CHARACTERIZATION AND PROCESS OPTIMIZATION OF *Melastoma malabathricum* L. EXTRACT

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Thesis submitted in fulfillment of the requirements
for the award of the degree of
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SUPERVISOR'S DECLARATION

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

Signature :
Name of Supervisor : Assoc. Prof. Dr. Mimi Sakinah Bt Abdul Munaim
Position :
Date :

STUDENT'S DECLARATION

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

Signature :
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Date :

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ABSTRACT

Melastoma malabathricum L. (MML) or local name as senduduk, have demonstrated the presence of phenolic compound. The phenolic compound in plants has a role to inhibiting the growth of microorganism. Antimicrobial activity in plants has become an important area in the food and beverages industry; therefore natural preservatives will be very potential for more natural and fresh-like foods. In this study, to obtain the phenolic compound from MML was used extraction. Conventional extraction as heating, boiling, or refluxing can be used to extract phenolic, however, the disadvantages are the loss of phenolics due to ionisation, hydrolysis and oxidation during extraction as well as the long extraction time. In this thesis, the potentionals of ultrasonic technology in extraction was investigated. Comparation between ultrasonic-assisted extraction and classical extraction (homogenizer extraction) with incubated waterbath as a control were investigated to provide understandings of influence of ultrasonic irradiation on the production of phenolic compound in MML. Extracts were analyzed for phenolic compound (gallic acid, ellagic acid and total phenols compound). Folin Ciocalteau method was chosen for total phenols determination, while the phenolic acids (gallic acid and ellagic acid) were analyzed by using HPLC. Results showed that ultrasonic-assisted extraction was more effective to produce phenolic compound (gallic acid, ellagic acid and total phenol) from MML extract than using the classical extraction.. Acid-hydrolysis method was found the best method for post-treatment process of MML extract to produce maximum phenolic compound. Some characterization studies also investigated to understandings about nutrient composition, chemical properties and antimicrobial activity of MML extract. MML extract showed inhibitory activity against microorganism, such as *B. cereus, B. subtilis*, *S. typhi* and *E. coli.* The extraction process of MML extract were studied in this thesis, namely solid-to-liquid ratio, extraction temperature and extraction time. Prior to conducting an experimental design approach, in this study was indentification the parameter range in extraction process. The experimental run and optimization were designed using Design Expert Software as suggested by Response Surface Methodology (RSM). The optimum extraction process for highest phenolic compound extracted were obtained at extraction temperature and extraction time of 59.96 °C and 92.55 min, respectively, whereas, the solid loading about 20.07 g. Under this condition, the yield of gallic acid, ellagic acid and total phenol was 1.79 mg/g, 0.16 mg/g and 15.10 mg GAE/g. The results obtained in this study have exposed capability of ultrasonic technology in extraction of phenolic compound. Further works are nevertheless required to provide deeper understanding the mechanisms involved to facilitate the development of an optimum system applicable to the industry.

ABSTRAK

Tesis ini membentangkan penyelidikan tentang penentuan dan pengoptimuman pengaruh dari kondisi ektrak (jumlah sample, suhu ektrak dan masa ektrak) dengan bantuan ultrasonik ektrak air dari kandungan phenol (asid gallik, asid ellagik dan total phenol) dalam upaya untuk menghasilkan *M. malabathricum* L. ektrak yang kaya phenol sehingga untuk mempersiapkan bahan yang dapat langsung dimasukkan ke dalam minuman sebagai tambahan pengawet. Kaedah *Folin Ciocalteau* dipilih untuk penentuan total phenol, manakala asid gallik dan asid ellagik dianalisis menggunakan Kromatografi Cecair Berprestasi Tinggi (HPLC). Satu faktor pada satu masa (OFAT) digunakan untuk menentukan pengaruh dari kondisi ektrak untuk menghasilkan kandungan phenol. Keputusan yang terhasil dari percubaan mendapati kandungan maksimum dari phenol dengan jumlah sample 20 g, suhu ektrak 60 °C dan masa ektrak selama 90 minit, dengan jumlah asid gallik, asid ellagik dan total phenol sebesar 1.72, 0.16 dan 14.78 mg/g. *Response surface methodology* (RSM) digunakan untuk mengoptimumkan kondisi ektrak kandungan phenol dari daun *M. malabathricum* L. Keputusan menunjukkan bahawa parameter jumlah sample mempunyai pengaruh signifikan terhadap kandungan phenol dan masa ektrak mempunyai kesan yang signifikan terhadap nilai asid gallik dan total phenol. Keadaan optimum dikenalpasti sebagai jumlah sample 20,07 g, suhu ektrak 59,96 °C dan masa ektrak sekitar 92,55 minit. Dalam keadaan ini, hasil dari asid gallik, asam ellagik dan total phenol 1,79 mg/g, 0,16 mg/g dan 15,10 mg GAE/g. Nilai percubaan pada pembolehubah respon pada keadaan optimum sesuai dengan baik dengan nilai ramalan.

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LIST OF ABBREVIATIONS AND SYMBOLS

AAS	Atomic absorption spectrometry
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
Ca	Calcium
CCD	Central composite design
CIP	Collection of Institut Pasteur
cm	Centimeter
Cu	Copper
DOE	Design of experiment
DNA	Deoxyribonucleic acid
EA	Ellagic Acid
Eq.	Equation
ET	Ellagitannins
FC	Folin Ciocalteau
FD	Folin Dennis
Fe	Ferrum
FTIR	Fourier Transfer Infrared
g	Gram

GA	Gallic Acid
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectra
GT	Gallotannins
h	Hour
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
HTs	Hydrolyzable tannins
H_2O	Water
IFO	Information
KBr	Potassium bromide
Kcal	Kilocalorie
KHz	Kilohertz
L	Litre
M	Molar
M. malabathricum L.	*Melastoma malabatricum* Linn
min	Minute
Mg	Magnesium
mg	Milligram

mg GAE/g	Milligram Gallic Acid Equivalent/gram
ml	Milliliter
mm	Micrometer
Mn	Manganese
Na	Natrium
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NCIMB	National Collections of Industrial, Marine and Food Bacteria
Nm	Nanometer
OFAT	One-factor-at-a-time
PB	Prussian blue
RSKK	Refik Saydam National Type Culture Collection
RSM	Response Surface Methodology
Std. Dev.	Standard Deviasi
TFA	Trifluoroacetic acid
TLC	Thin Layer Chromatography
TP	Total Phenol
UV	Ultraviolet
w/v	Weight/volume

y	Response
Zn	Zinc
3D	3 Dimensional
º	Degree
%	Percentage
µ	Micro/Micron
µl	Microlitre
µm	Micrometer
% w/w	Percentage weight/weight
β	Beta (Coefficient)

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Contamination and spoilage from microorganisms is a major problem in the food and beverage industry. One of the ways to inhibit the growth of microorganism in foods is to add chemicals that have antimicrobial activity, commonly called preservatives. Preservatives frequently used are acetic, benzoic, lactic, propionic, sorbic acid, nitrites and sulfites. The uses of chemical preservative are questioned in the food industry because they are suspected of having carcinogens. The use of natural preservatives in food will be of great potential for more natural and fresh-like foods. Therefore, the search for natural products with antimicrobial activity has become an important area in the food and beverage industry.

Antimicrobial activities in plants are partly due to the in phenolic compound, particularly tannins and flavonoids. In previous study that conducted by Zakaria (2007) concluded that the leaves of *Melastoma malabathricum* L. (MML) have demonstrated the presence of phenolic compound. MML is an erect shrub or small tree of 1.5 to 5 m in height, found more or less everywhere throughout Malaysia. *Melastomataceae* spp. belongs to the Family of *Melastomataceae*, Order Mrytales, Class Dicotyledon and Division Angiosperm (Sulaiman et al., 2007). It is commonly called “Straits Rhododendron” and locally known as “Senduduk”.

The studied that persent by Yoshida et al. (1992a) had succeeded in isolating several hydrolysable tannins from the dry leaves of MML. The main tannin was oligomers named nobotanin B, diMMLrs named malabathrins B, malabathrins C and malabathrins D, monomers named 1,4,6-tri-*O*-galloyl-β-D-glucoside, 1,2,4,6-tetra-*O*-galloyl-β-D-glucoside, strictinin, casuarictin, pedunculagin, nobotanin D, pterocarinin and oligomers nobotanin G, nobotanin H, nobotanin J. β-sitosterol, α-amyrin, uvaol, sitosterol-3-*O*-β-Dglucopiranoside, quercetin, quercitrin and rutin are also found in MML by Nuresti et al. (2003).

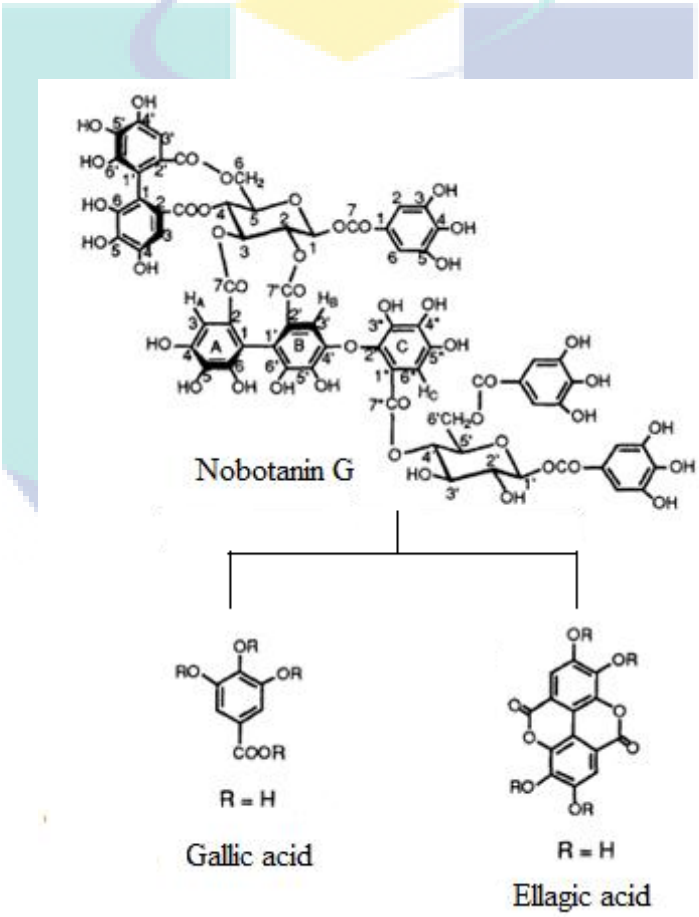


Figure 1.1: Structures of acid hydrolysates from Nobotanin G

Source: Yoshida et al. (1992b)

Acid hydrolysis of nobotanins produces the percentages of gallic acid and ellagic acid (Yoshida et al., 1992b). Figure 1.1 shows the chemical structure of gallic acid and ellagic acid. Thus this study was established to analyze gallic acid, ellagic acid and total phenol in leaves of MML. Gallic acid and ellagic acid were common constituents and known to have antimicrobial activity (Martini et al., 2009; Chanwitheesuk et al., 2007).

1.2 Statement of Problem

Phenolic compound from plants has been extracted using methanol, ethanol, acetone, water, ethyl acetate, propanol, dimethylformamide, and their combinations (Antolovich et al., 2000). However, it is difficult to find satisfactory extraction solvents that are suitable to extract all classes of phenolics. Methanol has been widely used to extract free and simple phenolics in plants, including fruits, vegetables and bitter melons for identification and quantification purposes (Budrat and Shotipruk, 2009; González-Montelongo et al., 2010). However, the use of methanol for extraction is not acceptable for food uses, due to its toxicity to humans. For these reasons, in this study was established using aqueous extraction.

Conventional extraction as heating, boiling, or refluxing can be used to extract phenolic, however, the disadvantages are the loss of phenolics due to ionisation, hydrolysis and oxidation during extraction as well as the long extraction time (Hui et al., 2005). The production of phenolic compound necessitates a search for economically and ecologically feasible extraction technologies. For this purpose, the application of power ultrasound seems to be very promising, as it was concluded from the studies on the extraction of proteins, medicinal compounds, tea solids, etc. (Mason et al., 1996). Recently, ultrasonication has been reported to improve significantly the phenolic compound in *Betula alleghaniensis* Britton, grape skin, *Foluim eucommiae*, *Rosmarinus officinalis* and *Saphora japonica* compare to the control extraction, such as maceration, waterbath incubated, reflux, microwaves and enzym-assisted extraction (Diouf et al., 2009; Corrales et al., 2008; Huang et al., 2009; Albu et al., 2004; Paniwnyk et al., 2001). In this studied was to compare the extractibility of the phenolic compound in MML, using aqueous as a solvent extraction,

by classical procedures (homogenizer) as well as by application of ultrasound, with incubated in waterbath as a control. The further purpose was to investigate the influence of ultrasonic irradiation on the production of phenolic compound in MML.

The MML extract was obtain and fully characterized of the nutrient composition, chemical properties; such as gallic acid and ellagic acid, and antimicrobial activity. The characterization studies to provide understandings of fundamental issues such as nutritional quality, fungtional group and retention time of chemical properties and inhibitation microorganism from MML extract.

The optimization from extraction process (solid loading, extraction temperature and extraction time) to produce the optimum yield of phenolic compound, have became a important area. Classical optimization studies use the one-factor-at-a-time approach, in which only one factor is variable at a time while all others are kept constant. This approach is time-consuming and expensive. In addition, possible interaction effects between variables cannot be evaluated and misleading conclusions may be drawn. The response surface methodology (RSM) can overcome these difficulties, since it allows accounting for possible interaction effects between variables (Khuri and Comell, 1996; Montgomery and Runger, 2003). If adequately used, this powerful tool can provide the optimal conditions that improve a process (Bas and Boyaci, 2007).

1.3 Research Objective

1. To extract the phyto-chemical of MML,
2. To characterize MML extract for the nutrient composition, chemical properties and antimicrobial activity
3. To optimize the extraction process for the production of optimum yield of phenolic compounds.

1.4 Scope of Study

1. The plant materials, leaves of MML with light pink-magenta petals were collected randomly around UMP campus, Gambang, Kuantan, Malaysia.
2. The characterization of MML extract was analyzed for their nutrient composition, chemical properties and antimicrobial activity. The characterization in nutrient composition was included to proximate value of ash compound, protein, fat, total carbohydrate, energy, dietary fiber, cholesterol, mineral compounds and trace element. The chemical properties of MML extract, namely gallic acid and ellagic acid, the characterization was included fungtional goup analyzes using FTIR spectrometer and retention time using HPLC. While the disc diffusion method was used to characterize the antimicrobial activity in MML extracts.
3. There are three extraction techniques was compare from determine the best extraction technique for produce phenolic from MML, namely ultrasonic-assisted extraction, homogenizer extraction and incubated in waterbath. The extraction was used aqueous as a solevent extraction.
4. The method being used to analyze total phenol compound was Folin Ciocalteau method that is by using UV-VIS spectrophotometer. The Folin Ciocalteau method was applied because it is the most recently established procedure for analysing total phenolic compound which has replaced the Folin-Denis reagent method (Singleton and Rossi, 1965). High-performance liquid chromatographic with UV detection at 280 nm was utilized to determine the phenolic acid (gallic acid and ellagic acid compounds). High-performance liquid chromatographic was a simple and rapid analytical method for the determination of phenolic acid (Amakura et al., 2000). The MML extract was tested with hydrolysis method prior analyze phenolic acid using HPLC.
5. Analyzed of optimization extraction process (solid loading, extraction temperature and extraction time) to produce the optimum yield of gallic acid, ellagic acid and total phenol was using Response Surface Methodology (RSM).

1.5 Significance of Study

Nowadays, an increasing awareness of the consumers for the use of synthetic preservative needs research for more efficient antimicrobials with fewer side effects on human health. Phenolic from various natural sources have been reported to have a variety of biological effects, including antimicrobial activities. The most important thing during the produced of phenolic from plants is to extract the plants. Thus extraction technique and extraction process have an important area to produce maximum yield of phenolic from plants.

It is expected the knowledge obtained from this study will affirm good extraction technique for extracted the phenolic compound from MML, also the characterization and optimization in extraction process for development and application in order to produce food grade MML extracts rich in phenols, thus as to prepare ingredients that can be directly be incorporated into flavored waters and/or fruit drinks as additional preservative.

1.6 Organization of Thesis

The organization of this thesis goes as follows:

1. Chapter 2

   In chapter 2 briefly describe the chemistry of major phenolic compound of MML extract such as gallic acid and ellagic acid, also their antimicrobial activity. The chapter also explains the extraction process of phenolic compound and the techniques for determination the total phenol and phenolic acid from MML extract. Previous work related to these techniques and the various applications considered in thesis are reviewed.

2. Chapter 3

   Methodology of extraction, characterization and optimization of MML extract were discussed in this chapter. Included the extraction technique, post-treatment of

extraction, nutrient composition, chemical properties, antimicrobial activity, parameter process range identification and CCD design for optimization were also discussed.

3. Chapter 4

   In chapter 4 describe the results of the best method and post-treatment of extraction phenolic compound from MML extract, characterization of MML extract and optimization of produce the optimum phenolic compound of MML extract, also the validation of empirical model and confirmation run of the optimum MML extract.

4. Chapter 5

   In these chapter summaries the major features of this thesis and proposes some research points that can be investigated for future work.

CHAPTER 2

LITERATURE REVIEW

This chapter has presented a detailed explanation of the topic. The previous works and researches have been provided to relate with this research, thus this research will be more relevant and supported. There are two sections in this chapter, namely chemistry and extraction of MML.

2.1 Chemistry of MML

This section are divided into two sub-sections, there are phenolics compound in leaves of MML and antimicrobial activity of phenolics compound in leaves of MML.

2.1.1 Phenolics Compound in MML

Phenolics are synthesized by plants as secondary metabolites (Balasundram et al., 2006) and occur as tannins or other phenolics (Naczk and Shahidi, 2006). Based on their chemical structures and behavior, tannins can generally be categorized into two large groups; hydrolysable tannins and condensed tannins (Chavan et al., 2001; Okuda et al., 1989).
The major phenolic compounds in the leaves of MML are gallic acid and ellagic acid. There are represent the polyphenolic parts in the molecules of hydrolysable tannins.

Hydrolysable tannins are compounds containing a central core of glucose or another polyol esterified with gallic acid, also called gallotannins, or with hexahydroxydiphenic acid (HHDP), also called ellagitannins. Hydrolysable tannins are hydrolyzed by acids, bases or

esterase. The hydrolysis of gallotannins with strong acids will yield gallic acid and the core polyol. Meanwhile, hydrolysis of ellagitannins will liberate HHDP which will spontaneously lactonized to ellagic acid in aqueous solution (Hagerman, 2002). In Figure 2.1 shown chemical sturture of Gallotanin and Ellagitannin where with acid hydrolysis may produce gallic acid and ellagic acid.

