1.000	0.201	0.014	0.9077	
BC	2.126	1.000	2.126	0.149	0.7073	
A^2	285.805	1.000	285.805	20.074	0.0012	
B^2	18.320	1.000	18.320	1.287	0.2831	
C^2	0.178	1.000	0.178	0.013	0.9131	
Residual	142.375	10.000	14.238			
Lack of Fit	94.900	5.000	18.980	1.999	0.2327	**not significant**
Pure Error	47.475	5.000	9.495			
Cor Total	1329.632	19.000				
C.V. =6.81%; R^2=0.90; Adjusted R2=0.80; Adeq. Precision=11.43.						

[a]Sum of squares.
[b]Degree of freedom.
[c]Mean Square

Table 4-56: ANOVA analysis for the optimization model of total flavonoid content.

Source	**SS[a]**	**Df[b]**	**MS[c]**	**F-Value**	**p-value**	
Model	2838.362	9.000	315.374	6.319	0.0040	**significant**
A-Ethanol Purity	448.778	1.000	448.778	8.992	0.0134	
B-Extraction Time	138.512	1.000	138.512	2.775	0.1267	
C-Power	1009.750	1.000	1009.750	20.232	0.0011	
AB	130.047	1.000	130.047	2.606	0.1376	
AC	222.388	1.000	222.388	4.456	0.0609	
BC	277.793	1.000	277.793	5.566	0.0400	
A^2	176.322	1.000	176.322	3.533	0.0896	
B^2	125.920	1.000	125.920	2.523	0.1433	
C^2	39.254	1.000	39.254	0.787	0.3960	
Residual	499.076	10.000	49.908			
Lack of Fit	198.454	5.000	39.691	0.660	0.6701	**not significant**
Pure Error	300.622	5.000	60.124			
Cor Total	3337.438	19.000				
C.V. =8.11%; R^2=0.85; Adjusted R2=0.72; Adeq. Precision=10.68.						

[a]Sum of squares.
[b]Degree of freedom.
[c]Mean Square

The R^2 for the model was ranging from 0.80 until 0.92, implying a good correlation between the observed and predicted values, as shown in Figure 4-31, Figure 4-32, Figure 4-33, Figure 4-34 and Figure 4-35. Based on the R^2 value, it indicates that not more than 20 % of the total variability was not explained by the model terms in the model. The good R^2 value represent the model obtained will be able to give a convincingly good estimate of response of the system within the range studied. The lack of fit test, which was not significant for the model, shows that the model satisfactorily fits the data. From all of these statistical tests, we can summarize that the developed model was suitable to represent the data. Furthermore, these data able to provide a good description on the relationship between the process variables and response.