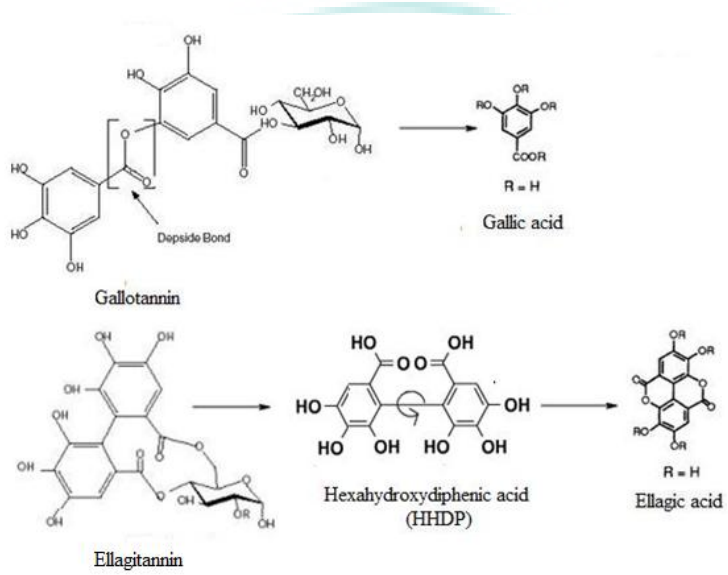


Figure 2.1: Structure of acid hydrolysis from Gallotannin and Ellagitannin

Source: Mueller-Harvey (2001)

Gallic Acid

Gallic acid is one of the important compounds that contribute the antimicrobial activity. The IUPAC name for Gallic acid is 3, 4, 5-trihydroxybenzoic acid. The chemical formula is $C_6H_2(OH)_3COOH$. Gallic acid is found both free and as part of tannins. This compound has a molecular weight of 170.12 g/mol. The chemical structure of gallic acid is aromatic as shown in Figure 2.2.

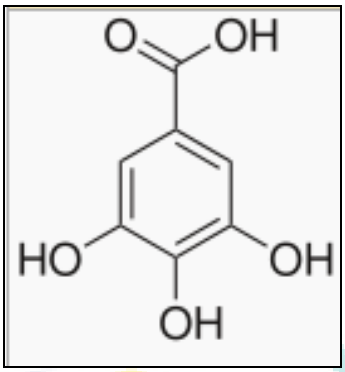


Figure 2.2: The chemical structure of gallic acid

Ellagic Acid

The IUPAC name for ellagic acid is 2,3,7,8-Tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione. The formula molecule for ellagic acid is $C_{14}H_6O_8$. This compound has a molecular weight of 302.197g/mol. The chemical structure of ellagic acid is aromatic as shown in Figure 2.3.

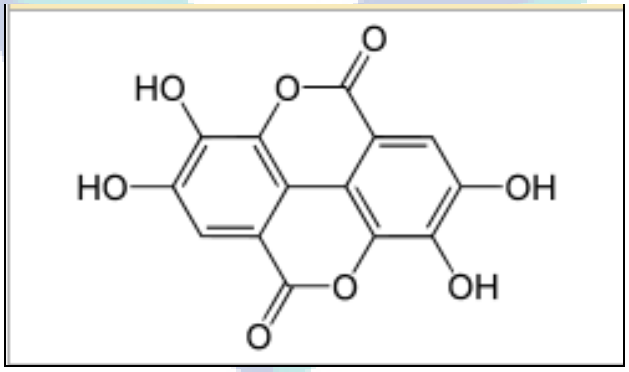


Figure 2.3: The chemical structure of ellagic acid

2.1.2 Antimicrobial Activity of Phenolic Compound in MML

Since many plant phenolics have been found to be responsible for several biological properties, including antimicrobial properties, it was expected that the antimicrobial activity

of the plant species would be related to its phenolic compounds. Phenolic compound have been found to exhibit antimicrobial has recently been discussed in many research works as shown in Table 2.1.

The mechamism of antimicrobial activity in gallic acid and ellagic acid have been discussed in previous study. Chung et al. (1998) concluded that antimicrobial mechanism of gallic acid could be iron deprivation which may work like a siderophore to chelate essential iron from the medium and make its iron unavailable to the microorganisms. This activity appears to depend on the structure of the plant polyphenols, i.e., the presence of a galloyl group (3,4,5-trihydroxybenzoyl group) (Taguri et al., 2004). Three hydroxyl groups in the B ring in gallic acid have more antimicrobial activity than other phenolic which have less hydroxyl group in the B ring. Gallic acids also bind to and precipitate proteins and enzymes. This mechanism could be explained by hydrogen binding of polygalloyl glucopyranose and hydrophobic interactions by gallic acid with surface proteins on bacteria cells. Labieniec and Gabryelak (2006) proved that there is a direct interaction between gallic acids and DNA or bovine serum albumin (BSA) and this interaction causes the conformational changes in DNA and BSA. Kawamato et al. (1997) also showed that a sufficient number of galloyl groups are needed to form a strong binding between ligand and BSA in a polyphenol molecule. An increased number of gallic acid could enhance the antimicrobial activity by increasing their protein binding capacity (Kawamato et al., 1997).

Polymeric phenolics such as ellagic acid have partial hydrophobicity that making effective to act efficiently at the membrane–water interface of bacteria. Molecules such as ellagic acid can possibly stack or embed itself in the membrane. This can severely impair the plasticity of the membrane and therefore can destabilize the cell by weakening membrane integrity which may result in the disruption of the bacterial membrane and also critical transport processes. Ellgic acid have been shown to inhibit the growth of microorganism by sequestering metal ions critical for the microbial growth and metabolism (Acamovic and Stewart, 1992; McDonald et al., 1996; Kainja et al., 1998) or by inhibiting critical functions of the bacterial membrane such as ion channels and proteolytic activity (Muhamed, 1999).

Table 2.1: Antimicrobial activity of phenolic compound from different extracts

Antimicrobial	Phenolic compound	Pathogen	Reference
Cistaceae aqueous extract	Punicalagins derivatives and gallic acid	*Staphylococcus aureus* and *Escherichia coli*	Barrajon-Catalan, E. et al., 2010
Thermally processed tannic acid	Gallic acid	*L. Monocytogenes 7694, (DA,DB); Salmonella Typhi 6539; Salmonella enterica serovar Typhimurium 19585, 14028; E. sakazakii MSDH, Fec39; E. coli 35150, 43890,* and *43895*	Kim et al., 2010
Cranberry pomace-fungal extracts	Ellagic acid	*Listeria monocytogenes,Vibrio parahaemolyticus*and *Escherichia coli-O157: H7*	Vattem et al., 2004
Blackberry leaves (*Rubus ulmifolius*)	Gallic acid, Caffeic acid, Ferulic acid, Coumaric acid, Ellagic acid, Rutin, Quercetin 3-O-β-d-glucopyranosid, Quercetin, Kaempferol	*Helicobacter pylori (CagA+ strain 10K* and *CagA− strain G21)*	Martini et al., 2009
Leaf and flower extracts from *Tamarix gallica*	Syringic acid and cathecin	*S. aureus ATCC 25923, S. epidermidis CIP106510, Micrococcus luteus NCIMB 8166, E. coli ATCC 35218, P. aeruginosa ATCC 27853, Candida albicans, Candida glabrata, Candida kefyr, Candida holmii, Candida sake*	Ksouri et al., 2009

Also, ellagic acid might be disrupting the cellular homeostasis by inhibiting the function of ion channels required by the bacteria to survive under conditions of high salt concentration. Disruption of such a mechanism in the bacterial cell membrane may be inhibitory to the microorganism (Vattem et al., 2004).

2.2 Extraction of MML

These sections were divided into two sub-sections; there are phenolic compound extraction and determination of phenolic compound in MML extract.

2.2.1 Phenolic Compound Extraction

It is generally known that the yield of chemical extraction depends on solid loading, type of solvents with varying polarities, extraction time and temperature as well as on the chemical composition and physical characteristics of the samples. Increasing solid loading was found to work positively for enhancing phenol yields (Cacace and Mazza, 2003). However, an equilibrium between the use of high and low solid loadings, involving a balance between high costs and solvent wastes and avoidance of saturation effects, respectively, has to be found to obtain an optimized value (Pinelo et al., 2006). The other, Pinelo et al., 2005a concluded that increasing solid loading could beget preferential flow channels and offside zones, promoting a decrease in the surface contact between solid and liquid. As a consequence, a decrease in mass transfer was observed (Pinelo et al., 2005a).

The solubility of phenolics is governed by the chemical nature of the plant sample, as well as the polarity of the solvents used. Previous study that condected by Harbourne et al., 2009b; Oliveira et al., 2008; Harbourne et al., 2009a; Marete et al., 2009 and Chanwitheesuk et al., 2007 have succeeded to extract phenolic compound in Meadowsweet, Walnut, Chamomile, Feverfew and *Caesalpinia mimosoides* Lamk. Using aqueous as a solvent extraction. The phenolic compound that they found was established as total phenol compound. Thus in this

study was used aqueous as a solvent extraction for safe and convenient for food usage as natural preservative.

The recovery of phenolic compounds from plant materials is also influenced by the extraction time and temperature, which reflects the conflicting actions of solubilization and analyte degradation by oxidation (Robards, 2003). An increase in the extraction temperature can promote higher analyte solubility by increasing both solubility and mass transfer rate. In addition, the viscosity and the surface tension of the solvents are decreased at higher temperature, which helps the solvents to reach the sample matrices, improving the extraction rate. However, many phenolic compounds are easily hydrolyzed and oxidized. Long extraction times and high temperature increase the chance of oxidation of phenolics which decrease the yield of phenolics in the extracts. For example, conventional extraction and concentration of phenolic is typically conducted at temperatures ranging from 20 to 50°C (Jackman et al., 1987), because temperatures > 70°C have been shown to cause rapid phenolic degradation (Cacace and Mazza, 2003). Therefore, it is of critical importance to select efficient extraction procedure/method and maintain the stability of phenolic compounds. Table 2.2 demonstrates the optimal temperature in producing phenolic compound from plants.

The conventional extraction methods such as maceration and soxhlet extraction have shown low efficiency and potential environmental pollution due to large volumes of organic solvent used and long extraction time required in those methods. A number of methods have been developed in recent years such as microwave, ultrasound-assisted extractions, and techniques based on use of compressed fluids as extracting agents, such as subcritical water extraction (SWE), supercritical fluid extraction (SFE), pressurized fluid extraction (PFE) or accelerated solvent extraction (ASE) were also applied in the extraction of phenolic compounds from plant materials. Ultrasound-assisted extraction (UAE) is a potentially useful technology as it does not require complex instruments and is relatively low-cost. It can be used both on a small and large scale in the phytopharmaceutical extraction industry (Vinatoru, 2001). Its feasibility for the extraction of secondary metabolites such as tea, ginger, olive fruit, orange peel,

strawberries etc has been highlighted in many research works (Xia et al., 2006; Balachandran et al., 2006; Jerman et al., 2010; Khan et al., 2010; Herrera and Castro, 2005).

Table 2.2: Temperature optimum extraction in different raw materials

Raw material	Extraction	Bioactive compound	Temperature optimal of extraction	Reference
Meadowsweet (*Filipendula ulmaria* L.)	Aqueous extraction	Total phenol, quercetin and salicylic acid	At or above 90 °C for 15 min	Harbourne et al., 2009b
Grape (*Vitis labrusca* B.) peel	Supercritical fluid extraction	Total phenols and total anthocyanins	45–46 °C	Ghafoor et al., 2010
Roasted wheat germ	Supercritical carbon dioxide extraction	Total phenolic and tocopherol contents	58 °C	Gelmez et al., 2008
Chamomile (*Matricaria chamomilla* L.)	Aqueous extraction	Total phenols and apigenin 7-glucoside	90 °C for 20 min	Harbourne et al., 2009a
Gardenia (*Gardenia jasminoides* Ellis) fruits	Ethanol extraction	Total phenols, crocin and geniposide	70.4 °C for 28.6 min	Yang et al.,2009
Wheat bran	Ultrasound-assisted extraction	Total phenol	60 °C for, 25 min	Wang et al., 2008a

The benefit of using ultrasound in plant extraction has already been demonstrated for a number of compounds of interest to both the pharmacology and food industries (Vinatoru et al., 1999). Specific example of the benefit include the extraction of grape skin with 50 % ethanol using ultrasonic which had increased phenolic compounds recovery approximately two-fold higher compare to the control extraction which had used waterbath incubated method (Corrales et al., 2008). An ultrasonic method for polyphenol extraction from twigs of *Betula*

alleghaniensis Britton had been developed and had resulted in an increased yield by 51% relative to the conventional polyphenol extraction method (maceration) (Diouf et al., 2009). Ultrasound is defined as sound waves of frequency > 18-20 kHz. When ultrasound is applied to liquid, it imposes an acoustic pressure in addition to hydrostatic pressure. In an infinite medium, the acoustic pressure (*Pa*) of sound wave is typically considered to be a sinusoidal wave and dependent on frequency (*f*), time (*t*) and maximum pressure amplitude of the wave (*Pa, max*), which can be written as:

$$Pa = Pa\ max\ sin2\pi ft \quad (2.1)$$

Pa, max is directly proportional to power input of the transducer. At low ultrasound intensity, the pressure wave induces motion and mixing within the liquid (Leighton, 1998), while at high intensities, the sound wave propagates into the liquid medium creating alternating compression and rarefaction cycles as shown in Figure 2.4 (Zheng and Sun, 2006).

The negative pressure in the rarefaction cycle causes liquid to fracture, which ultimately results in the formation of small bubbles or cavities (Ashokkumar and Grieser, 1999; Gong and Hart, 1998). During the subsequent compression and rarefaction cycle, bubbles contract and expand, resulting in an increased in bubble size over each ultrasound cycle (Zheng and Sun, 2006). After a number of compression and rarefaction cycles, the bubbles attain a critical size in which sonic energy is unable to keep the vapor phase inside. As a consequence during the following compression cycle, vapor suddenly condenses and bubbles implode (Mason et al., 1996). The implosion of cavitations bubbles results in many physical, mechanical and chemical effects due to generation of macro-turbulence, high velocity inter particle collisions and perturbation in micro-porous particles of the material treated resulting in the acceleration of eddy diffusion and internal diffusion. Macro turbulence has been used to enhance the heat and mass transfer in many processes (McClement, 1995). The physical effect that cavitation provides had been used to accelerate the extraction kinetics and to enhance the extraction yield of intracellular materials (Ma et al., 2009; Shah et al., 2005). The improvement in extraction performance of ultrasound-assisted extraction achieved in food processing can probably be

attributed to diffusion through the plant cell walls, disruption and washing out of the cell compounds, reduction in particle size of the vegetal material as enhanced by ultrasonic cavitation. The disruption of tissue surface structure of caraway seeds and soybean flakes due to cavitation was revealed by microscopic examination (Chemat et al., 2004; Haizou et al., 2004).

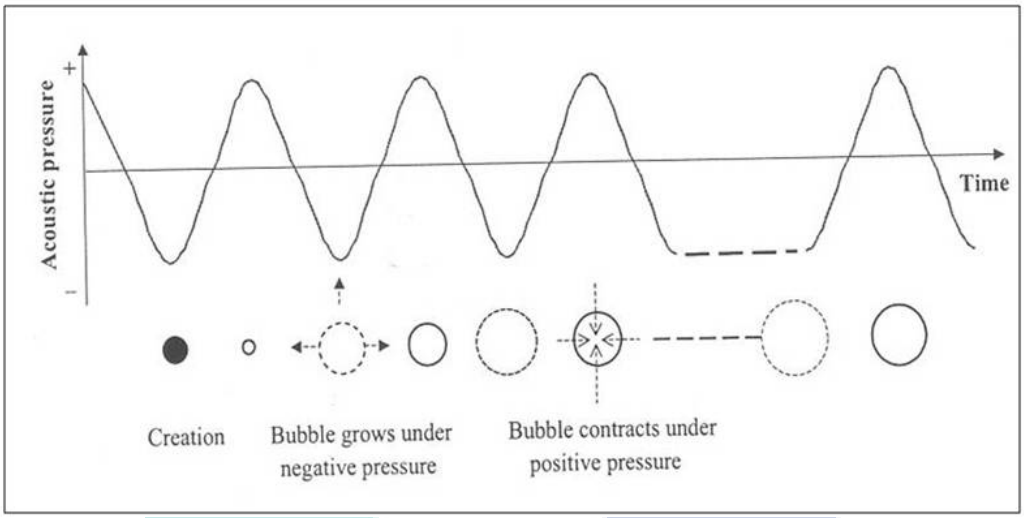


Figure 2.4: Motions of bubbles during cavitation

Source: Zheng and Sun (2006)

2.2.2 Determination of Phenolic Compound in MML extract

These parts were divided into two parts. The first part was discussed about determination of total phenol using UV-VIS spectroftometry. While the second part was discussed about determination of phenolic acid (gallic acid and ellagic acid) used HPLC method.

2.2.2.1 Determination of Total Phenol

Reaction of phenolic compounds with a colorimetric reagent has been used frequently as a method to study phenolic compound (Vermerris and Nicholson, 2006). Phenolic compound or total phenolic can be determined using the method of Folin Dennis (FD), Folin Ciocalteau (FC), and Prussian blue (PB) assay (Shahidi and Nazck, 2004).

Folin Denis Assay

The Folin Denis assay (FD) was used to determine phenolic compounds (Shahidi and Nazck, 2004) such as tannin. In 1912, it was claimed that FD was the surprising sensitive assay which detected blue (color) reaction (Folin et al., 1912). In this study, the color was formed by the reduction of phosphotungstic and phosphomolibdic reagents by polyphenolic compound and its reaction to uric acid. Schlesinger and Hasey (1981) reported that FD assay could determine total soluble phenolic compound like tannin. Folin Denis was reported to be the best assay available for phenolic determination. This assay was used widely for the determination of total phenolic in plant materials and beverages (Sahidi and Nzack, 2004). According to Singleton and Rossi (1965), there were some limitations in using FD assay, such as unreliability in results if the samples ware inadequately mixed or not in accordance to proper order of reactant addition, instability of blue color formation and the difficulties faced in experiments using a large sample. White and dense precipitates were formed in the presence of high concentration of FD reagents which would interfere in the quantification of color intensity.

Folin Ciocalteau Assay

Folin Ciocalteau assay is a substitution method of the FD assay (Shahidi and Nzack, 2004). Folin and Ciocalteau (1927) modified the FD assay by the addition of lithium sulfate and bromine to the reagents which had prevented any precipitation. According to Singleton and Rossi (1965) and George et al. (2005), this assay involves reaction of the Folin Ciocalteau

(FC) reagent with phenolic compounds in alkaline solution. In the assay, the FC reagent oxidizes phenolic compounds through their hydroxyl sites (transferring electrons from phenolic compounds) resulting in the production of complex molybdenum (Mo_8O_{23}) and tungsten blue (W_8O_{23}) complex which is known by phosphomolybdic/phosphotungstic acid complexes (Ainsworth and Gillespie, 2007). The intensity of the blue color formed would be directly proportional to the amount of phenolic compounds present.

The FC assay was claimed to be the easiest and the most consistent method to yield total phenolic compound in brown algae dissolved in 80 % methanol (Alstyne 1994). Singleton and Rossi (1965) had mentioned that the FC assay had more advantages for total phenolic determination compared to the FD assay. However, Shahidi and Nazck (2004) reported that FC assay reacted with not only phenolic compound but also with those found in the extractable protein including ascorbic acid. The FC was used widely for the determination of total phenolic in agricultural products. The assay has been used to quantify total concentration of phenolic hydroxyl groups present in wine by-products (Alonso et al., 2002), bitter melon (Horax et al., 2005), tomato extract (Luthria et al., 2006), vegetables and fruit waste (Peschel et al., 2006), red wine grape (Thimothe, 2007), and green coffee powder (Ramalaksmi et al., 2007).

Prussian blue Assay

Prussian blue assay was also used to quantify polyphenolic compounds as tannin (Despandhe and Cheryan, 1987). The reaction involved in Prussian blue assay was based on the formation of ferricyanide-ferrous ion complex by the reduction of ferric to ferrous ion by polyphenolic compounds (Shahidi and Nzack, 2004). This assay would give a higher total phenolic compound of dry bean than did the FC assay (Despandhe and Cheryan, 1987). Carmona et al. (1991) reported that although FD had given a similar result as the Prussian blue assay, Prussian blue assay was recomended for tannin determination due to its convenience.

2.2.2.2 Determination of Phenolic Acid (Gallic Acid and Ellagic Acid)

Determination of phenolic acid have devided in two steps, fisrt step is hydrolysis the MML extract for liberated the phenolic acid from cell wall. The seconds step is analyzed the hydrolyzed MML extract using HPLC method.

2.2.2.2.1 Hydrolysis Method for Phenolic Acid (Gallic Acid and Ellagic Acid)

Phenolic acids also exist as insoluble bound complexes, which are coupled to cell wall polymers through ester and glycosidic links (Escarpa and Gonzalez, 2001; Mattila and Kumpulainen, 2002; Nardini and Ghiselli, 2004). Bound phenolic acids are typically liberated using base hydrolysis, acid hydrolysis or enzymatic treatmens (Mattila and Kumpulainen, 2002; Yu et al., 2001).

Mattila and Kumpulainen (2002) showed that acid hydrolysis liberated significant yield of gallic acid from red raspberries and strawberries. Soong and Barlow (2006) indicated that subjecting mango seeds to an acid hydrolysis procedure allowed for the release of substantial yield of gallic acid and ellagic acids.

The basic of hydrolysis is it liberates additional phenolics acids from the cell walls (Kosar et al., 2005). This was evident in Fallico et al. (1996) and Peleg et al. (1991) research whereby the level of free acids as determined by direct extraction of orange juice was very low compared to that of bound phenolic acids released by hydrolysis. As shown in Figure 2.5 and 2.6, the concentration of gallic acid and ellagic acid in mango kernel increased after the acid hydrolysis treatment (Soong and Barlow, 2006). The work of Soong and Barlow (2006) showed a dramatic difference in the amount of gallic acid and ellagic acid measured in mango kernels that were subjected to acid hydrolysis (gallic acid: 838 mg/100 g and ellagic acid: 74.5 mg/100 g and) compared to methanol extraction (gallic acid: 20 mg/100 g and ellagic acid: 11.7 mg/100 g). Hydrolysis of hydrolysable tannins present in the mango kernels was cited as the reason for the increase in gallic acid levels.

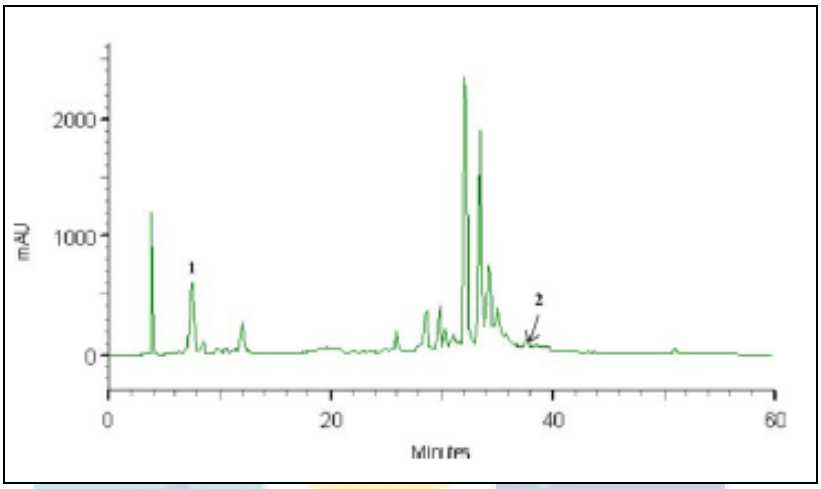


Figure 2.5: HPLC chromatogram (monitored at 280 nm) of (1) Gallic Acid and (2) Ellagic Acid extracted from mango kernel with methanol (at 70 °C for 1 h) (Soong and Barlow, 2006)

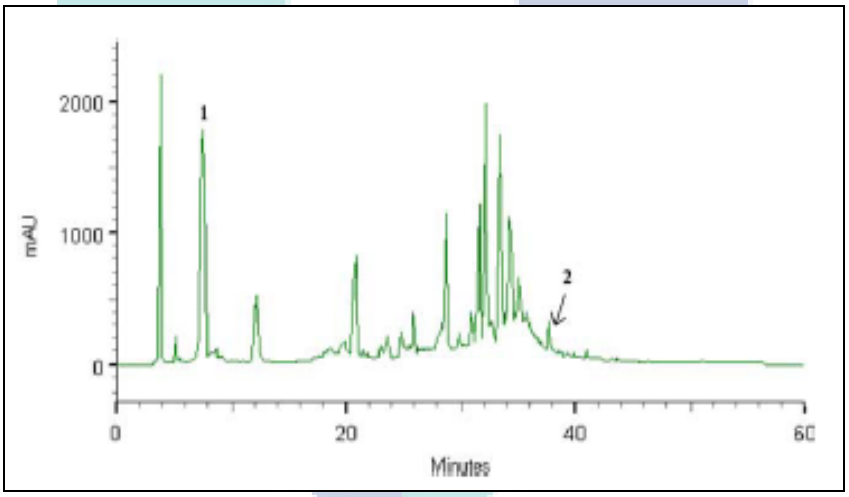


Figure 2.6: HPLC chromatogram (monitored at 280 nm) of (1) Gallic Acid and (2) Ellagic Acid from mango kernel methanolic extract hydrolyzed at 85 °C for 2 h (Soong and Barlow, 2006)

In this study acid hydrolysis methods had been used based on the work presented by Harbourne et al. (2009b) for liberated amounts of gallic acid and ellagic acid from MML extract. Various methods of hydrolysis with different raw materials are being shown in Table 2.2.

2.2.2.2.2 HPLC Analysis of hydrolyzed MML extract

HPLC with UV detector currently represents the most popular and reliable technique for analysis of phenolic compounds. HPLC techniques offer a unique chance to analyze simultaneously all components of interest together with their possible derivatives or degradation products (Sakakibara et. Al., 2003; Downey and Rochfort, 2008). The introduction of reversed-phase (RP) columns has considerably enhanced HPLC separation of different classes of phenolic compounds and RP C18 columns are almost exclusively employed. It was found that column temperature may affect the separation of phenolics and constant column temperature is recomended for reproducibility (Oh et al., 2008).

Acetonitrile and methanol are the most commonly used organic modifiers. In many cases, the mobile phase was acidified with a modifier such as acetic, formic, and phosphoric acid to minimize peak tailing. Both isocratic and gradient elution are applied to separate phenolic compounds. According to Pussayanawin and Wetzel (1987), detection at 280 nm was found to be the best alternative for the determination of phenolic compounds. Most of the researchers have been published on application of HPLC methodologies for the analysis of phenolics (Table 2.3).

Table 2.2: Method of acid hydrolysis in plant

Sample	Method of acid hydrolysis	Reference
Andean mashua tubers	A 20 mg sample of dried fraction was hydrolysed using 5 ml of a 50% methanol acidified solution (1.2 M HCl) for 2 h at 90°C. The solution was then adjusted to a final volume of 15 ml with 50% methanol and was cooled to room temperature.	Chirinos et al., 2007
Berries	The sample (0.5 g of freeze-dried berry) was rinsed with 25 ml of methanol into the bottle. To this mixture, 10 ml of 6 M HCl was added by careful mixing (final HCl concentration 1.2 M) and the solution was sonicated for 2 min. The remaining air in the bottle was replaced by nitrogen gas. The mixture (total volume 50 ml) was shaken in 35°C waterbath in dark. After 16 h, the extract was allowed to cool and was then filtered. A 15ml portion of the filtrate was evaporated to dryness using rotary evaporator and 35 °C waterbath. The residue was dissolved in 1.5 ml of methanol and filtered.	Haekkinen et al., 1999
Meadowsweet extracts	Briefly, 4.5 ml of extract, 4.5 ml of methanol and 1 ml of HCl (35%) were mixed and heated at 90°C under reflux for 2 h.	Harbourne et al., 2009b
Longan seed and mango kernel	10 ml of 6 M hydrochloric acid was added to extract by careful mixing (final HCl concentration 1.2 M). The solution was stirred using a magnetic stirrer at 35° C for 16 h and refluxed at 85°C for 2 h	Soong and Barlow, 2006
Green and white tea	A mixture of 1 ml of filtered tea extract and 4 ml of hydrochloric acid (2 M) was boiled in a waterbath for 30 min. After cooling, the mixture was extracted three times with diethyether (4 + 4 + 3 ml). The ethereal phases were collected and evaporated. Residue was dissolved in 1 ml of 96% ethanol, filtered through the nylon filter (0.22 μm) and stored at −20 °C.	Rusak et al., 2008
Andean purple corn (*Zea mays* L.)	A 20 mg of the freeze dried water fraction was dissolved in 15 mL 6 M HCl and heated at 100 °C in a closed vial for 40 min.	Pedreschi and Zevallos, 2007

Table 2.3: High-performance liquid chromatography of tannin and flavonoid groups in extract

Bioactive Compounds	Extract Sampel	Coloumn	Solvent System	Detection	Reference
Ellagic acid	Roots of *Decalepis hamiltonii*	Supelco, reverse-phase C18	A: 0.1% TFA in water B: methanol 70% A in B	UV, 216 nm	Srivastava et al., 2006
Quercetin and Salicylic acid	Dried aerial parts of meadowsweet (*Filipendula ulmaria* L.)	150 x 4.6 mm Agilent Zorbax Eclipse XDB C18	A: 0.025 M phosphoric acid B: Acetonitrile 0–15 min, from 20% to 40% B; 15–20 min, 20% B	UV, 210 nm	Harbourne et al., 2009b
Afzelechin, Epiafzelechin, Catechin, Epicatechin, Gallocatechin and Epigallocatechin	Leaves of *Maytenus ilicifolia*	250 × 4.6 mm Synergy Fusion RP-C18	H2O:acetonitrile:acetic acid (89:10:1, v/v)	UV, 280 nm	Souza et al., 2008
Gallic acid and Ellagic acid	Longan (*Dimocarpus longan* Lour.) and mango (*Mangifera indica* L.)	250 × 4.6 mm Shim-Pack VP-ODS column C18	A: water-acetic acid (97:3 v/v) B: methanol 0-10 min, from 0-10% B; 40 min, 70% B	UV, 280 nm	Soong and Barlow, 2006
Gallic acid, Ellagic acid and Corilagin	*Terminalia chebula* Retz Fruits	reverse-phase column C18	A: 0.1% formic acid B: methanol 0 min, 4% B; 27min, 80% B	UV, 270 nm	Rangsriwonga et al., 2009

Table 2.3: Continued

Bioactive Compounds	Extract Sampel	Coloumn	Solvent System	Detection	Reference
Catechins, Caffeine and Gallic Acid acids	Green tea; Oolong tea; Pu-erh tea; Black tea	Alltech adsorbosil C18 reverse-phase column	A: water–acetic acid, 97:3 v/v B: methanol 1 min, 100% A; 27 min, 63% B	UV, 280 nm	Zuo et al., 2002
Ellagic acid, Quercetin and Kaemferol	Leaves of *Rubus* (raspberry, blackberry)	3.9×150 mm C18 SyMMLtry C18	A: methanol; B: 0.5% orthoposporic acid in water 0 min 40% A in B, 0-0.5 min 40%to 60% A in B, 0.5-2.5 min 65% A in B, 2.5-6.0 min 65% to 45% A, 6.0-8.0 min 40% A in B	Flavonoid: UV, 370 nm Ellagic acid: UV, 254 nm	Tomczyk and Gudej, 2004
Gallic acid, Protocatechuic acid, Epigallocatechin, Catechin, Caffeic acid, Procyanidin B2, Epicatechin, Epigallocatechin Gallate, p-Coumaric acid, Ferulic acid, Piceid, Epicatechin gallate, Catechin gallate, Resveratrol, Quercetin	Peanut skin	4.6 x 250 mm C18 reverse-phase column	A: formic acid in water, 0.1% v/v B: formic acid in acetonitrile, 0.1% v/v 0–7 min: 5–7% B; 7–75 min: 7–17% B; 75–110 min: 17–45% B	Benzoic acid derivatives: UV, 250 nm Cinnamic acid derivatives: UV,320 nm Flavanols: UV, 280 nm Flavonols: UV, 370 nm Stilbenes: UV, 306 nm	Francisco and Resurreccion, 2009

CHAPTER 3

METHODOLOGY

This chapter has presented a detailed about the method was used in this study, also the procedure to implement the methodology. There are three sections in this chapter, namely extraction of phenolic compound from MML, characterization of MML extract, also experimental design and process optimization of MML extract.

3.1 Extraction of Phenolic Compound from MML

In this section was divided in three sub-section, methododlogy, materials and procedure of extraction.

3.1.1 Methodology of MML Extraction

There are three technique of extraction process from MML, namely ultrasonic-assisted extraction, homogenizer extraction and incubated in waterbath as a control. The aim of the study was to determine the best method of extracting phenolic compound from MML. That have been discussed in Chapter 2 that ultrasonic irradiation may increased the production of phenolic compound (as determined by total phenol) in many plants. Thus in this study was investigated the influence of ultrasonic irradiation on the production of phenolic compound in MML.

3.1.2 Materials of MML Extraction

Sample of Extraction

Fresh leaves of MML were randomly collected around Universiti Malaysia Pahang, Gambang, Kuantan, Malaysia, in November 2009. Sampling was performed only once. The plant was identified Biodiversity unit of Biosience Institute of Universiti Putra Malaysia (see Appendix A). A voucher specimen (SK 1783/10) was deposited in the Herbarium of UPM, Malaysia. MML leaves were cutting to small pieces and dried in an oven at 60 °C until a constant weight was gained. The dried MML were then ground in the laboratory with a blade mixer and sieved (50μ) and stored in a bottle for further analysis. Details picture of preparation the MML showed in Appendix B.

Solvent of Extraction

In this study used distilled water as a solvent of extraction.

3.1.3 Procedure of MML Extraction

Ulttrasonic-assisted extraction

The grounded MML of 20 g were first loaded into a 500 ml erlenmeyer flask which was added 100 g of distilled water and sealed by plastic film. The flasks were iMMLrsed into the ultrasonic bath for irradiation under 60 °C for 90 min. The extraction processes were carried out in an ultrasonic bath with the frequency of 20 KHz. All experiments were performed in triplicates. Figure 3.1 shows the photo of ultrasonic waterbath which was used in this study.

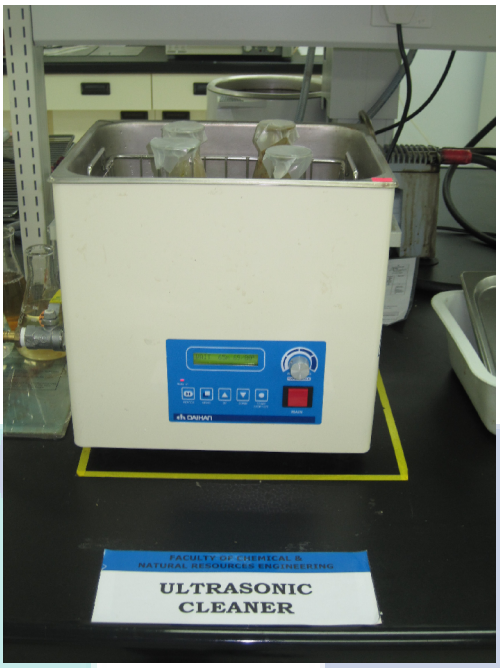
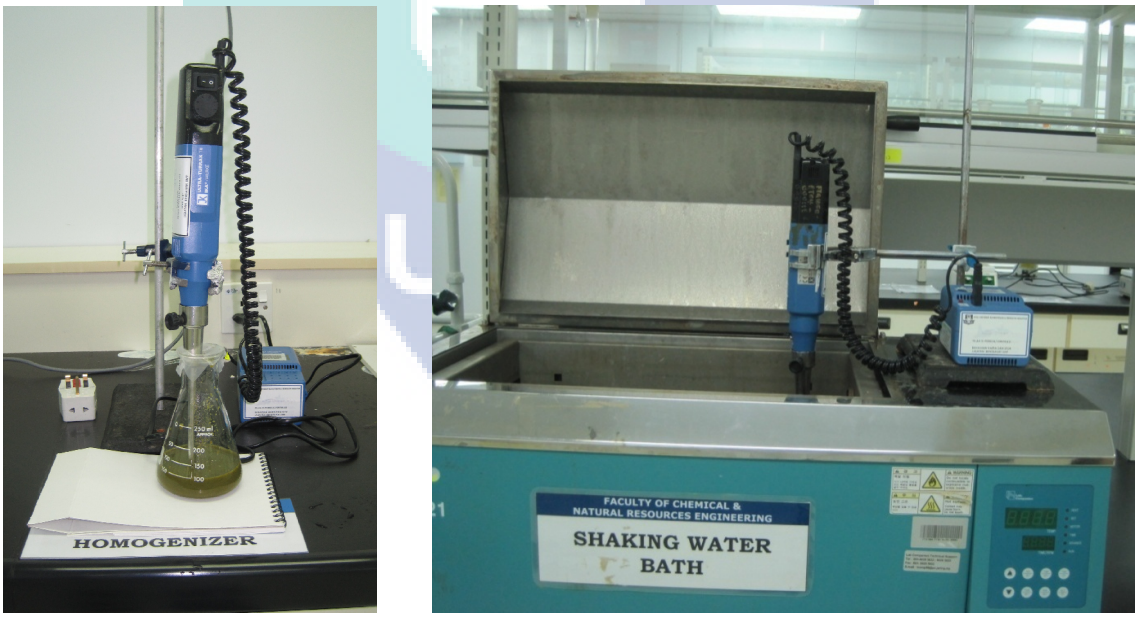


Figure 3.1: Ultrasonic waterbath



a b

Figure 3.2: Preparation of homogenizer extraction (a) and iMMLrsed homogenizer in waterbath as a technique of homoenizer extraction (b)

Homogenizer extraction

The grounded MML of 20 g were first loaded into a 500 ml erlenmeyer flask which was added 100 g of distilled water and sealed by plastic film. The homogenizer was included in the sample flasks and then iMMLrsed into a waterbath for 90 min at 60 °C. All experiments were performed in triplicates. Figure 3.2 shows the photo of homogenizer which was used in this study.