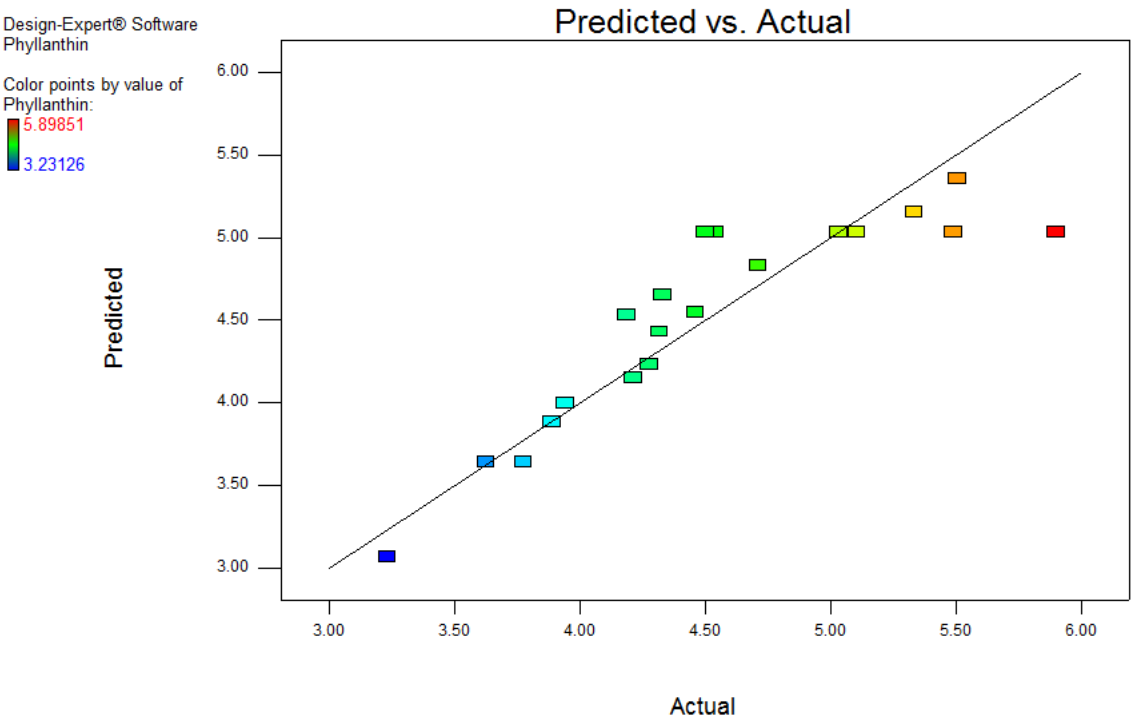


Figure 4-31: Predicted vs. actual phyllanthin colored by standard order

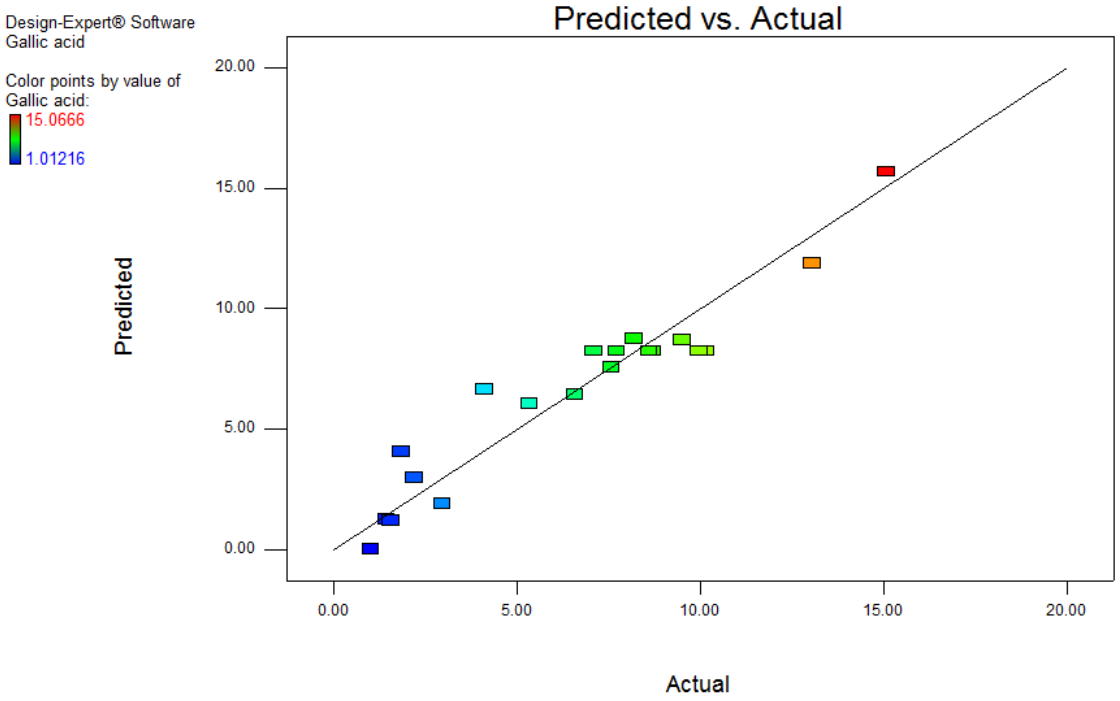


Figure 4-32: Predicted vs. actual gallic acid colored by standard order

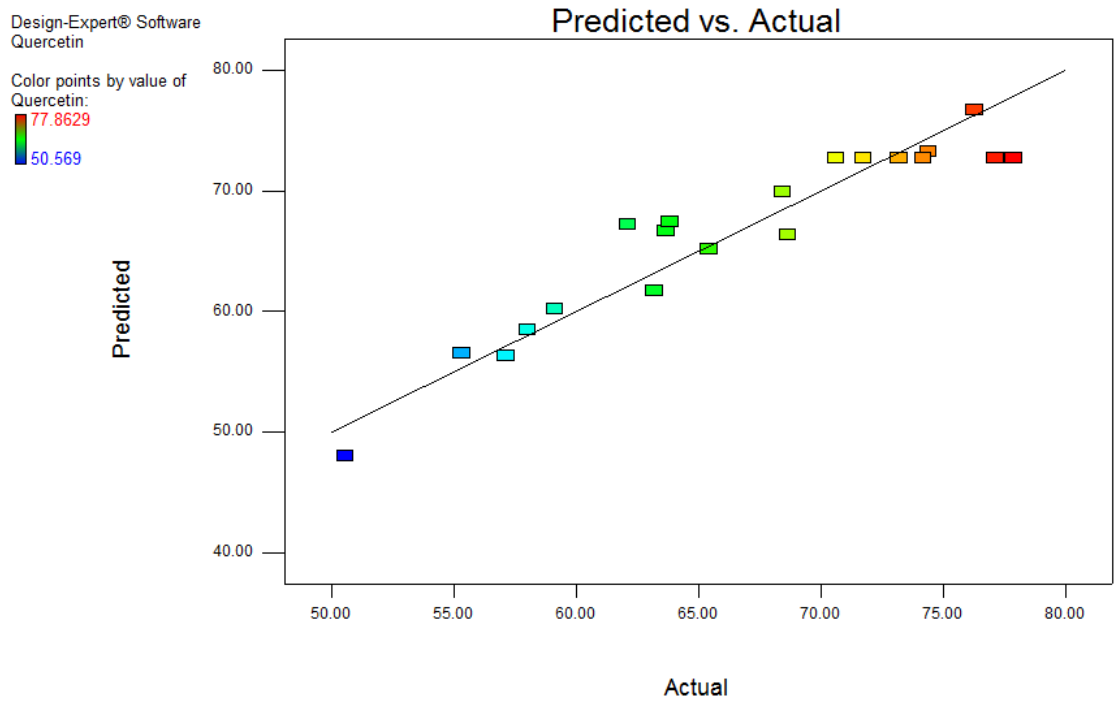


Figure 4-33: Predicted vs. actual quercetin colored by standard order

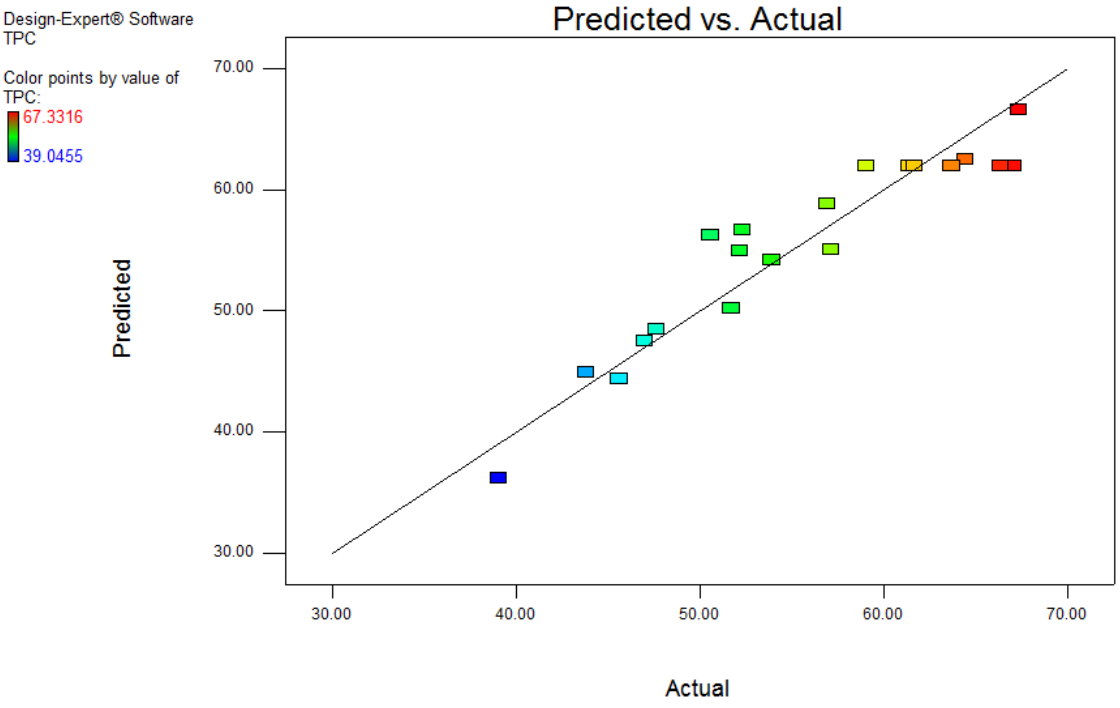


Figure 4-34: Predicted vs. actual total phenolic content colored by standard order