Incubated in Waterbath (Control)

The grounded MML of 20 g were first loaded into a 500 ml erlenmeyer flask which was added 100 ml of distilled water and sealed by plastic film. The flasks were iMMLrsed into a waterbath for 90 min with temperature control led at 60 °C. All experiments were performed in triplicates. Figure 3.3 shows the photo of waterbath which was used in this study.

Figure 3.3: Waterbath

3.2 Characterization of MML Extract

In this section was divided in three sub-section, there are nutrient composition, chemical properties and antimicrobial properties of MML extract. The aim of the study was determine the characterization of the nutritional quality of MML extract, fungsional group of phenolic compound that was in previous study concluded that the major of phenolic compounds of MML extract are gallic acid and ellagic acid, also determine the antimicrobial activity of MML extract.

3.2.1 Nutrient Composition

Characterizations the nutrient composition in MML extract to order incorporate into flavored waters and/or fruit drinks as an additional preservative was an important area for a number of reasons:

- *Nutritional labeling.* The concentration of nutrition and type of minerals present must often be stipulated on the label of a food.
- *Microbiological stability.* High mineral contents are sometimes used to retard the growth of certain microorganisms.
- *Nutrition.* Some minerals are essential to a healthy diet (*e.g.,* calcium, phosphorous, potassium and sodium) whereas others can be toxic (*e.g.,* lead, mercury, cadmium and aluminum).

There are eight nutrient composition was studied, namely ash content, protein, fat, total carbohydrate, energy, dietary fiber, cholesterol and mineral contents. The methodology and procedure to determine the nutrient composition was described below.

Ash Content

Methodology

Dry ashing methods have been officially recognized for the determination of the ash content of various foods/extracts (AOAC Official Methods of Analysis). Dry ashing procedures use a high temperature muffle furnace capable of maintaining temperatures of between 500 and 600 ^{o}C. This advantage of the methods is safe, few reagents are required, many samples can be analyzed simultaneously, not labor intensive, and ash can be analyzed for specific mineral content.

Procedure

Sample: MML extract

Assay: 2.5-3 g sample was weighed and put into a crucible. The sample was charred on a heating mantle until no smoke was present. Ashing was carried out in a muffle furnace (MeMMLrt, Germany) at 550°C for about 8 hours or until grey ash was obtained. The sample was then cooled in a dessicator. The ash was calculated after the constant weight was obtained (AOAC, 1984).

% of Ash = Weight of ash / Weight of sample x 100 (3.1)

Fat

Methodology

Soxhlet extraction is an accepted technique for extracting fat from samples. This method was involves drying of sample, extraction dried sample with petroleum ether, and solvent evaporation for a gravimetric determination.

Procedure

Sample: MML extract

Reagent: Petroleum ether BP 40 – 60°C

Assay: Sample (3-4g) was placed into an extraction thimble. The thimble was then placed in a beaker and dried in an electric oven for 5 hours at 70-80°C. Dried sample was extracted with petroleum ether using Soxhlet extraction apparatus for 6-8 hours. The solvent was evaporated and the residue was dried in an electric oven for 30 minutes at 105°C. The sample weight was then measured (AOAC, 1980).

$$\% \text{ Fat} = \frac{(W2-W1) \times 100}{\text{Sample weight in g}} \quad (3.2)$$

W1 = weight of evaporating flask

W2 = weight of evaporating flask + compound after drying

Fiber

Methodology

Acid-detergent fiber has been accepted as an official method for feed by the AOAC. The acid-detergent fiber method is a rapid procedure to determine crude fiber. The principle of acid-detergent fiber method that crude fiber is loss on ignition of dried residue remaining after digestion of sample with 1.25% H2SO4 and 1.25% NaOH solutions under specific conditions. Method is applicable to grains, meals, flours, feeds, fiber-bearing material, and pet foods from which fat can be extracted to leave workable residue.

Procedure

Sample: MML extract

Reagent: 0.255N Sulphuric acid (A.R Grade), 0.313N Sodium hydrochloride (A.R Grade), Hydrochloric acid (1% in water v/v) were used.

Assay: Defatted sample (1-3g) was weighed (W_0) and placed in a beaker. 200 ml of sulphuric acid was added and it was boiled for 30 minutes. The sample was filtered using Whatman paper no. 1 and the residue was washed with hot water until it was free from acid. The residue was then washed with 200 ml of warmed sodium hydroxide (0.313N), boiled for 30 minutes and filtered through a crucible. The residue was washed with hot water, 1% HCL and hot water again until it was neutralized, then followed by using ethanol. The sample was dried in an oven at 105 °C for 1 hour. The crucible with the residue was weighed (W_1) and ignited in a muffle furnace at 450 °C for 4 hours. The cooled crucible was then weighed again (W_2) (AOAC, 1984).

$$\% \text{ Crude fiber} = W_1 - W_2 / W_0 \times 100 \quad (3.3)$$

Protein

Methodology

For many years, the protein content of foods has been determined on the basis of total nitrogen content, while the Kjeldahl (or similar) method has been almost universally applied to determine nitrogen content (AOAC, 2000). Nitrogen content is then multiplied by a factor to arrive at protein content. This approach is based on two assumptions: that dietary carbohydrates and fats do not contain nitrogen, and that nearly all of the nitrogen in the diet is present as amino acids in proteins.

Procedure

Sample: MML extract

Reagent: Concentrated sulphuric acid (A.R Grade), Sodium hydroxide (A.R Grade 40%), 0.05M Hydrochloric acid, 4% Boric acid with bromocresol green indicator and catalyst, Kjeltabs (1.5 g K2S04 and 0.0075 g Se) were used.

Assay: 0.2-1 g sample was weighed and mixed with 2 pieces of Kjeltabs and 10 ml of sulphuric acid in a digestion tube. The mixture was digested for 1 hour or until a clear solution was obtained at 420 °C. The sample was cooled and distilled using Kjeltec 1026 Distilling Unit with 25 ml of 4% boric acid solution. Bromocresol indicator was placed at 62 on the receiver flask. The sample was then titrated with 0.05M Hydrochloric acid (HCL) to neutral grey.

Calculation:

$$\% \text{ N} = \frac{14.01 \times (\text{ml of titrant of sample} - \text{ml of titrant of blank}) \times \text{conc. of std acid}}{\text{g of sample} \times 10} \quad (3.4)$$

$$\% \text{ Protein} = \% \text{ N} \times \text{factor specific for different product (6.25)} \quad (3.5)$$

Carbohydrate and Energy

Methodology

Total carbohydrate content of foods has, for many years, been calculated by difference, rather than analysed directly. Under this approach, the other constituents in the food

(moisture compound, ash, fat, protein, crude fiber) are determined individually, suMMLd and subtracted from the total weight of the food. This is referred to as total carbohydrate and is calculated by the following formula:

Total carbohydrate (%) = 100% - (moisture compound (%) + ash (%) + fat(%) + protein(%) + crude fiber(%)). (3.6)

Energy was calculated using the factors 4.0, 4.0 and 9.0 kcal/g for protein, carbohydrate and fat, respectively (Abdurahman et al., 1998).

Energy (Kcal)= (4 kcal/g x amount of protein, g) + (4 kcal/g x amount of carbohydrate, g) + (9 kcal/g x amount of fat, g) (3.7)

Mineral Compound

Methodology

In this studied the determination of mineral compound was used AAS (Atomic Absorption Spectroscopy). The determination of mineral type and concentration by atomic spectroscopy is more sensitive, specific, and quicker than traditional wet chemistry methods.

Procedure

0.1 g of plant material was weighed accurately in a test tube and placed in an aluminium block or sand bath containing a thermometer (0 – 400°C). 5 mL of mixed nitric and perchloric digesting acid (1 mL 70% HClO4 and 4 mL 70% HNO3) was added. The block was heated for 2 hours at 120°, and then the temperature was slowly increased to 180°C over a 3 hour period to drive off the nitric acid. White fumes from the perchloric acid will indicate the end of the digestion procedure. It is important not to allow the digestate to dry out.

The digestion was carried out under strict supervision in a protected fume hood. On completion of the digestion, the compounds of the test tube were rinsed into a 25 mL volumetric flask and made up to the mark with distilled deionised water. The digestate was normally clear and does not require filtering; if a small amount of solid material was

present this could be removed by filtering the digestate through a Whatman 541 filter paper with some distilled deionised water. The filtrate was used for the analysis of minerals compounds by using Atomic Absorption Spectrometry (AAS) (Miah and Chino, 1999). Figure 3.4 shows the photo of the AAS instrument which was used in this study.

Figure 3.4: Atomic Absorption Spectroscopy

3.2.2 Chemical Properties

Characterization of chemical properties of MML extract was used FT-IR. The aim of this studied was known the fungsional group of phenolic compounds in MML extract.

The acquisition of the infrared spectra of MML extracts was carried out in the FT-IR spectrometer (Thermo Nicolet Corporation, Madison, WI) equipped with a DTGS KBr detector. The software OMNIC version 6.0a from Thermo Nicolet was used for spectra acquisition. All spectra were smoothed using the 'automatic smooth' function of the above software.

The hydrolyzed MMLextract was mixed with 100 mg of dried Potassium Bromide (KBr) and compressed into a salt disc. The disc was then read spectrophotometrically. Hydrolyzed MML extract was read in triplicates. The same procedure was followed for the standards (gallic acid and ellagic acid). Figure 3.5 shows the photo of FTIR instrument which was used in this study.

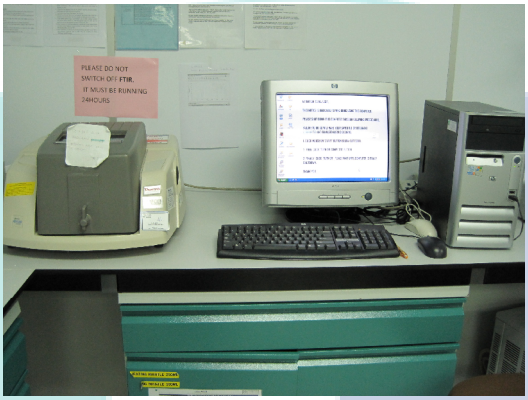


Figure 3.5: Fourier Transfer Infrared Spectroscopy

3.2.3 Antimicrobial Activity

Methodology

Determination of antimicrobial activity in this studied by disc diffusion method. The disc diffusion method was employed to determine the antimicrobial activities of the phenolic compounds. Disc-assay was found tobe a simple, cheap and reproducible practical method (Maidment et al., 2006).

Procedure

Sample: MML extract

Microbial Cultures: Several strains of *Escherichia coli* ATCC 8739, *Salmonella thyphi* ATCC 13311, *Bacillus subtilis* ATCC 6633 and *Bacillus cereus* ATCC 14579 were obtained from the culture collection of Universiti Malaysia Pahang. Stock cultures were

maintained at -70 °C. Prior to the antibacterial activity test, all strains were sub cultured at least three times in Nutrient Agar. Cultures were incubated at 37 °C for 16 to 24 h.

Growth Inhibition Studies: Each microbial suspension (100 µl) was spread on a nutrient agar plate. A sterilized filter disk (6 mm in diameter) was dipped into filtered extract (30 µl of each test sample) and placed in one sector of the plate. After incubation at 37°C for 24 h, the antimicrobial activity of each extract was measured as a zone of inhibition (mm) of the bacterial growth around the disk (Chanwitheesuk et al, 2007).

3.3 Experimental Design and Process Optimization of MML

In this section was divided in three sub-section, there are analyzed the MML extract, process parameter range identification and the last is optimization the extraction condition in order to produce optimum yield of phenoliccompound in MML.

3.3.1 Analyzed the MML extract

Analyzed of MML extracts consists of two methods, namely total phenols by using spectrofotometer and phenolic acids by HPLC.

3.3.1.1 Determination of Total Phenol Compound

Methodology

Determination of total phenols from aqueous extract of MML in this study had used the most common method, Folin Ciocalteau method based on the work presented by Rangsriwonga et al. (2009). Folin Ciocalteu assay is simple, reproducible and has been widely used for quantification of phenolic compounds in plant materials and extracts (Singleton and Rossi, 1965).

Procedure

The total phenolic compound of the extracts was determined by the Folin–Ciocalteu method (Rangsriwonga et al., 2009) using UV spectrofotometry. Figure 3.3 shows the

photo of Hitachi U-1800 spectrofotometer instrument which was used in this study. Briefly, the extract (0.1 ml) was added to 10 ml of deionized water and mixed with 2 ml of 2% aqueous sodium carbonate solution (Thermo Fisher Scientific). After 3 min, 0.1ml of 50% Folin-Ciocalteu reagent (Thermo Fisher Scientific) was added to the mixtures and left at room temperature for 30 min, after which the absorbance was measured at 750nm (each sample n = 3) using distilled water as a reference. The compound of total phenolic was calculated on the basis of calibration curve of gallic acid (Sigma Aldich). Figure 3.6 shows the photo of UV spectrofotometer which was used in this study.

Figure 3.6: UV Spectrofotometer

3.3.1.2 Determination of Phenolic Acid

Determinations of phenolic acids have two steps. The first step was sample preparation included hydrolysis method prior analyzed of phenolic acid. The second step was analyzed of phenolic acid using HPLC assay.

Hydrolysis

Methodology

MML extracts were hydrolysed using a acid hydrolysis method according to Harbourne et al. (2009) by using reflux apparatus. Acid hydrolysis was a suitable method to hydrolyze the phenolic acid of MML extract (gallic acid and ellagic acid) (Soong and Barlow, 2006)

Figure 3.7: Reflux apparatus

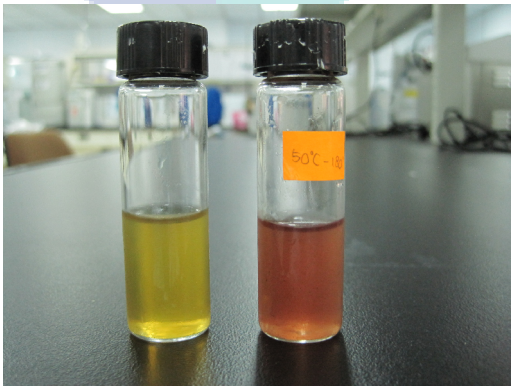


Figure 3.8: *M. malabathricum* extract before hydrolysis (left) and after hydrolysis (right)

Procedure

Briefly, 4.5 ml of MML extract, 4.5 ml of methanol and 1 ml of HCl (35%) (Thermo Fisher Scientific) were mixed and heated at 90°C under reflux for 2 h. Figure 3.7 shows the photo of reflux apparatus which was used in this study. After heating, the samples were cooled in an ice-bath and then filtered through Whatman No. 1 filter paper. Figure 3.8 shows the different colors of the sample before and after acid hydrolysis.

HPLC assay

Methodology

In this study gallic acid and ellagic acid were analyzed using HPLC system with gradient elution at UV detection of 280 nm, with 0.085% ortophosphoric acid and acetonitrile as a mobile phase.

Procedure

Sample Preparation: Hydrolyzed MML extract was filtered through a 0.2-µm membrane filter and 10 µl was injected directly onto the HPLC column.

Standard Preparation: Standard phenolic acids: gallic acid and ellagic acid (Sigma Aldich) were used in this experiment. Stock solution was prepared at a concentration of 0.5 mg/ml each in methanol. Methanol with HPLC grade (99.99%) (Thermo Fisher Scientific) was being used. The standard solution was then diluted into mg/ml to give a linear range for the preparation of standard curve. The solutions were filtered through 0.45-µm membrane filter and 10 µl of each standard was injected into the HPLC.

HPLC Condition: HPLC analyses were carried out with an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA), in combination with an Ascentis RP-Amide (15cm x 4.6mm i.d.; 5µm) column consisting of a vacuum degasser, an autosampler, a quaternary pump, a diode array detector, and a column heater. The solvents used were 0.085% ortophosphoric acid (A) and acetonitrile (B). The separations were performed at 30 °C by gradient elution at a flow rate of 1.0 ml/min. UV detection was set at 280 nm. The following gradient was used: 0–30 min, from 85% to 65% A; 30–35 min, from 65% to 15% A. Identification was based on retention times by comparison with a coMMLrcial standard.

Calibration was performed by injecting the standards three times at five different concentrations. Samples were injected in triplicates. Results were expressed as mg/100 g sample dry weight (DW). (Harbourne et al., 2009b; Tomczyk and Gudej, 2004). Figure 3.9 shows the photo of Agilent HPLC instrument which was being used in this study.

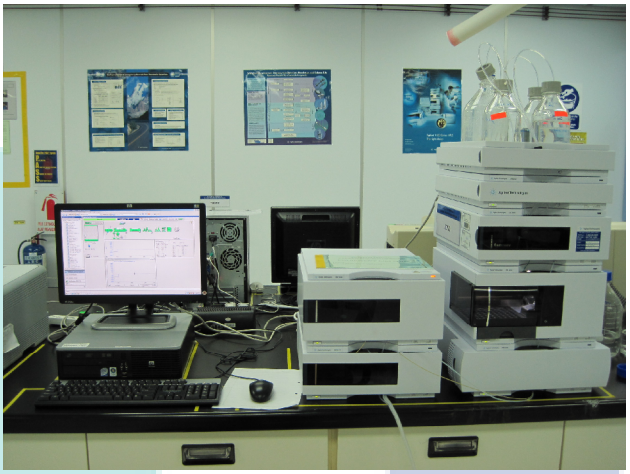


Figure 3.9: HPLC apparatus

3.3.2 Process parameter range identification of MML extract

In this studied have three parameter of extraction process, there are solid-liquid ratio, extraction temperature and extraction time. Prior optimize the parameters of extraction process, in this study was done in preliminary study, namely process parameter range identification.

Procedure

Solid loading: The grounded MML of of 5, 10, 15, 20 and 25 g were first loaded into a 500 ml erlenmeyer flask which was added 100 g of distilled water and sealed by plastic film. The flasks were iMMLrsed into the ultrasonic bath for irradiation under 60 °C for 90 min. The extraction processes were carried out in an ultrasonic bath with a frequency of 20 KHz. All experiments were performed in triplicates.

Extraction Temperature: The grounded MML of 20 g were first loaded into a 500 ml erlenmeyer flask which was added 100 g of distilled water and sealed by plastic film. The flasks were iMMLrsed into the ultrasonic bath for irradiation under 40, 50, 60, 70, 80, 90 and 100 °C for 90 min. The extraction processes were carried out in an ultrasonic bath with a frequency of 20 KHz. All experiments were performed in triplicates.

Extraction Time: The grounded MML of 20 g were first loaded into a 500 ml erlenmeyer flask which was added 100 g of distilled water and sealed by plastic film. The sample flasks were iMMLrsed into the ultrasonic bath for irradiation under 60 °C for 30, 60, 90, 120, 150 and 180 min. The extraction processes were carried out in an ultrasonic bath with a frequency of 20 KHz. All experiments were performed in triplicates.

Analysis of Data in Process Range Identification

The data analysis has been done by statistical analysis. Homogeneity (or lack of homogeneity) of the samples obtained were determined for each sampling method, where the parameters were measured in three replications. Statistical analysis was performed with SPSS software using analysis of variance (ANOVA), and differences between means was analyzed using Tukey's B method of statistical significance at $P < 0.05$ (Morgan et al., 2007). All data are expressed as the mean ± S.D. unless otherwise stated. The differences between two means were analyzed by one-way analysis of variance (ANOVA). The results for analysis of data in process range identification were shown in Appendix C1 - 4.

3.3.3 Optimization of MML extract

Optimization was being done with the Response Surface Methodology (RSM) developed based on the Central Composite Design (CCD) with gallic acid, ellagic acid and total phenol compound as the dependent variables (responses) while the parameter in solid loading, extraction time and extraction temperature as the independent variables.

Table 3.1: Independent variables and concentration levels for response surface study

Factors	Unit	Levels		
		-1	0	+1
A (X_1) Solid loading	g	15	20	25
B (X_2) Temperature	°C	50	60	70
C (X_3) Time	min	60	90	120

Table 3.2: Experimental layout central composite design

	Factors		
Std order	Solid loading (g) (A)	T (°C) (B)	Time (min) (C)
1	15	50	60
2	25	50	60
3	15	70	60
4	25	70	60
5	15	50	120
6	25	50	120
7	15	70	120
8	25	70	120
9	10	60	90
10	30	60	90
11	20	40	90
12	20	80	90
13	20	60	30
14	20	60	150
15	20	60	90
16	20	60	90
17	20	60	90
18	20	60	90
19	20	60	90
20	20	60	90

The CCD was conducted with a 20 experiment central composite designs of combination factors at two levels (high, +1 and low, −1 levels), included with six star points (axial)

corresponding to an α value of 2 and six replicates at the center points (coded level 0, midpoint of high and low levels). The range and levels of the processing parameters involved are tabulated in Table 3.1 while the central composite design matrices and experimental response of each individual experiment are shown in Table 3.2.

The quadratic model for predicting the optimal point was according to Eq. (3.8). Where *Y* is the response variable, *b* is the regression coefficient of the model, *x* is the coded levels of the independent variables. In general, the primary objective of RSM is to optimize the response (*Y*) based on the factors investigated (Montgomery and Runger, 2007). The Design Expert software 6.0.4 was used to develop the experimental plan and optimize the regression equation (Eq. (1)). The statistical significance of the second order model equation was determined by performing Fisher's statistical test for analysis of variance (ANOVA). In particular a good model must be significant based on the *F*-value and *P*-value as opposed to the Lack of Fit (insignificant). Moreover the proportion of variance exhibited by the multiple coefficient of determination R^2 should be close to 1 as this would demonstrate a better correlation between the experimental and the predicted values.

$$y = b_o + \sum b_i x_i + \sum b_{ii} x_i^2 + \sum b_{ij} x_i x_j \quad (3.8)$$

Validation

Adequacy of the developed empirical model needs to be verified or validated in order to confirm the prediction accuracy, which is generated by the regression equation in predicting the concentration of phenolic compound in *MML* extract, such as gallic acid, ellagic acid and total phenol compound at any particular solid loading, extraction time and temperature of extraction within the range of level defined previously. The obtained actual values and its associated predicted values from the selected experiments were compared for further residual and percentage error analysis. The percentage error between actual and predicted value of both responses over a selected range of operating levels are calculated based on Eqs. (3.9) and (3.10).

Residual = (Actual value − Predicted value) (3.9)

$$\% \text{ Error} = \frac{\text{Residual}}{\text{Actual value}} \times 100\% \quad (3.10)$$

3.4 Summary of experimental work

There are three parts that focused in this research, namely extraction of phenolic compound from MML, characterization of MML extract and process optimization of MML extract. Figure 3.10 was shown the operational framework of this research.

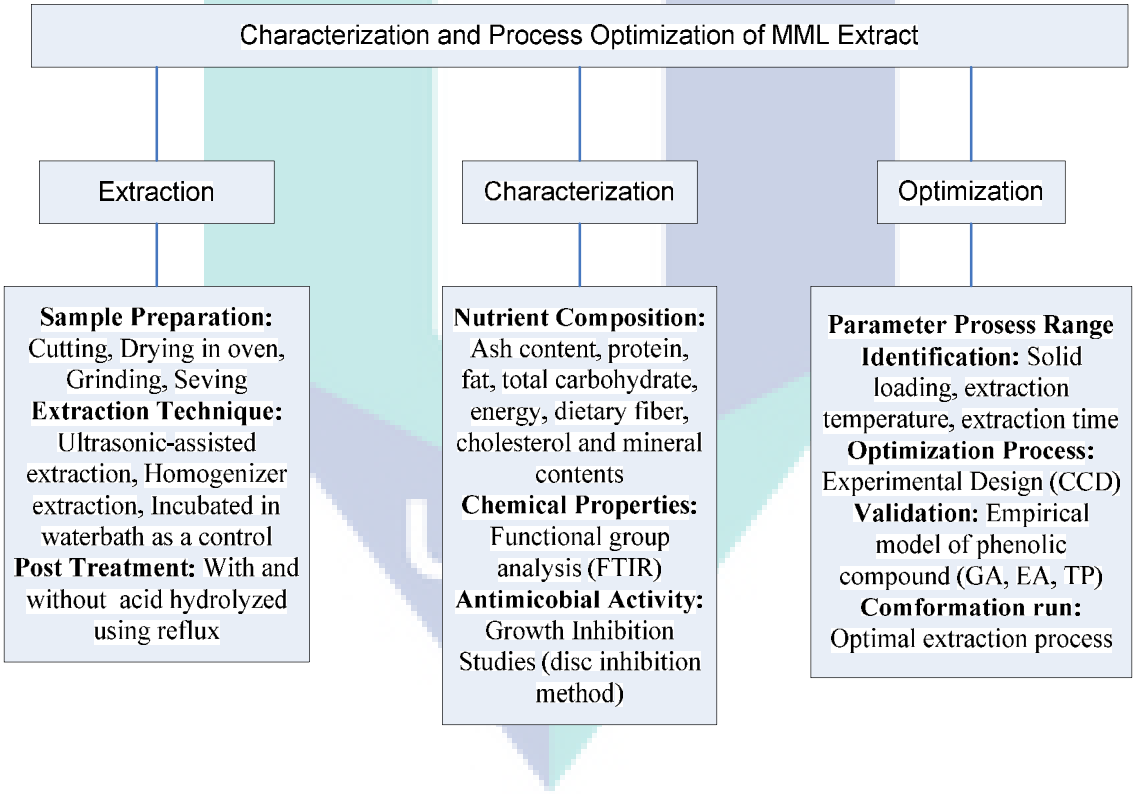


Figure 3.10: Operational Framework

CHAPTER 4

RESULTS AND DISCUSSION

This chapter was divided into three parts of results and discussion, namely extraction, characterization and optimization of MML extract.

4.1 Extraction of MML Extract

4.1.1 Extraction Technique of MML Extract

Homogenize process is needed in the extraction because homogenize has a goal to decrease the particle size and to obtain a greater degree of uniformity and stability (Troy, 2006). According to Kasapis et al. (2009), that there are many types of homogenize equipment, such as high-speed mixer, colloid mill, high-pressure homogenizer, microfluidizer, ultrasonic and membrane processing. The differences in the types of homogenize equipment create the energy density and the relative energy efficiency of their respective mixing to be different.

Figure 4.1, 4.2 and 4.3 were shown the effect of extraction technique. The graph in this studied represent the means (n =3). Details of triplicate data were shown in Appendix D1. The vertical bars in represent the standard deviation (n = 3), bars with same letters (a-e) were not significantly different at $p \geq 0.05$. The vertical bars represent the standard deviation (n = 3), bars with same letters (a-f) were not significantly different at $p \geq 0.05$.

Figure 4.1 shown that the ultrasonic-assisted extraction could produce higher gallic acid yield MML extract than either the homogenizer or control. This result was in accordance

with the mechanism action of ultrasonic, whereby ultrasound produces cell disruption, particle size reduction and ultrasonic jet towards solids surfaces which would lead to a greater contact area between solid and liquid phase; thus, it created better access of solvent to valuable compound (Fang et al., 2007).

The mechanism action of ultrasonic had also been discussed by Sarker et al. (2006) which stated that ultrasound was used to induce a mechanical stress on the cells through the production of cavitations in the sample. The cellular breakdown would increase the solubilization of metabolites in the solvent and would improve extraction yields. When the cavitation bubbles collapse, an ultrasonic jet would be produced and would act as a micro pump solvent that could force a solvent into the cell to dissolve the compounds (Albu et al., 2004). Ultrasonic method is commonly applied to facilitate the extraction of intracellular metabolites from plant cell cultures (Sarker et al., 2006).

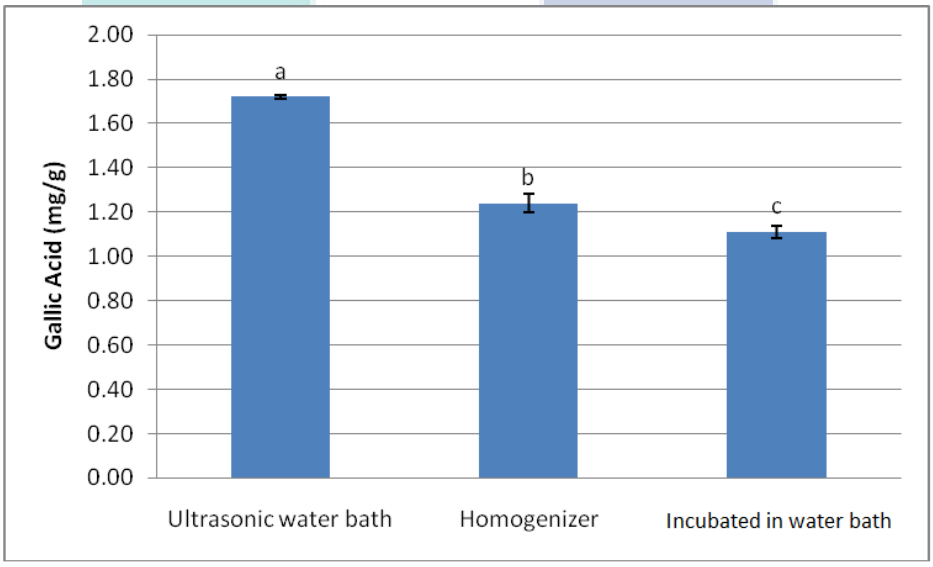


Figure 4.1: Effect of types of extraction on gallic acid in MML extract

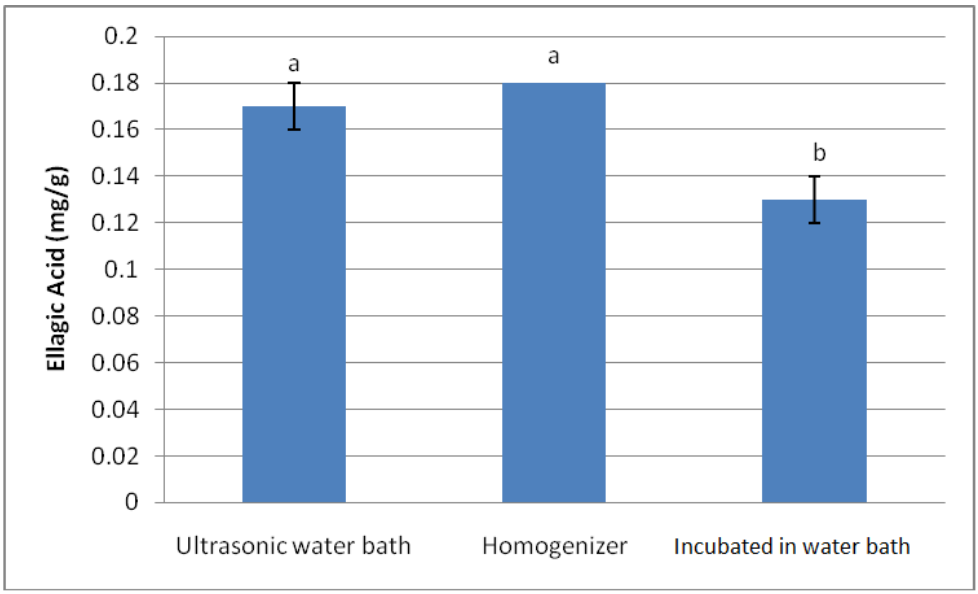


Figure 4.2: Effect of the types of extraction on ellagic acid in MML extract

Ellagic acid (Figure 4.2) from ultrasonic-assisted extraction showed no significant differences ($p>0.05$) compared to the homogenizer extraction but there were significant differences when compared to the control. Total phenol yield (Figure 4.3) had also showed the maximum results of ultrasonic-assisted extraction, followed by homogenizer and incubated in waterbath as a control, with the amount of 0.1665 mg/g of ellagic acid and 14.05 mg GAE/g of total phenol. This finding was well supported by the study carried out by Huang et al. (2009), who reported that the ultrasonic-assisted extraction of flavonoids from *Folium eucommiae* could produce 17% flavonoids extraction ratio and 41% extract yield, which was more efficient compared to heating, microwaves-assisted and enzyme-assisted extraction methods. In a study by Albu et al. (2004) it was found that the use of ultrasonic in the extraction of carnosic acid and rosmarinic acid from *Rosmarinus officinalis* was more effective than using the conventional method (heating process/reflux).

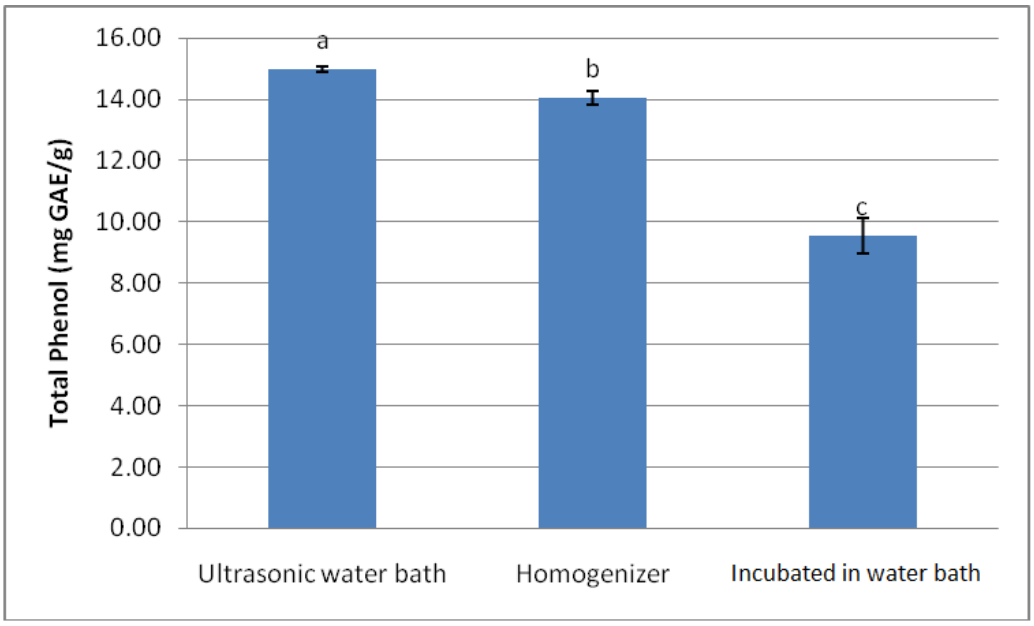


Figure 4.3: Effect of the types of extraction on total phenol in MML extract

According to Paniwnyk et al. (2001) the application of ultrasonic extraction of rutin from leaves of *Sophora japonica* had produced significant increase in the maximum extraction yield. Besides being a mechanism action of ultrasonic that had been described above, Kasapis et al. (2009) also explained that the energy density and the relative energy efficiency for ultrasonic were more compared to homogenizer.

4.1.2 Post-Treatment of MML Extract

Gallic acid (GA) and Ellagic acid (EA) are derivative from hydrolyzable tannins (HTs), which exist either in free form or bound, as gallo- (GT) and ellagitannins (ET), respectively. HTs are easily hydrolyzed in vivo by the action of acid, base and/or enzymes, releasing GA or EA units (Clifford and Scalbert, 2000). In this study the acid hydrolysis was implemented. An acid-catalyzed hydrolysis process was employed to liberate phenolic acids from their bound forms but it required a relatively high concentration of mineral acids under refluxing conditions (Hertog et al., 1992; Merken and Beecher, 2000).

As shown in Table 4.1, the concentration of gallic acid and ellagic acid in MML extract increased with acid hydrolysis. It is found that the findings support those of a study by Soong and Barlow (2006) in longan seed and mango kernel where gallic acid and ellagic acid yield increased with the increasing severity of hydrolysis.

In Figure 4.4 shown that the HPLC spectrum of extract without acid hydrolysis. Whereas Figure 4.5 shows that the HPLC spectrum of extract with acid hydrolysis; Figure 4.13 had also proven that the HPLC spectrum using acid hydrolysis resulted in a better separation of the spectrum thus the concentration of gallic acid and ellagic acid was easier to compare.