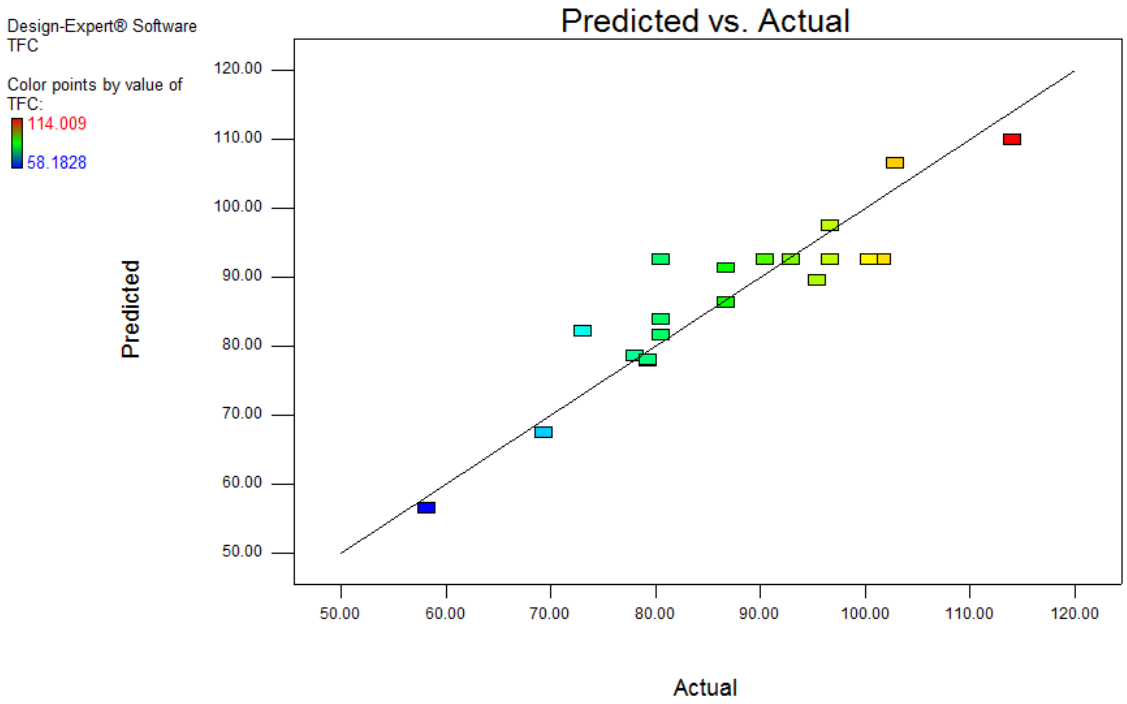


Figure 4-35: Predicted vs. actual total flavonoid content colored by standard order

4.6.2.3 Effect of ethanol purity, power and extraction time on polyphenol extraction.

Three effects of the three polymerization conditions (ethanol purity, power, and extraction time) on polyphenol extraction were analyzed using RSM. Three-dimensional response surface and contour plots were generated in order to investifate the interactive effects of any two variables on the response via evaluating two variables at a time while holding the other one constant at central level. A three-dimensional plot can give a clearer geometrical representation of the nature and extent of the interaction between the variables and response within the experimental range studied

The effect of non-interaction factors ethanol purity (A), power (B) and extraction time (C) on polyphenol extraction is depicted in Figure 4-36 Figure 4-37, Figure 4-38, Figure 4-39, Figure 4-40, Figure 4-41, Figure 4-42, Figure 4-43, Figure 4-44, Figure 4-45, Figure 4-46, Figure 4-47, Figure4-48, Figure 4-49 and Figure 4-50 for all the response. Interaction effects has p-value higher than 0.100 indicating both interaction were non-significant to the response. This was also demonstrated in previously discussed factorial analysis. The non-existent of interaction can be explained in a simple manner. Interactions cannot be seen because the factors were not affecting the other factors in a same process but two different processes.

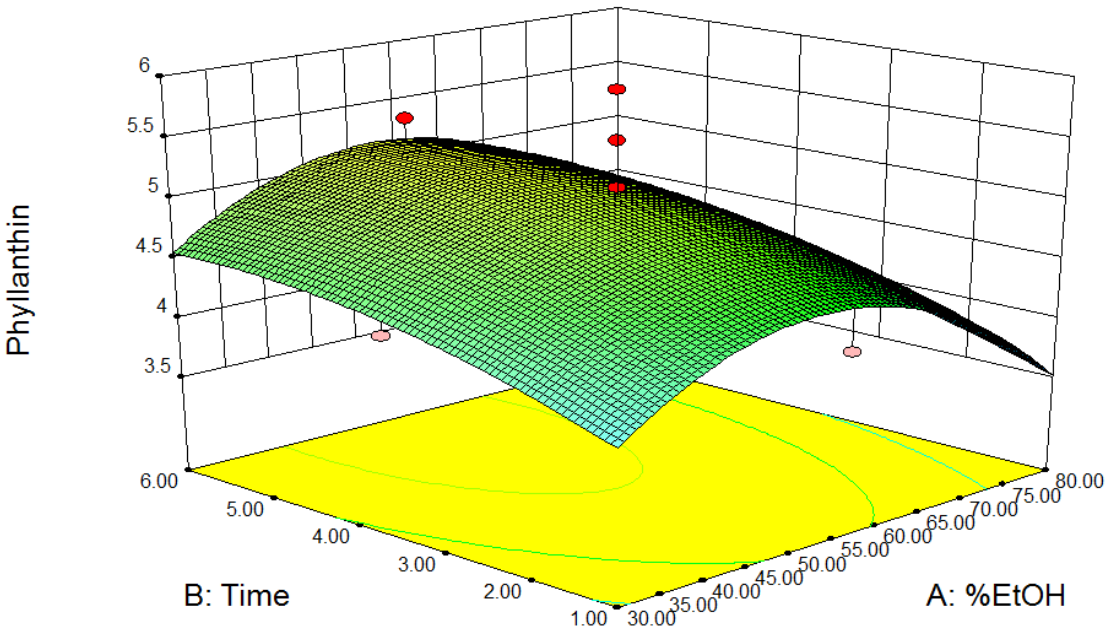


Figure 4-36: Three-dimensional response surface plot for the effect ethanol purity (A) and extraction time (B) on phyllanthin extraction at a constant power (C) at 140W.

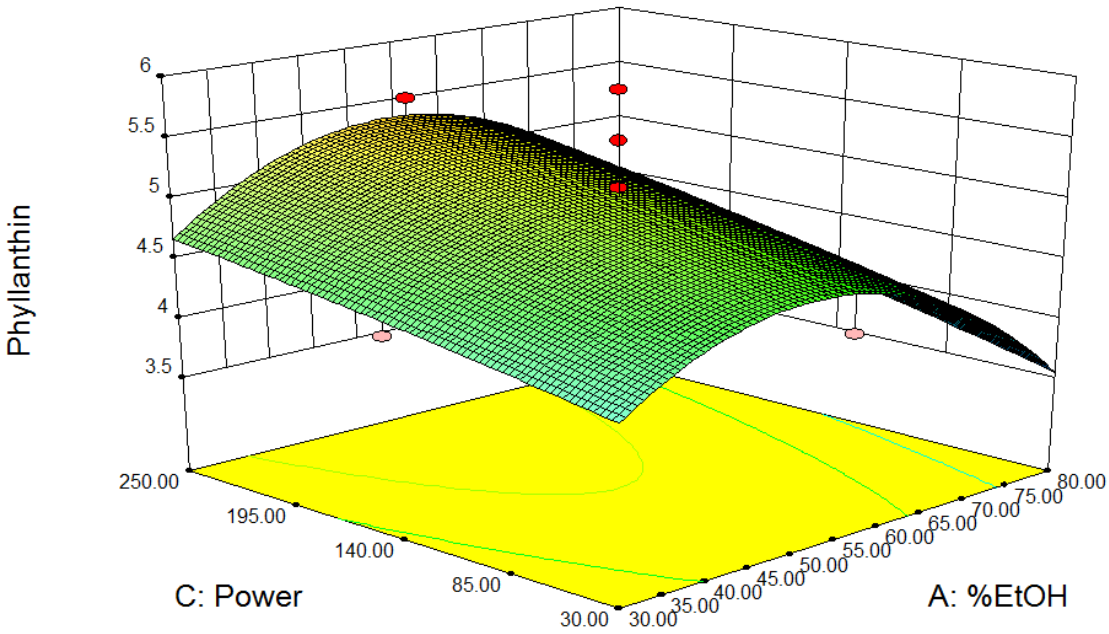


Figure 4-37: Three-dimensional response surface plot for the effect ethanol purity (A) and extraction time (C) on phyllanthin extraction at a constant time (B) at 3.5minutes.