Table 4.1: Concentrations of Gallic Acid and Ellagic Acid Recovered from MML extracts

Sample	GA[a] (mg/g)	EA[a] (mg/g)
Aqueous extraction at 60 °C, 90 min	0.47	ND
Aqueous extract and acid hydrolysis at 90 °C, 2 h	1.74	0.17

[a]Mean of three determinations

ND: Not Detected

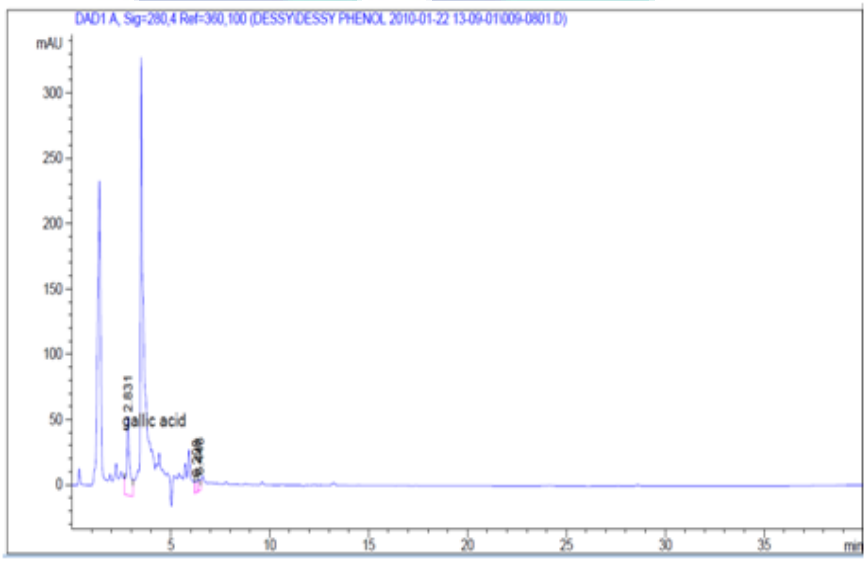


Figure 4.4: HPLC spectrum of aqueous extract of MML

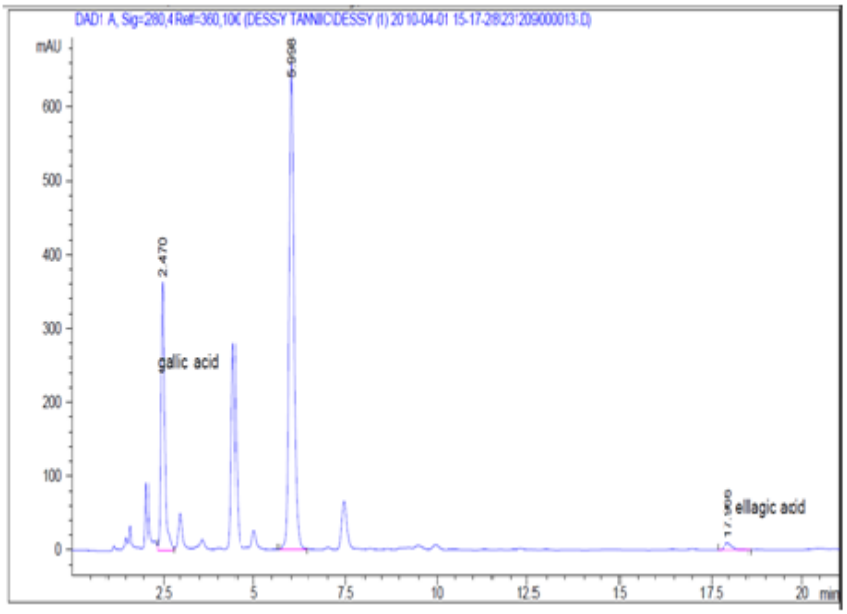


Figure 4.5: HPLC spectrum of hydrolyzed MML extract

4.2 Characterization of MML Extract

4.2.1 Nutrient Composition of MML Extract

A balanced human diet is required to maintain optimum health (Potter, 1999) and to protect from chronic diseases (Hunter and Fletcher, 2002). Thus, the nutritional quality is also associated with the greater changes in consumers' acceptance. The proximate composition and trace elements compound in MML extract were investigated. Table 4.1 shows the proximate values of MML extract. As observed, the amount of nutrient components in MML was very low and/or below human requirements. For example, staple foods with protein compound below 3% do not meet the protein requirements in human, but a diet of cereals with an 8 to 10% protein compound; provide enough to supply caloric requirements of adults (Cheftel et al., 1985). Fats serve as concentrated source of energy compared to protein and carbohydrate. Unfortunately, fat in the extract was detected at only less than 0.2 %. Similarly, Prasad et al. (2000) reported that the fruit based products such as pineapple beverage powder contained negligible amounts of both protein and fat.

As shown in the data, MML extract provide a good source of mineral and trace elements. Potassium was found as major components (469.91mg/L), followed by phosphorus (33.79 mg/L) and sodium (11.69 mg/L). According to food U.S. RecoMMLnded Dietary Allowances (USRDA) (National Research Council, 1993), the optimum daily dietary intakes of adults for phosphorus, magnesium, iron, zinc, sodium and potassium are about 800 mg, 300 to 350 mg, 10 to 18 mg, 15 mg, 1100 to 3300 mg and 1875 to 5625 mg, respectively. Potassium (intracellular cation) and sodium (extracellular ion) are regulated osmotic equilibrium and pressure, and also maintained body-fluid volume. Phosphorus is involved in the enzymes-controlled energy-yielding reactions of metabolism and helps control the acid-alkaline reaction of the blood (Potter, 1999). Table 4.1 also demonstrated that the amount of zinc and iron traced in MML extract were 1.09 and 4.79 mg/L. Zinc, one of the essential nutrients, strongly inhibits lipid peroxidation, which is possibly to be due to altering or preventing iron binding property.

No selenium was detected in MML extract. Selenium plays a major role in the synthesis and activity of glutathione peroxidase, a primary cellular antioxidant enzyme (Madhavi et al., 1996). Since the intakes of trace elements may cause toxicity, the maximum levels of selenium for adults should not exceed 0.05-0.2 mg (Potter, 1999). Potentially harmful metals such as lead, mercury, cadnium, zinc and selenium which naturally present in soil, water and plant foods. However, according to Potter (1999), some undesirable minerals and certain natural toxicants are largely removed or inactivated when foods are processed. From the above information it is indicated that the *MML* extract is safe to be added into beverages as additional preservative.

Table 4.2: Nutrient composition of MML

No	Parameter	Unit	Results
1	Proximate value		
	Ash	% w/w	< 0.1
	Protein as N x 6.25	% w/w	< 0.1
	Fat	% w/w	< 0.2
	Total Carbohydrate	% w/w	1
	Energy	Kcal/100g	4
	Dietary Fiber	% w/w	< 0.2
	Cholestrol	% w/w	< 0.01
2	Mineral compound		
	Zn	mg/L	1.09
	P	mg/L	33.79
	Fe	mg/L	4.79
	Na	mg/L	11.68
	K	mg/L	469.91
3	Trace element		
	Se	mg/L	ND
	Al	mg/L	149.38
	Pb	mg/L	0.44
	Mg	mg/L	10.27

***** ND – Not detected

4.2.2 Chemistry Properties of MML Extract

The IR spectra of MML extract was recorded at the range of 500 to 4000 cm^{-1}. The FTIR spectrum of MML extract in Figure 4.6 shown the intense peak of all was nearby 3600 cm^{-1}, which was due to the hydroxyl groups stretching vibration. The bands in the region of 2970 and 2664 cm^{-1} were the characteristic of C–H antisyMMLtrical stretching vibration. The absorption band from 1800 cm^{-1} to 800 cm^{-1} is called the “finger print” region. The bands around 1700 cm^{-1} represented the aromatic combination bands. The band in the region of 1500 cm^{-1} could be correlated to the stretching of aromatic C=C (Nakanishi andSolomon, 1977) in gallic acid and ellagic acid compounds. The spectral region around

1053cm^{-1} allowed the infrared absorption of aromatic C−H in-plane and aromatic C−H out-of-plane around 877cm^{-1}.

The FTIR spectrum of gallic acid standard (Figure 4.6) contained prominent absorption band of around 3674.16 cm^{-1} can be associated with O−H stretching vibrations. The peak in the region of 2970 cm^{-1} was the characteristics of C–H antisyMMLtrical stretching vibration (Coates, 2000). The peaks between 800 cm^{-1} and 1800 cm^{-1}, which is knowing as the fingerprint zone, could be attributed to C=C−C aromatic ring stretching (1580−1615 cm^{-1}; 1450−1510 cm^{-1}) and several aromatic C−H out-of-plane (670−900 cm^{-1}) and in-plane (950−1225 cm^{-1}) bending vibrations, among others (Coates, 2000). Whereas the FTIR spectrum of the MML extract also recorded the same number of peaks lying at 3683.03, 2970.84, 2884.87, 1557.94, 1053.63 and 877.47 cm^{-1} respectively. This finding had indicated that MML extract contained gallic acid.

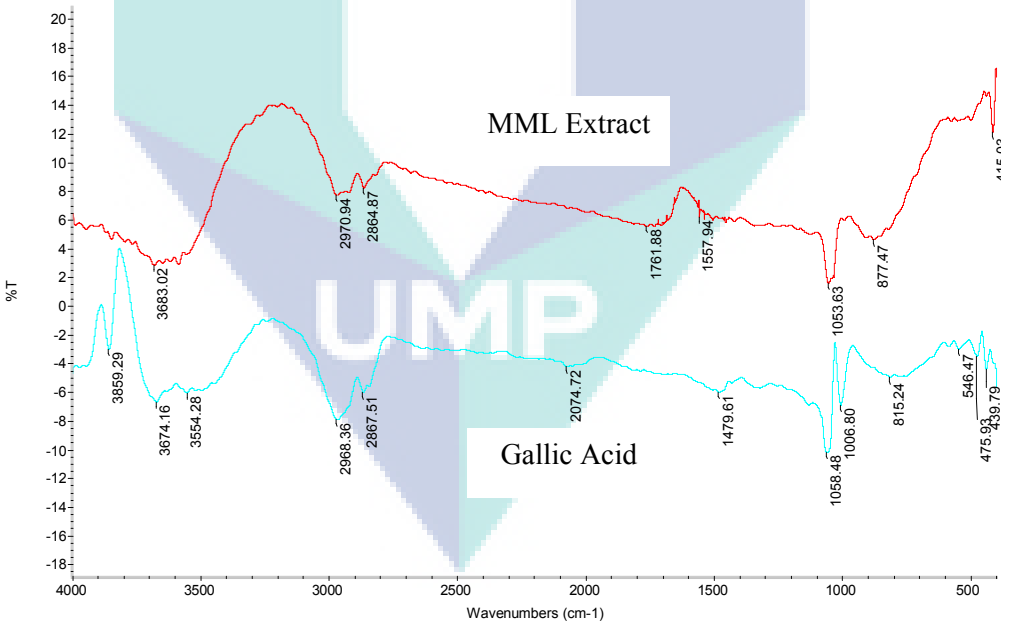


Figure 4.6: Infrared spectra of hydrolyzed MML extract and gallic acid standard

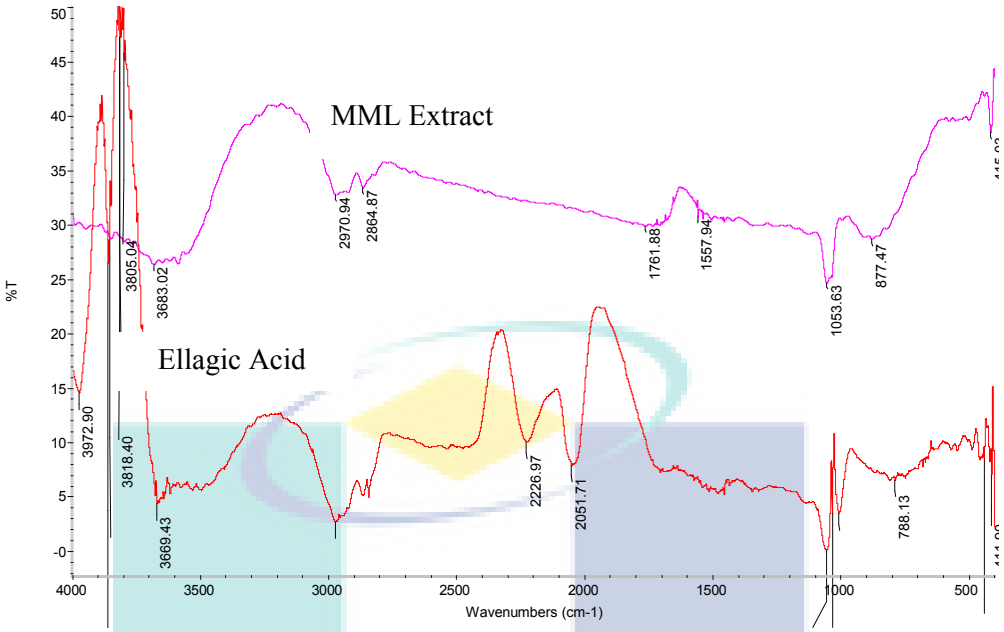


Figure 4.7: Infrared spectra of hydrolyzed MML extract and ellagic acid standard

The FTIR spectrum of the ellagic acid standard (Figure 4.7) contains eight major peaks at 3850.57, 3669.43, 2973.98, 2880.87, 2226.97, 2051.71, 1007.77 and 788.13 cm^{-1}. Whereas the FTIR spectrum of the MML extract was also recorded the same number of peaks lying at 3883.02, 2970.84, 2884.87 1053.63 and 877.47 cm^{-1} respectively. This finding indicates that the MML extract contained ellagic acid.

4.2.3 Antimicrobial Acticity of MML Extract

Some foodborne pathogens and food spoilage bacteria were chosen as indicators for antimicrobial activity of the optimum extract from MML Antimicrobial activities were calculated using paper discs. Antibacterial activitiy was indicated by the existence of clear inhibition zone around the disks after incubation for 18 hours at 37 °C. Results were shown as diameters of inhibition (mm) in Table 4.2 and Figure 4.8.

Extract of MML showed inhibitory activity against all gram-positive bacteria and gram-negative. The maximum zone of inhibition in MML extract was determined against

Bacillus cereus which was about 13.6 mm and *Bacillus subtilis* at 13 mm. Whereas for gram-negative bacteria, there existed a lower zone of inhibition than gram positive, which were 7.5 mm for *Salmonella typhi* and 7.83 mm for *Escherichia coli*. This fact was in accordance with the results of the study by Shan et al. (2007).

Table 4.2: Antimicrobial activity of aqueous extracts from leaves of *MML* against microorganisms tested, based on disc diffusion method

Type of microorganism	Microorganism	Inhibition zone in diameter (mm)[a]
Gram negative	*Escherichia coli*	7.83 ± 0.28
Gram negative	*Salmonella typhi*	7.5 ± 0.5
Gram positive	*Bacilluc subtilis*	13 ± 1.73
Gram positive	*Bacillus cereus*	13.6 ± 2.88

[a]Values are means ± SD (mm) of three separate experiments

This finding is probably caused by the differences in the composition of bacterial cell walls. Gram-positive bacteria have two layers consisting of peptidoglican and cell walls, while gram negative bacteria have three layers consisting of cytoplasmic, peptidoglican, and the outer membrane layer which acts as a barrier to antimicrobial compounds (Lugtenberg and Alpen, 1983). The mechanism action of inhibition from gallic acid and ellagic acid of MML extract was discussed in Chapter 2.

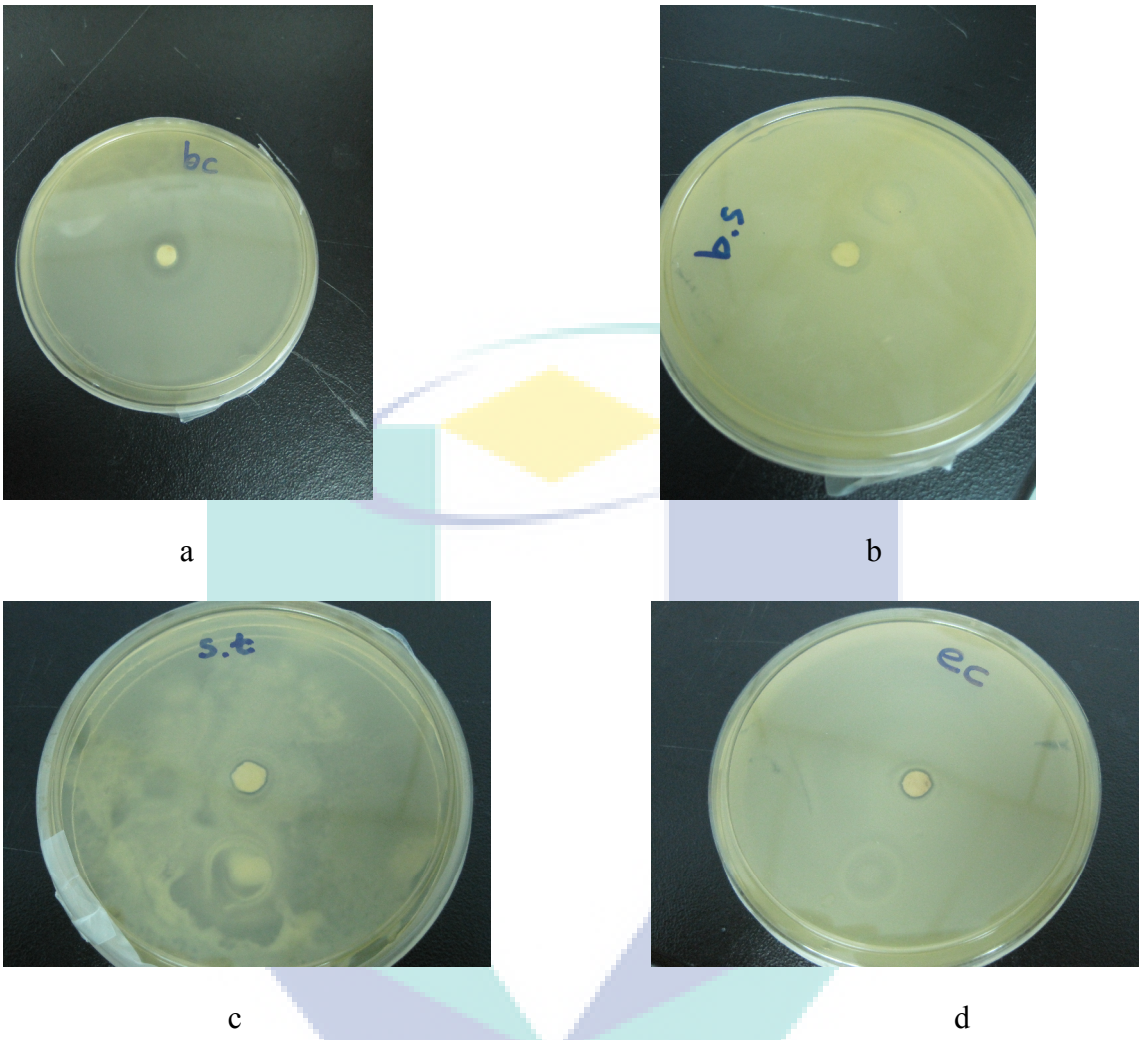


Figure 4.8: Inhibition activity of *MML* extracts towards *Bacillus cereus* ATCC 14579 (a), *Bacilluc subtilis* ATCC 6633 (b), *Salmonella thyphi* ATCC 13311 (c), *Escherichia coli* ATCC 8739 (d)

4.3 Process Parameter Range Identification of MML Extract

This part would present the results on the effect of extraction process (solid loading, extraction temperature and extraction time) of MML extract using the ultrasonic waterbath and identification the maximum range that produce optimum phenolic compounds. The

phenolic compounds analyzes were carried out to determine the concentration of gallic acid, ellagic acid and total phenol compounds.