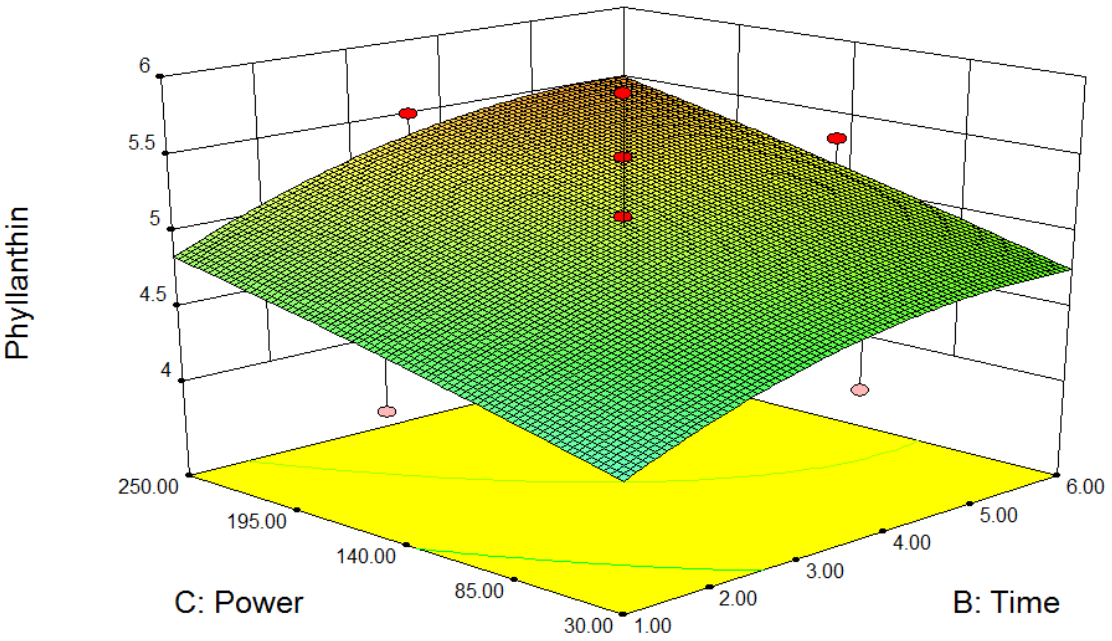


Figure 4-38: Three-dimensional response surface plot for the effect extraction time (B) and power (C) on phyllanthin extraction at a constant power (A) at 55%.

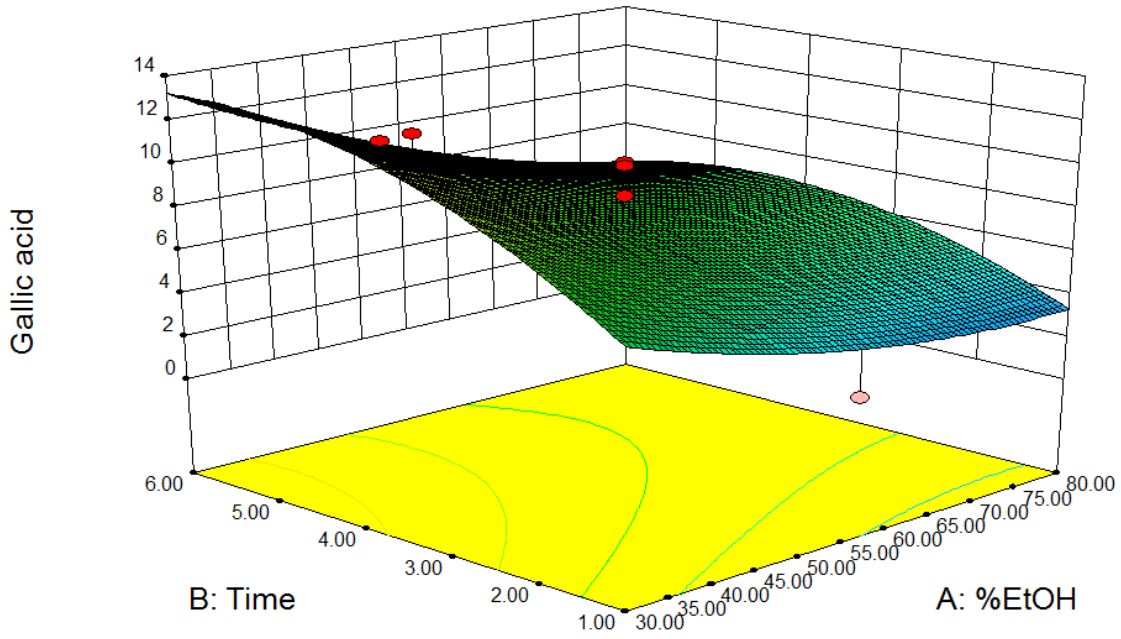


Figure 4-39: Three-dimensional response surface plot for the effect ethanol purity (A) and extraction time (B) on gallic acid extraction at a constant power (C) at 140W

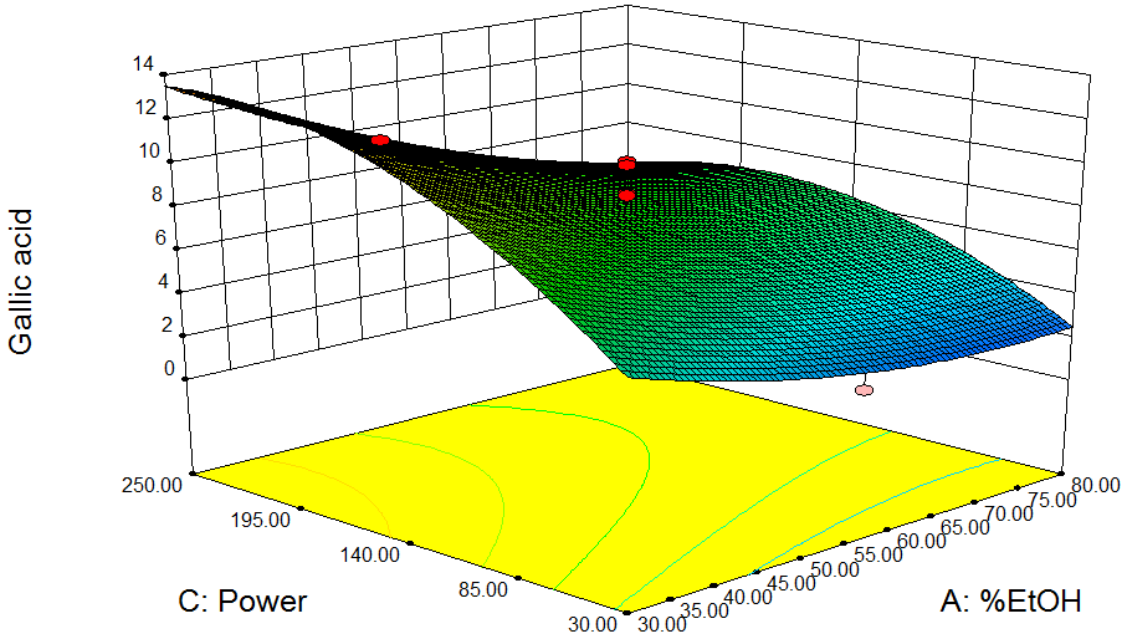


Figure 4-40: Three-dimensional response surface plot for the effect ethanol purity (A) and extraction time (C) on gallic acid extraction at a constant time (B) at 3.5minutes.

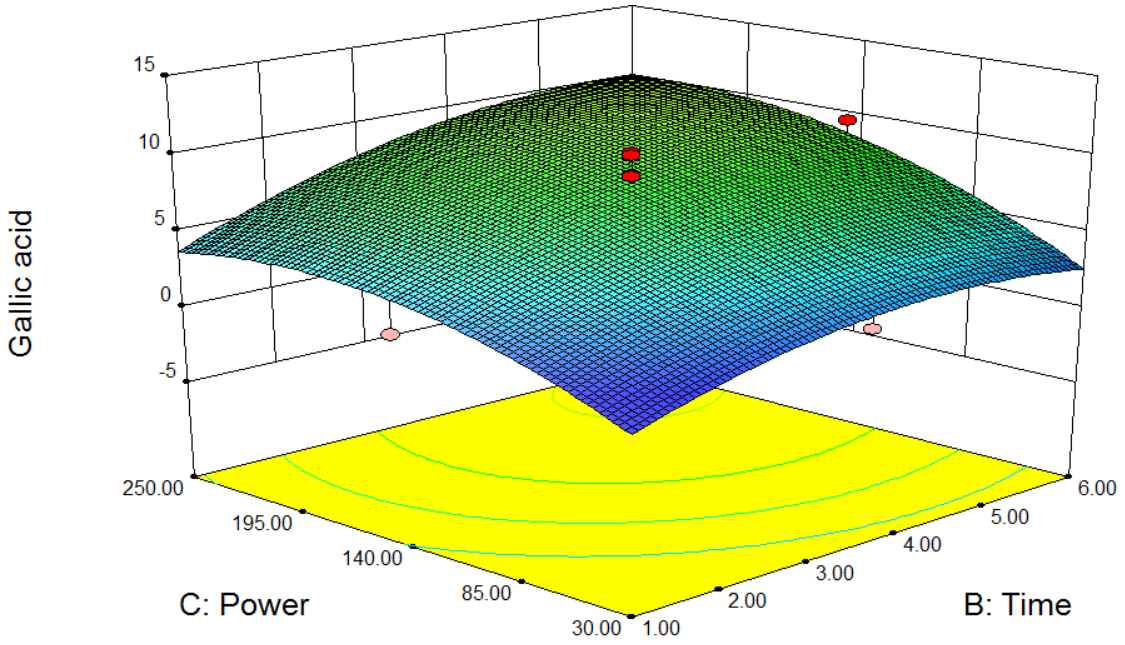


Figure 4-41: Three-dimensional response surface plot for the effect extraction time (B) and power (C) on gallic acid extraction at a constant power (A) at 55%.

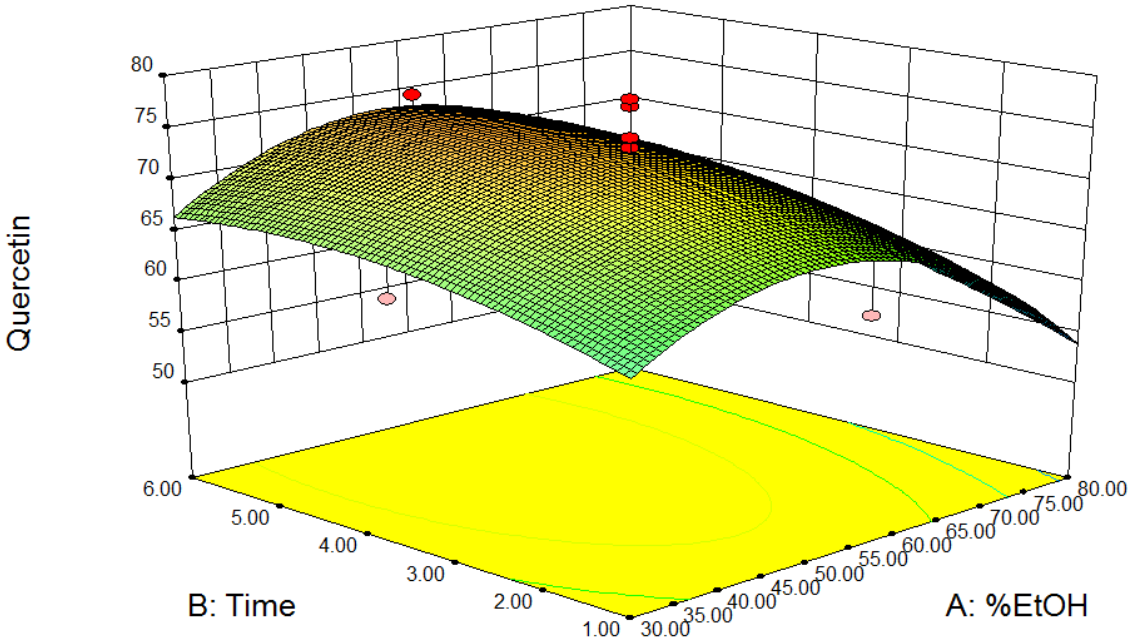


Figure 4-42: Three-dimensional response surface plot for the effect ethanol purity (A) and extraction time (B) on quercetin extraction at a constant power (C) at 140W

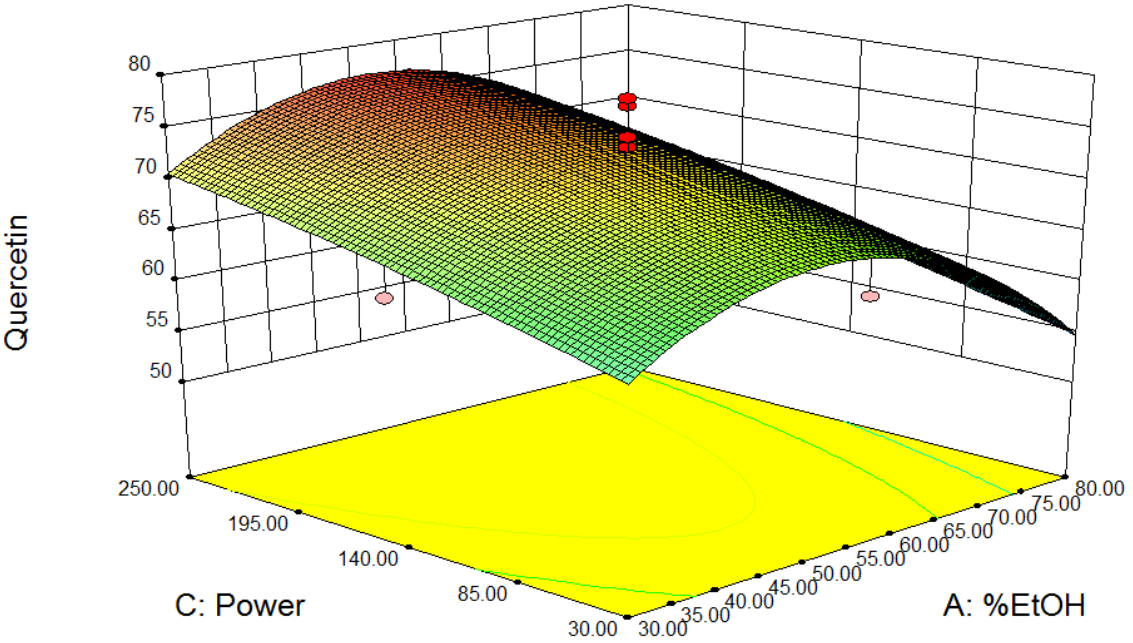


Figure 4-43: Three-dimensional response surface plot for the effect ethanol purity (A) and extraction time (B) on quercetin extraction at a constant power (C) at 140W