The determination of phenolic compound from the MML extracts had used the spectrophotometry-uv and HPLC. Spectrophotometry UV was used to determine total phenols from the extract, while HPLC was used to determine phenolic acid, namely gallic acid and ellagic acid.

The graph in this studied represent the means (n =3). Details of triplicate data were shown in Appendix D2 – D4. The vertical bars in represent the standard deviation (n = 3), bars with same letters (a-e) were not significantly different at $p \geq 0.05$. The vertical bars represent the standard deviation (n = 3), bars with same letters (a-f) were not significantly different at $p \geq 0.05$.

4.3.1 Effect of Solid Loading of MML Extract

To determine the best yield of solid loading of gallic acid, ellagic acid and total phenol on five different solid loading which ranged from 5 to 25 g which were tested. 100 ml of water was treated with different solid loading at constant duration of 90 minutes. The results obtained were plotted in Figure 4.14, 4.15 and 4.16. From the figureit could be seen that there existed solid loading-dependent phenolic compound in MML extract: increased solid loading of the extract had caused an increased yield of phenolic compound from the extract of MML While with an increase of solid loading over 20 g, there was no obvious effects on the bioactive compound yield.

Figure 4.9 shows 20 g of solid loading exhibited a significant ($p<0.05$) higher gallic acid compared to other solid loading. The yield of gallic acid was 1.75 mg/g, respectively. An increased in the solid loading to 20 g had significantly ($p<0.05$) decreased the gallic acid yield, but solid loading of 25 g would still produce gallic acid yield which was higher than 15 g. The extraction rate showed a slow down trend which indicated the exhaustion of cell walls being ruptured.

Figure 4.10 shows that the yield of ellagic acid showed significantly ($p<0.05$) increased from 5 to 20 g of solid loading. 25 g of solid had no significant ($p>0.05$) effect in increasing ellagic acid yield compared to 20 g of the solid loading.

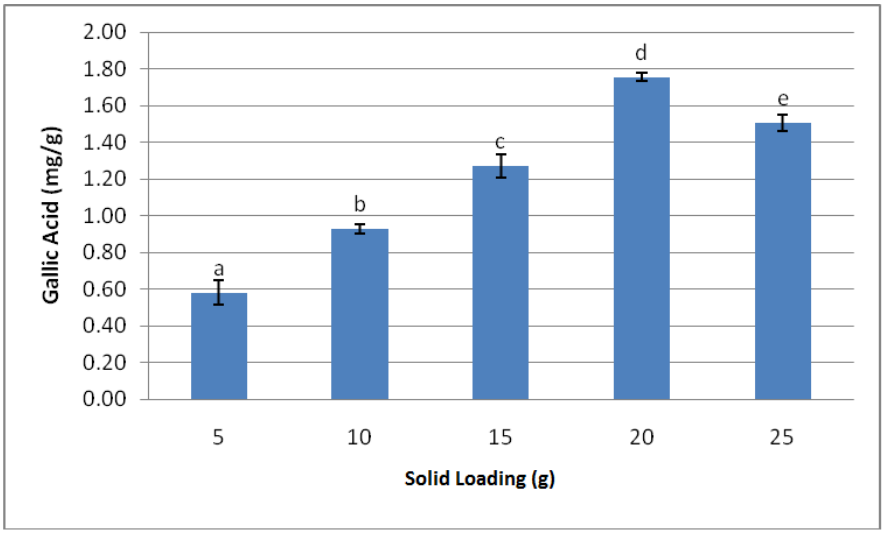


Figure 4.9: Effect of solid loading on gallic acid in MML extract

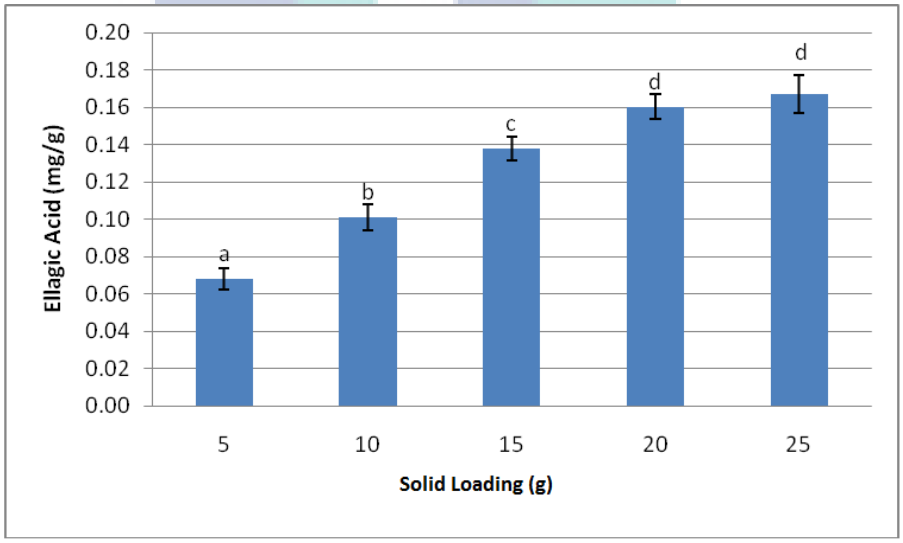


Figure 4.10: Effect of solid loading on ellagic acid in MML extract

Figure 4.11 shows the effect of solid loading in total phenol compound. The significance was ($p<0.05$) higher in the yield of total phenol of the 20 g solid, with the amount of 14.78 mg GAE/g, as compared to other solid loading. 25 g of solid loading showed a significant ($p<0.05$) decrease of total phenol yield.

The increase of the solid loading did not only prevent the expected increase in phenol concentration, but also promoted a decrease in this parameter. This difference could be justified by the occurrence of packaging phenomena during extraction. High solid loading could beget preferential flow channels and offside zones, promoting a decrease in surface contact between solid and liquid. As a consequence, a decrease in mass transfer was observed (Pinelo et al., 2005a)

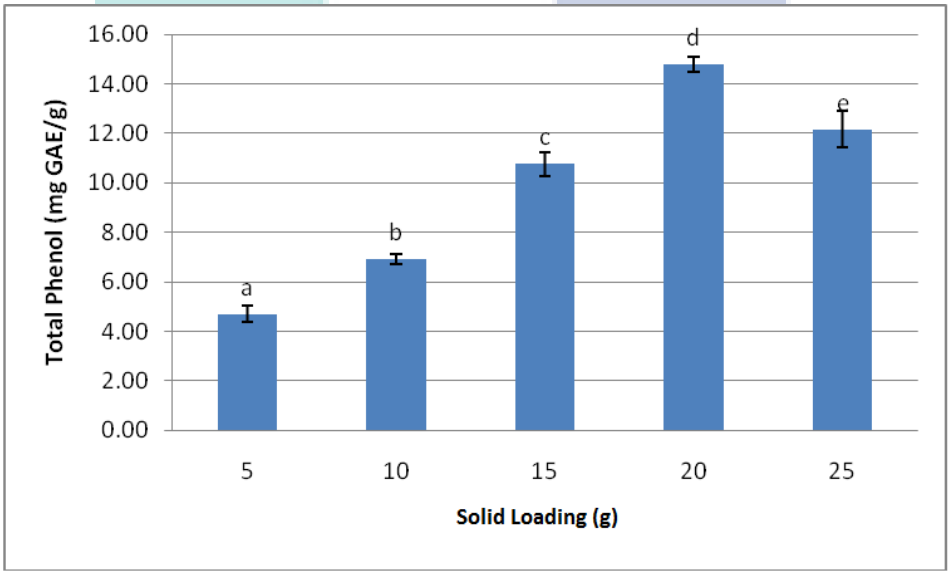


Figure 4.11: Effect of solid loading on total phenol in MML extract

4.3.2 Effect of Extraction Temperature of MML Extract

The influence of temperature on the phenolic compound in MML extract was investigated between the ranges of 40 °C to 100 °C. Research was conducted with the same sample of 20 g in 100 ml water, by using ultrasonic waterbath at a frequency of 20 KHz and the

extraction carried out for 90 minutes. Results from the study could be seen in Figure 4.12, 4.13 and 4.14.

The effect of extraction temperatures on the gallic acid is shown in Figure 4.12. Results showed that extraction temperature at 60 °C was significantly ($p<0.05$) higher in gallic acid yield compared to other temperatures. Increasing the temperature from 40 to 60 °C had resulted in the increase of the gallic acid yield significantly ($p<0.05$), with the highest amount of 0.176 ± 1.07 mg/g GAE (extracted at 60°C). An increase of the temperature from 70 to 100 °C had significantly decreased ($p<0.05$) on the yield of gallic acid.

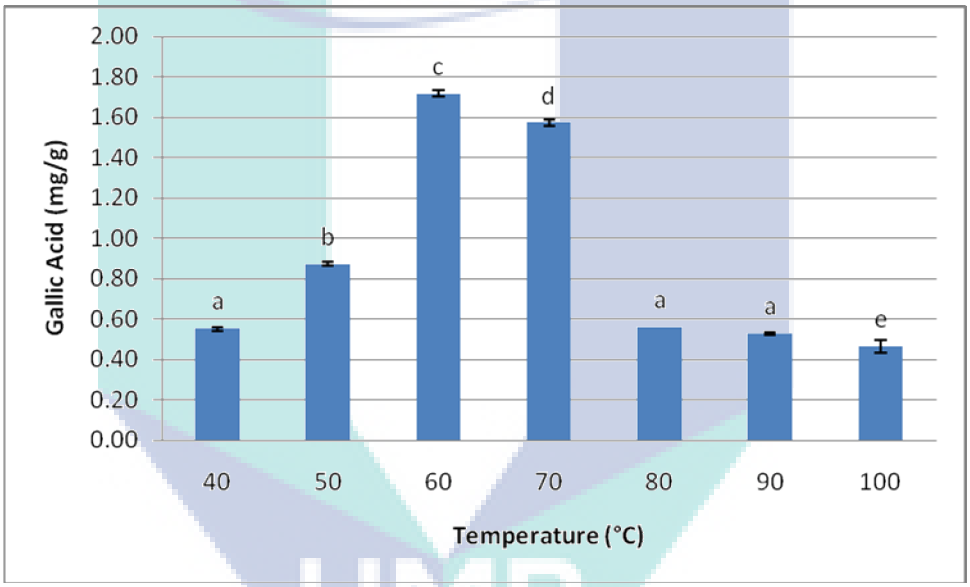


Figure 4.12: Effect of extraction temperature on gallic acid in MML extract

These results were in accordance to the research conducted by Wang et al. (2008a), which obtained the result that temperatures of 60 °C was the maximum temperature to obtain the total phenols from wheat bran by using the ultrasound-assisted extraction. While the research conducted by Liyana-Pathirana and Shahidi (2005) with research on wheat to determine the phenolic compounds also found that the result had an optimum temperature at either 61 ° or 64 °C. The extraction from *Inga edulis* leaves showed that the maximum temperature of 65.2 °C was needed to produce maximum phenolic compound (Silvia et al., 2007).

Figure 4.13 shows the increase of extraction temperature from 40 ° to 60 °C had significantly ($p<0.05$) increased the extraction yield of ellagic acid and temperatures of over 60°C resulted significantly ($p<0.05$) decrease in ellagic acid. Although an increase in temperature favored extraction of phenols by enhancing both the solubility of solute and the diffusion coefficient, it could not be increased indefinitely; since the denaturation of phenolic compounds might take place at temperatures above 50 °C (Pinelo et al., 2005b). However, there exists a possibility that polymerization reactions could occur due to the combination of various phenols by themselves, which could have an effect on the analytical quantification (Pinelo et al., 2005b). Other works showed that polymerization in phenols happened widely (Yilmaz and Toledo, 2004; Manthey and Grohmann, 2001). Thus, there was a question whether the decrease in the yield of *MML* phenols by ultrasound-assisted extraction was due to the degradation or the polymerization process.

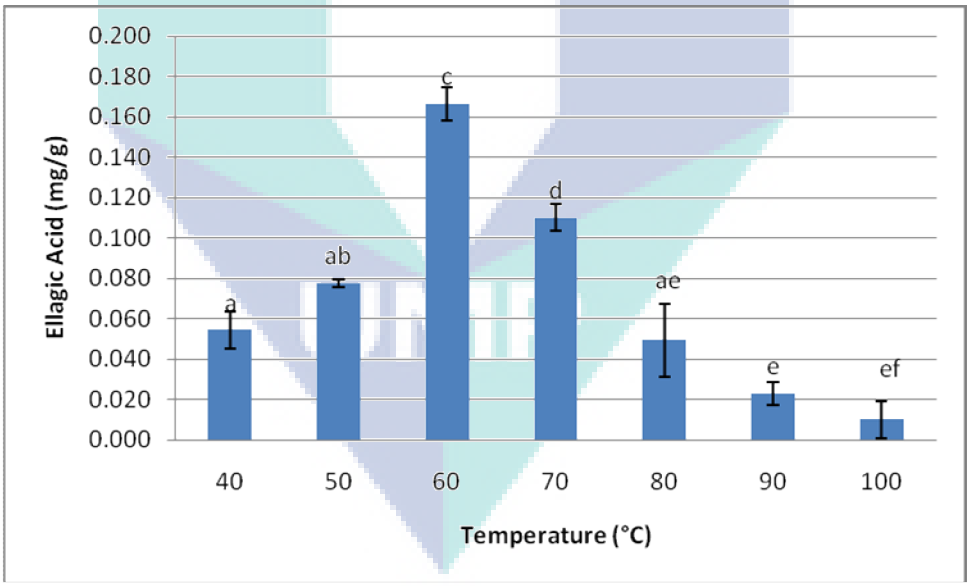


Figure 4.13: Effect of extraction temperature on ellagic acid in MML extract

Figure 4.14 shows that extraction temperature of 60 °C was the most significant ($p<0.05$) as it produced the highest total phenol from *MML* leaves. Pinelo et al. (2005b) reported that the yield of phenolic compound from grape pomace depended significantly on extraction

temperature and time. On the other hand, higher temperatures beyond 50 °C induced the instability of phenolic compound (Herrera and Castro, 2005). The indicated high temperature may have resulted in the degradation of some phenolic acids. As had been reported before (Herrera and Castro, 2005), the degradation (close 100%) of phenolic compounds from strawberries had occurred by the ultrasound method.

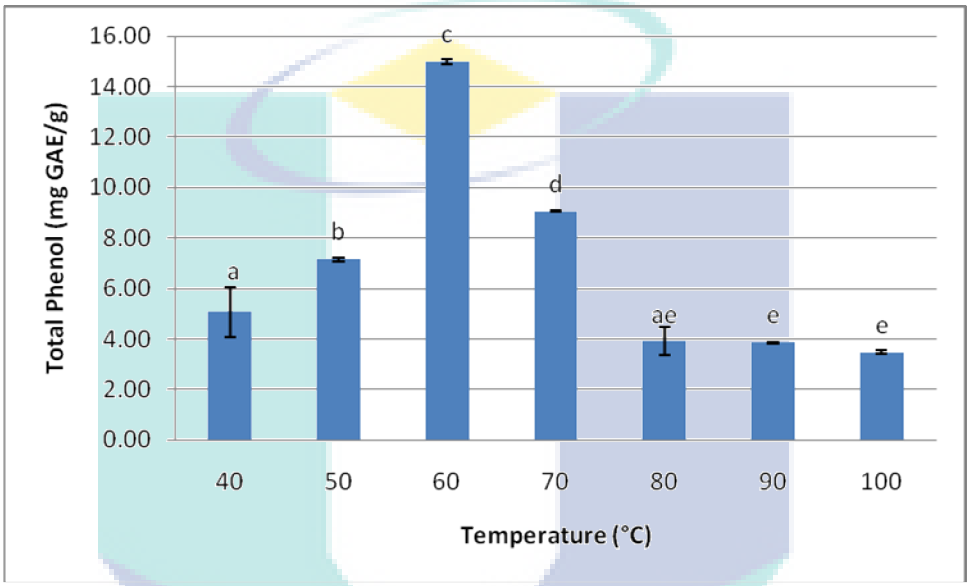


Figure 4.14: Effect of extraction temperature on total phenol in MML extract

4.3.3 Effect of Extraction Time of MML Extract

The effect of extraction time in gallic acid, ellagic acid and total phenol yield could be seen in Figure 4.15, 4.16 and 4.17. These figures showed that gallic acid, ellagic acid and total phenol yield were time-dependent. Extraction time of 90 min produced significantly ($p<0.05$) higher gallic acid, ellagic acid and total phenol compared to other times. The increase of ultrasonic time for extraction from 30 to 90 min had increased the extraction yield of phenolic compound (gallic acid, ellagic acid and total phenol). But there was a slight decrease in extraction yield if ultrasonic times were more than 90 min. It could be explained that, as the extraction time prolonged, the chemical decomposition of bioactive compound present in extract might occur, which would result in a decrease in the extraction

yield (Lu et al., 2008). Many studies had stated that the extraction temperature and extraction time are highly significant variables in influencing the total phenol yield from various plants (Gelmez et al., 2008; Silvia et al., 2007 and Alu'datt et al., 2010).