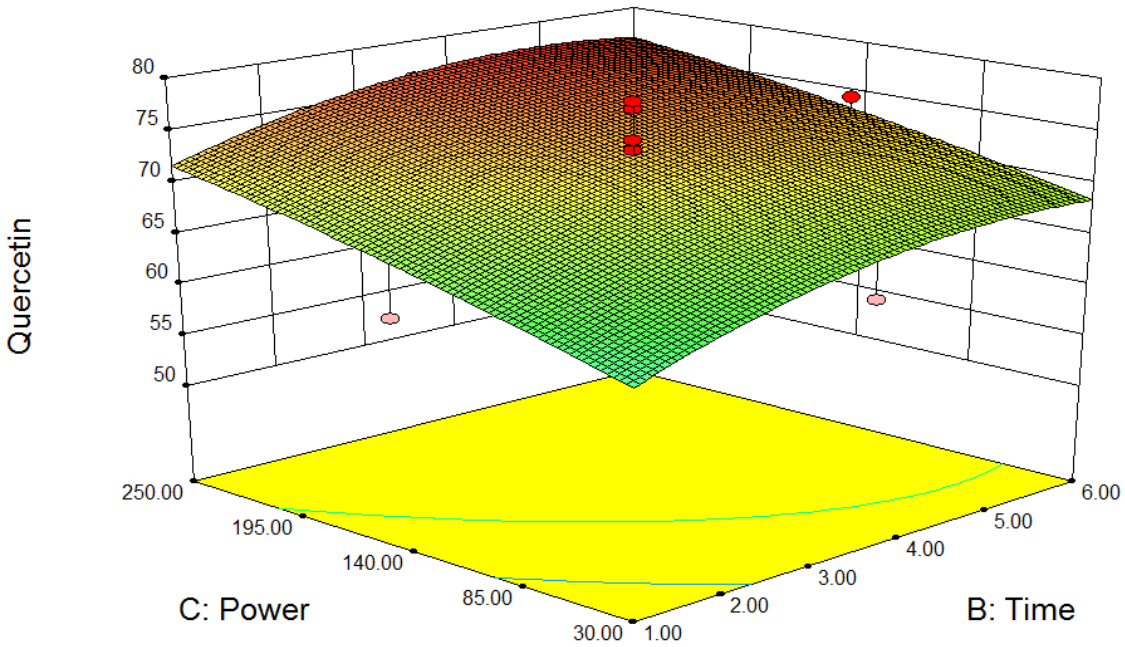


Figure 4-44: Three-dimensional response surface plot for the effect extraction time (B) and power (C) on quercetin extraction at a constant power (A) at 55%.

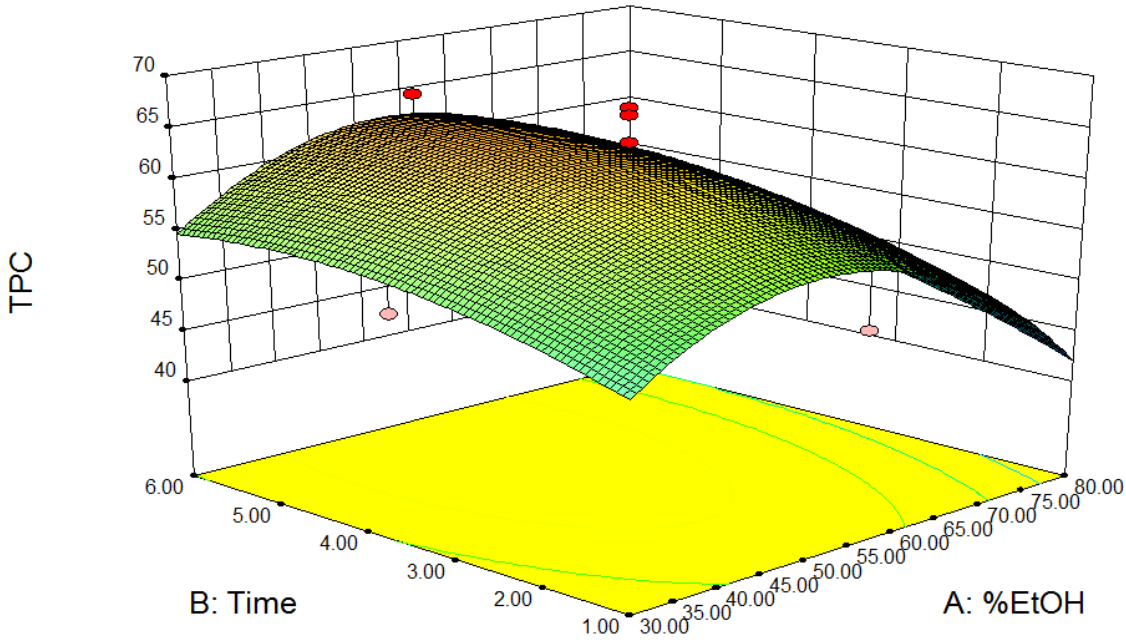


Figure 4-45: Three-dimensional response surface plot for the effect ethanol purity (A) and extraction time (B) on total phenolic content extraction at a constant power (C) at 140W

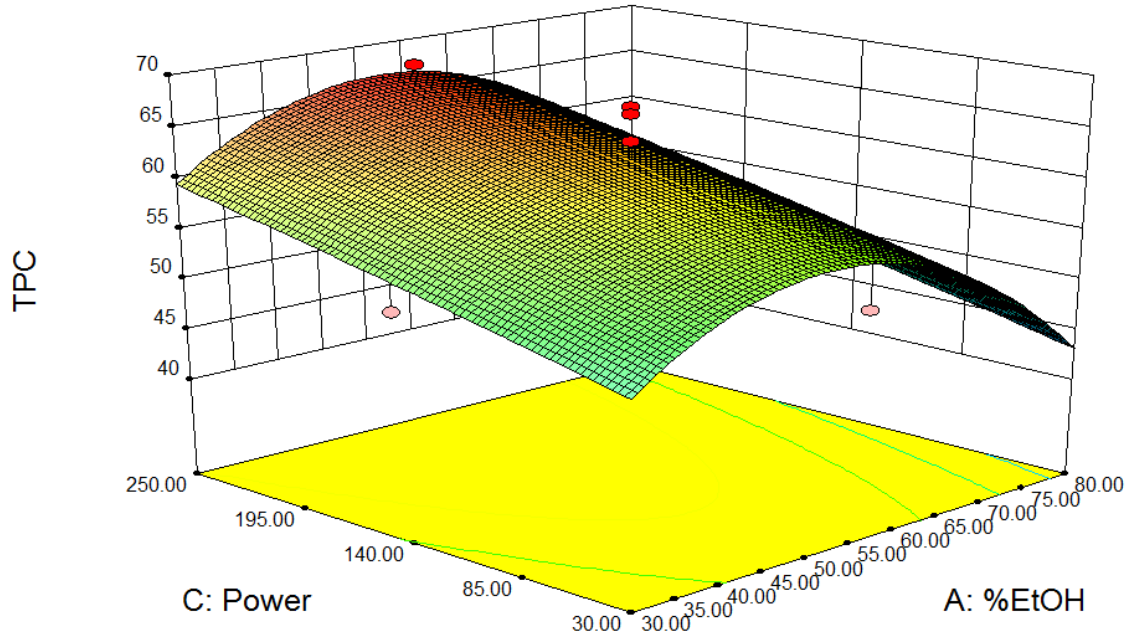


Figure 4-46: Three-dimensional response surface plot for the effect extraction time (B) and power (C) on total phenolic content extraction at a constant power (A) at 55%.

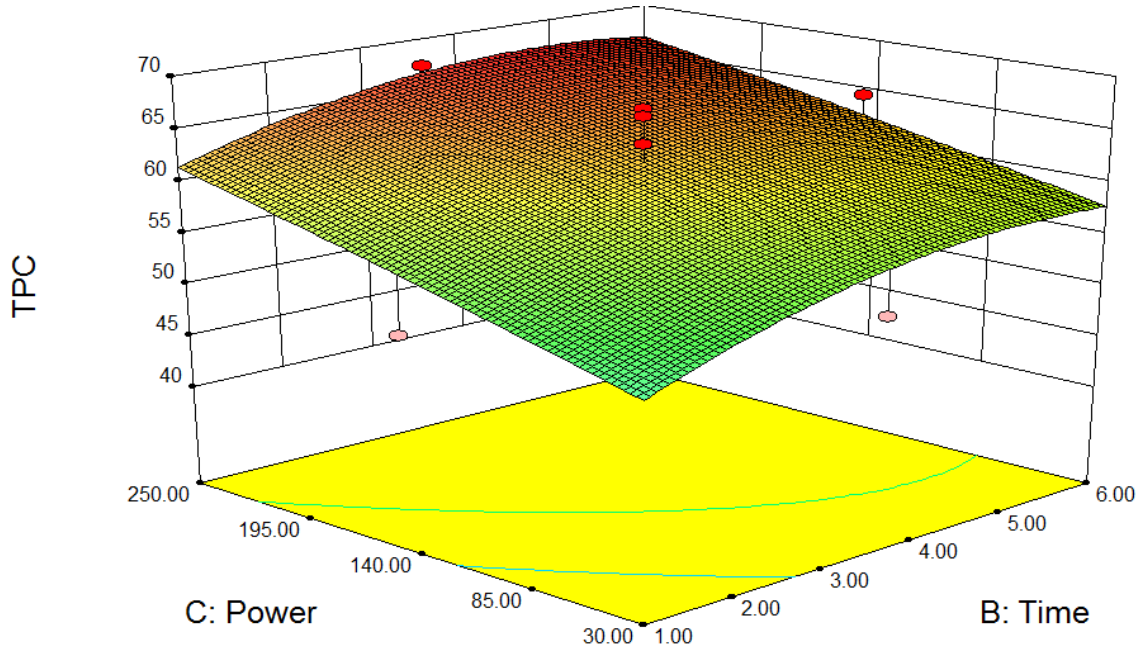


Figure 4-47: Three-dimensional response surface plot for the effect and extraction time (B) and power (C) on total phenolic content extraction at a constant ethanol purity (A) at 140W.

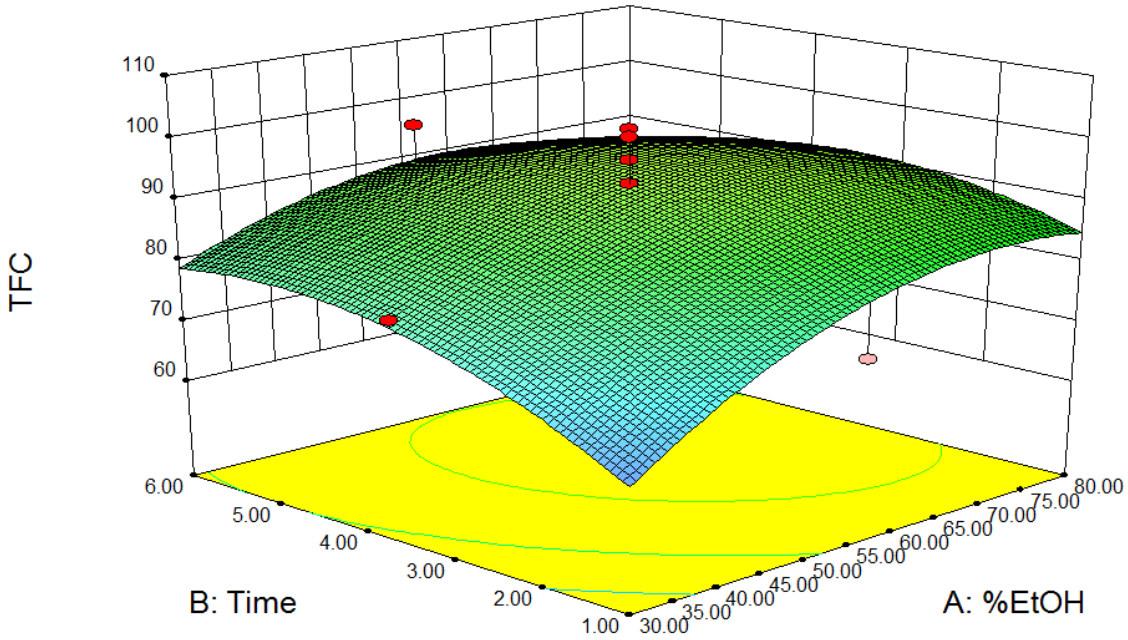


Figure 4-48: Three-dimensional response surface plot for the effect ethanol purity (A) and extraction time (B) on total flavonoid content extraction at a constant power (C) at 140W.

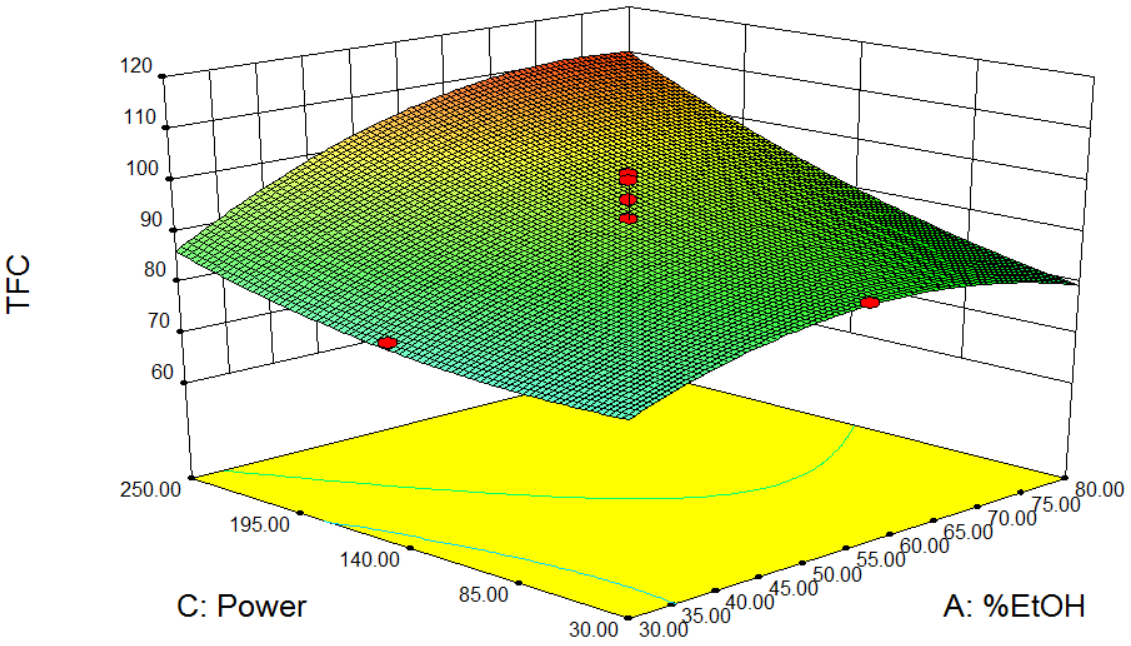


Figure 4-49: Three-dimensional response surface plot for the effect extraction time (B) and power (C) on total flavonoid content extraction at a constant power (A) at 55%.

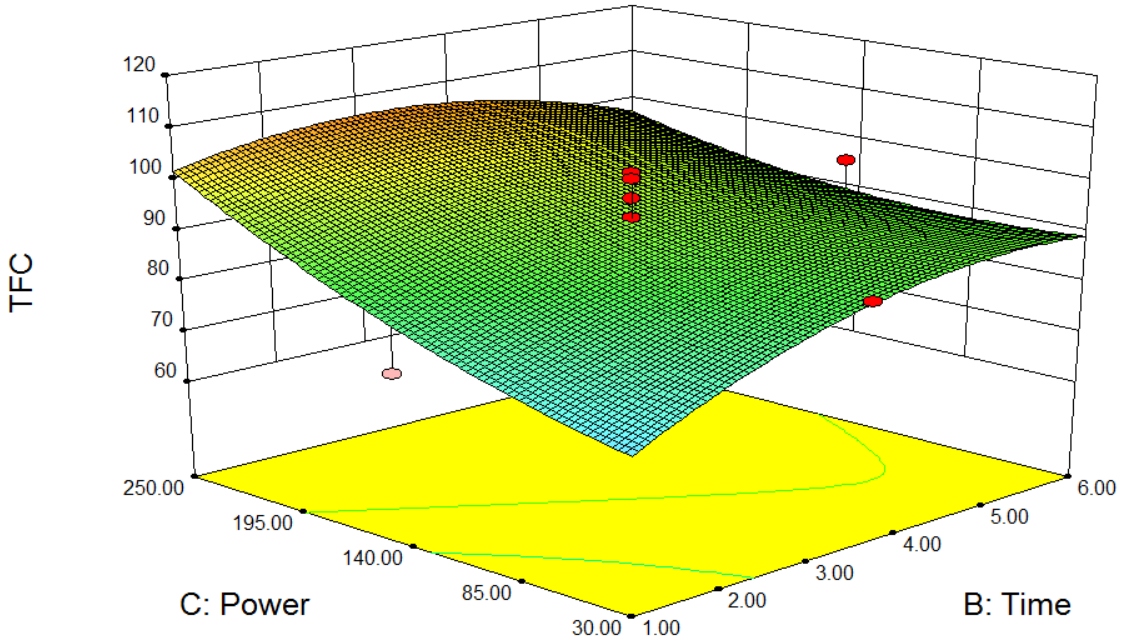


Figure 4-50: Three-dimensional response surface plot for the effect and extraction time (B) and power (C) on total flavonoid content extraction at a constant ethanol purity (A) at 140W.

4.6.2.4 Validation of model

Optimization can be performed by using mathematical (numerical) or graphical (contour plot) approaches. Graphical optimization is limited to cases due to few responses. Simon, (2003) explained that numerical optimization requires defining an objective function (called a desirability or score function) that reflects the levels of each response in terms of minimum (zero) to maximum (one) desirability.

Table 4-57: Condition for factors in optimizing polyphenol extraction.

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

To determine the suitability of the model equation, prediction on the optimum response value was tested under the optimum conditions as described in Table 4-57. The

experiments were performed based on the suggested best condition in Table 4-57 and the result is presented in Table 4-58. The validation experiments were conducted at the suggested best conditions and the error from these runs were range from 0.474% to 4.355%. Referring on the predicted and experimental results presented, the experimental values were in good agreement with the predicted values proposed by the model with an error less than 5 % and proved to be an adequate model.

Table 4-58: Comparison between predicted and experimental value of UAE and MAE at optimum condition

Response	Run	Ultrasonic Assisted Extraction			Microwave Assisted Extraction		
		Predicted Value	Experimental Value	Error	Predicted Value	Experimental Value	Error
Phyllanthin	Run 1	5.026	5.241	4.102	5.513	5.331	3.414
	Run 2	5.026	4.999	0.54	5.513	5.487	0.474
	Run 3	5.026	5.105	1.548	5.513	5.764	4.355
Gallic Acid	Run 1	10.498	10.103	3.91	15.149	15.394	1.592
	Run 2	10.498	10.706	1.943	15.149	15.531	2.46
	Run 3	10.498	10.468	0.287	15.149	15.031	0.785
Quercetin	Run 1	17.212	17.524	1.78	77.656	76.905	0.977
	Run 2	17.212	17.692	2.713	77.656	77.097	0.725
	Run 3	17.212	17.236	0.139	77.656	75.364	3.041
Total Phenolic Content	Run 1	48.791	51.139	4.591	67.355	66.214	1.723
	Run 2	48.791	46.582	4.742	67.355	68.135	1.145
	Run 3	48.791	50.139	2.689	67.355	66.097	1.903
Total Flavonoid Content	Run 1	76.175	79.881	4.639	112.023	113.496	1.298
	Run 2	76.175	79.535	4.225	112.023	112.946	0.817
	Run 3	76.175	77.024	1.102	112.023	110.651	1.24

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 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, 4.41mg Phy/g of phyllanthin, 9.86mg GA/g gallic acid, and 5.48mg Que/g of quercetin. From the RSM study 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 30.03% to 76.31 % subjective to which response is required. The best power is suggested at 250W. 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 preferable method for polyphenol extraction as it required lesser than and able to obtain higher yield compared to UAE method.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In conclusion from the findings of research, the yield of the bioactive component is dependent on the solvent polarity used in the extraction. The highest yield value of 4.56mg Phy/g DW of phyllanthin was obtained by using 20% aqueous Isopropanol; 10.14mg Que/g DW of quercetin was obtained by using 20% aqueous ethanol; and 15.44mg GAE/g DW of gallic acid was obtained by using water. This showed that the polarity of solvent enhances the extraction of both hydroxylated and methoxylated compounds from the P. Niruri. Besides, it is concluded that microwave assisted extraction is shown a faster extraction compared to ultrasonic assisted extraction. From the central composite design analysis, microwave assisted extraction at extraction power at 250W, extraction time at 3.62 minutes and ethanol concentration of 52.58% able to obtain the optimum yield of polyphenol extraction with the desirability of 83.70%.

5.2 Recommendation

The research is recommended continue in the aspect retention time of bioactive component after extraction. 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 thermal degradation over a period exposure of high temperature. Therefore, the research could be continued for the bioactive component retention period by using spray drying microencapsulation technology.

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APPENDIX