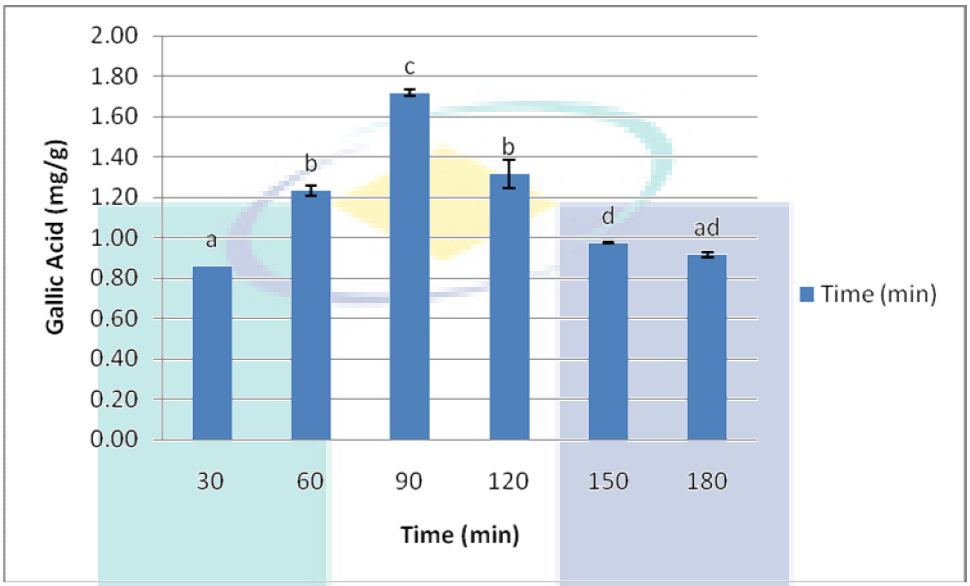


Figure 4.15: Effect of extraction time on gallic acid in MML extract

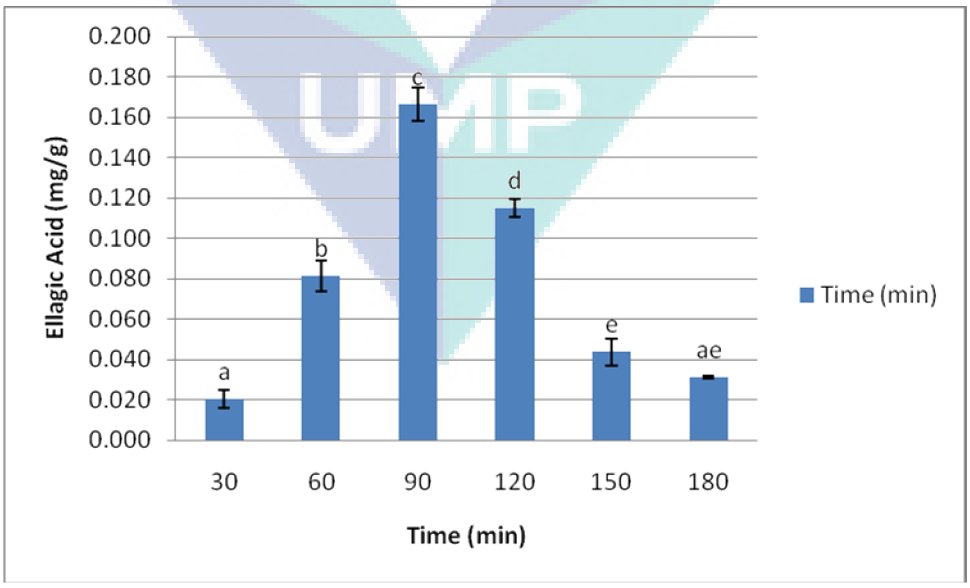


Figure 4.16: Effect of extraction time on ellagic acid in MML extract

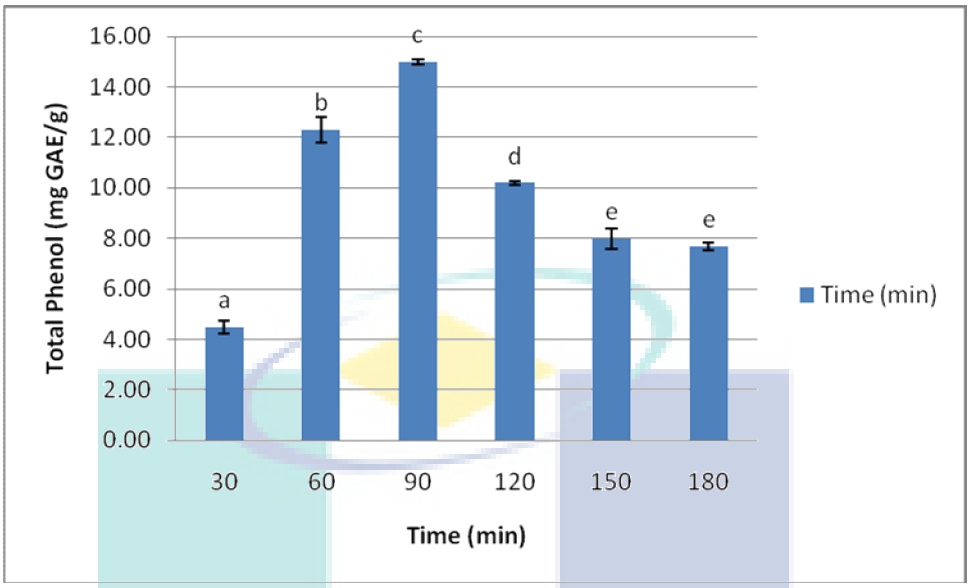


Figure 4.17: Effect of extraction time on total phenol in MML extract

4.4 Optimization of MME Extract

This part discusses the optimization of phenolic compound from the leaves of MML extract. Gallic acid, ellagic acid and total phenol were selected as the marked compounds in the extract. Response Surface Methodology (RSM) with a two factor – three levels Central Composite Design (CCD) was employed to obtain the optimum conditions. Three independent variables selected in this study were solid loading, temperature and time. Dependent variables were the total phenol, gallic acid and ellagic acid compound.

The results were analyzed using Analysis of Variance (ANOVA) by Design Expert software. Three-dimensional plots were obtained based on the effects of the levels of the two factors. From these three-dimensional plots, the simultaneous interactions of the three factors on the responses were studied. The optimum region was also identified based on the main parameters in the overlay plot. The experiment was repeated for 3 times and each result obtained was compared to the predicted values in order to determine the validity of the model.

4.4.1 Determination of The Relevant Variables and Experimental Ranges

At the beginning of this study, the factors solid loading, temperature and time of contact were investigated to determine the appropriate experimental ranges to be considered during the optimization process. The three factors together with lower, middle and upper design points for RSM in coded and non-coded values were presented in Table 4.3. In the present study, the relationship between response (gallic acid, ellagic acid and total phenol compound) and three independent variables (solid loading, extraction temperature and time of extraction) were being studied. The results at each point were based on the experimental design, as shown in Table 4.4. The results were represent the means (n =3). Details of triplicate data were shown in Appendix D5.

Table 4.3: Variables and levels used for central composite design

Factors	Unit	Levels		
		-2	0	+2
(A) Solid loading	g	10	20	30
(B) Temperature	°C	40	60	80
(C) Time	min	30	90	150

Table 4.4: Rotatable central composite design setting

Std order	Factors			Responses[a] (mg/g)		
	Solid loading (g) (A)	T (°C) (B)	Time (min) (C)	Gallic Acid	Ellagic Acid	Total Phenol
1	15	50	60	0.9824	0.098	8.2202
2	25	50	60	1.0652	0.081	9.2141
3	15	70	60	0.9069	0.082	8.2205
4	25	70	60	0.9883	0.061	8.5608
5	15	50	120	1.0575	0.082	9.1121
6	25	50	120	1.0621	0.083	9.9342
7	15	70	120	1.0794	0.102	9.0535
8	25	70	120	1.0898	0.072	10.0791
9	10	60	90	0.9323	0.094	6.711
10	30	60	90	1.0196	0.064	9.6608
11	20	40	90	0.562	0.054	5.0762
12	20	80	90	0.5914	0.07	3.8981
13	20	60	30	0.8956	0.021	4.4774
14	20	60	150	0.9707	0.044	7.9821
15	20	60	90	1.7065	0.185	14.9232
16	20	60	90	1.7316	0.158	14.8921
17	20	60	90	1.7024	0.163	15.0176
18	20	60	90	1.7204	0.168	15.0486
19	20	60	90	1.7565	0.166	15.0334
20	20	60	90	1.7121	0.17	14.7398

[a]responses are means (n=3)

4.4.2 Analysis of Variance (ANOVA) and Statistical Analysis

4.4.2.1 Response Surface Analysis of Gallic Acid

Table 4.5 shows the ANOVA table for gallic acid analysis of *MML* extract after transformation as recommended by Box-Cox plot (State-Ease, Inc., 2000) using non transform (lambda = 1) (Appendix E1). The experimental data had a correlation coefficient (R^2) of 0.9971. That means the calculated model was able to explain 99.71% of the results in the case of gallic acid yield. The results had indicated that the model used to fit the reponse variable was significant (p<0.0001) and adequate to represent the relationship

between the reponse and the independent variables. Moreover the "Lack of Fit" value was found insignificant (Prob > F = 0.1167) which denoted that the model was desirably fit.

Table 4.5: ANOVA for the regression model and respective model terms for gallic acid

Source	Sum of squares	Degree of freedom	Mean of square	F-value	Prob>F[a]
Model	2.90	9	0.32269	387.38	< 0.0001
A	0.0078	1	0.00782	9.39	0.0119
B	1.21E-04	1	1.21E-04	0.1453	0.7111
C	0.0154	1	0.01539	18.47	0.0016
A^2	0.8933	1	0.89329	1072.38	< 0.0001
B^2	2.09	1	2.08983	2508.82	< 0.0001
C^2	0.9976	1	0.99758	1197.59	< 0.0001
AB	2.42E-06	1	2.42E-06	0.0029	0.9581
AC	0.0028	1	0.00278	3.34	0.0975
BC	0.0051	1	0.0051	6.12	0.0329
Residual	0.0083	10	0.00083		
Lack of Fit	0.0063	5	1.26E-03	3.15	0.1167
Pure Error	0.0020	5	0.0004		
Cor Total	2.91	19			
Std. Dev.	0.0289		R^2		0.9971
Mean	1.18		Adj R^2		0.9946
Adeq Precision	56.777		Pred R^2		0.9812

[a]Prob>F-value less than 0.05 is significant

The model is significant whereby A and C have the significant effects in this model term. A and C in this ANOVA table have the values of 'Prob>F' less than 0.05 which indicates the model is significant at a 95% confidence level. From the ANOVA table, it is shown that the C is the most significant effect, followed by A and BC. The difference between adjusted R^2 and predicted R^2 is lower than 0.2 whereby the result for this experiment is at 0.0134 and it is acceptable. Adequate precision also indicates an adequate signal, whereby the ratio obtains was 56.777 which are greater than 4. The normal probability plot of residuals, plot

of predicted versus actual, plot of residual vs. predicted, and outlier T plot were performed and these plots is shown in Appendix E1.

In the model graph shown in Figure 4.18, the significant effects that influence gallic acid result were the extraction temperature (B) interaction with the extraction time (C). It could be seen that the maximum gallic acid corresponded in a positive correlation which indicated that interaction in synergistic effect. In particular, the gallic acid increased when temperature (B+) was increased from 50 ° to 60 °C and as time (C+) was increased from 60 to 120 min. When the temperature (B-) setting was decreased from 70 ° to 60 °C and time (C-) was decreased from 120 to 60 min, the gallic acid had decreased too. While with the increase of temperatures over 60 °C, there was a gradual decline in the response. It could be explained that, as the extraction temperature prolonged, the chemical decomposition of bioactive compound present in the extract might occur, resulting in a decrease in the extraction yield (Lu et al., 2008). Figure 4.19 shows the 3D surface graph of gallic acid with respect to the temperature extract and solid loading.

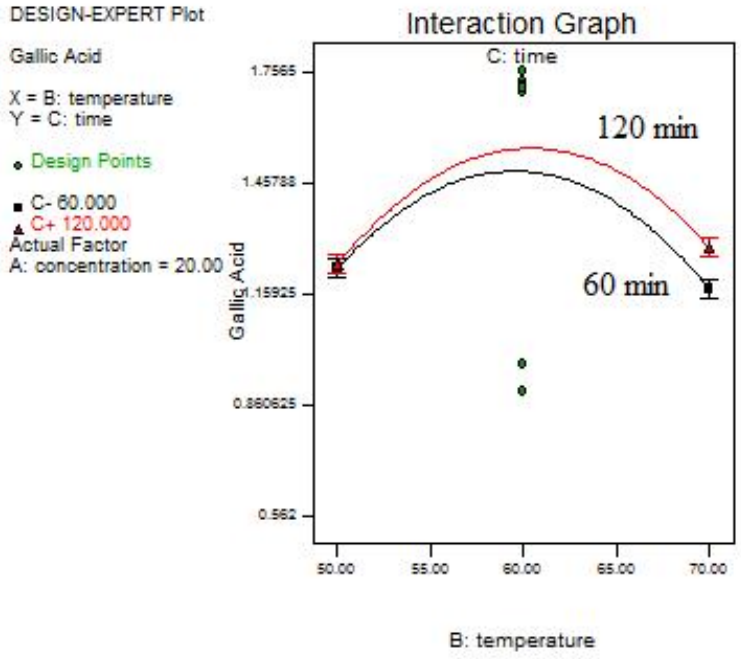


Figure 4.18: Interaction graph plot of gallic acid

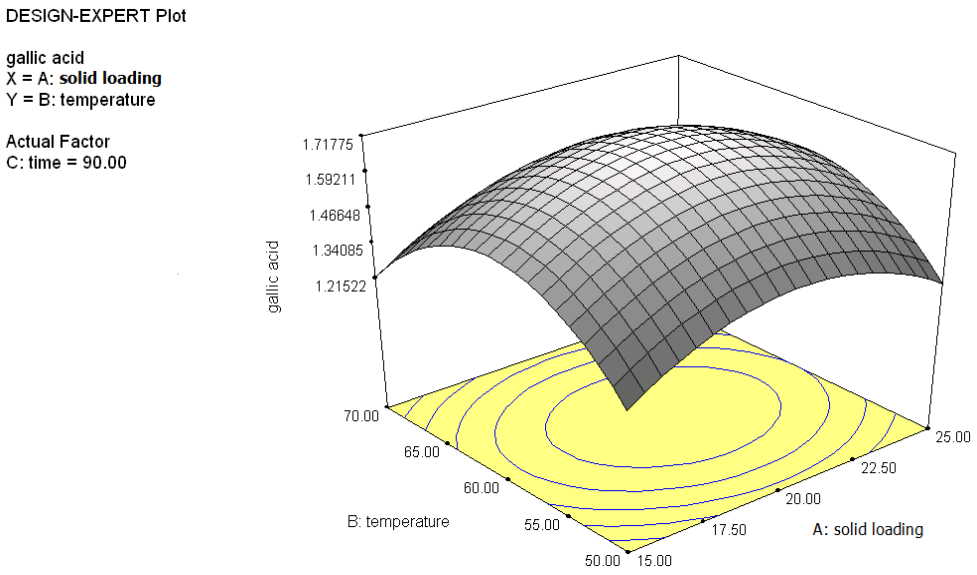


Figure 4.19: 3D surface graph of gallic acid with respect to the temperature extract and solid loading

The following equations were the final empirical models in terms of coded factors and actual factors for gallic acid respectively. These equations were generated by the Design Expert 6.0.4 software after the transformation had been carried out.

Final equation in terms of coded factors:

$$\text{Gallic acid} = +1.7174 + 0.0221*A - 0.0027*B + 0.0310*C - 0.1885*A^2 - 0.2883*B^2 - 0.1992*C^2 + (0.0005*A*B) - (0.0187*A*C) + (0.0253*B*C) \quad (4.2)$$

Final equation in terms of actual factors:

$$\text{Gallic acid} = -13.39109 + (0.31654*\text{Concentration}) + (0.33789*\text{Temperature}) + (0.038308*\text{time}) - (7.53959.10^{-3}*\text{Concentration}^2) - (2.88302.10^{-3}*\text{Temperature}^2) - (2.21322.10^{-4}*\text{time}^2) + (1.10000.10^{-5}*\text{Concentration}*\text{Temperature}) - (1.24333.10^{-4}*\text{Concentration}*\text{time}) + (8.41667.10^{-5}*\text{Temperature}*\text{time}) \quad (4.3)$$

4.4.2.2 Response Surface Analysis of Ellagic Acid

Table 4.6 shows the ANOVA table for ellagic acid analysis of MML extract after transformation, as recommended by Box-Cox plot (State-Ease, Inc., 2000) using non transform (lambda = 1) (Appendix E2). The multiple correlation coefficient of R^2 was calculated to be 0.9823, which indicated a good agreement existed between the experimental and predicted value as well as depicting that 98.23% of the variability in the response could be well explained by the model while only 1.77% of the total variation was poorly described by the model. Moreover the "Lack of Fit" value was found insignificant (Prob > *F* = 0.5309) which denoted that the model was desirably fit.

Table 4.6: ANOVA for the regression model and respective model terms for ellagic acid

Source	Sum of squares	Degree of freedom	Mean of square	F-value	Prob > F[a]
Model	0.0448	9	0.0050	61.72	< 0.0001
A	0.0010	1	0.0010	12.27	0.0057
B	1.82E-06	1	1.82E-06	0.0226	0.8835
C	0.0003	1	0.0003	3.22	0.1032
A^2	0.0128	1	0.0128	158.52	< 0.0001
B^2	0.0179	1	0.0179	222.04	< 0.0001
C^2	0.0294	1	0.0294	365.21	< 0.0001
AB	1.64E-04	1	1.64E-04	2.03	0.1845
AC	1E-05	1	1E-05	0.1256	0.7304
BC	0.0003	1	0.0003	3.22	0.1028
Residual	0.0008	10	8.1E-05		
Lack of Fit	0.0004	5	7.77E-05	0.9296	0.5309
Pure Error	0.0004	5	8.4E-05		
Cor Total	0.0456	19			
Std. Dev.	0.0090		R^2		0.9823
Mean	0.1008		Adj R^2		0.9664
Adeq Precision	22.828		Pred R^2		0.9187

[a]Prob>F-value less than 0.05 is significant

The model is significant whereby A had the significant effect in this model term. A in the ANOVA table has the values of 'Prob>F' less than 0.05 which indicated the model is significant at a 95% confidence level. From the ANOVA table, it is shown that the A is the most significant effect. The R^2 value is 0.9823 which is high and almost reaching the value of 1, thus indicated that it is desirable. The difference between adjusted R^2 and predicted R^2 is supposed to be lower than 0.2 whereby the result for this experiment was 0.0477 which is acceptable. Adequate precision also indicated an adequate signal, where the ratio obtained was 22.828 which are greater than 4. The normal probability plot of residuals, plot of predicted versus actual, plot of residual vs. predicted, and outlier T plot were performed and these plots is shown in Appendix E2.

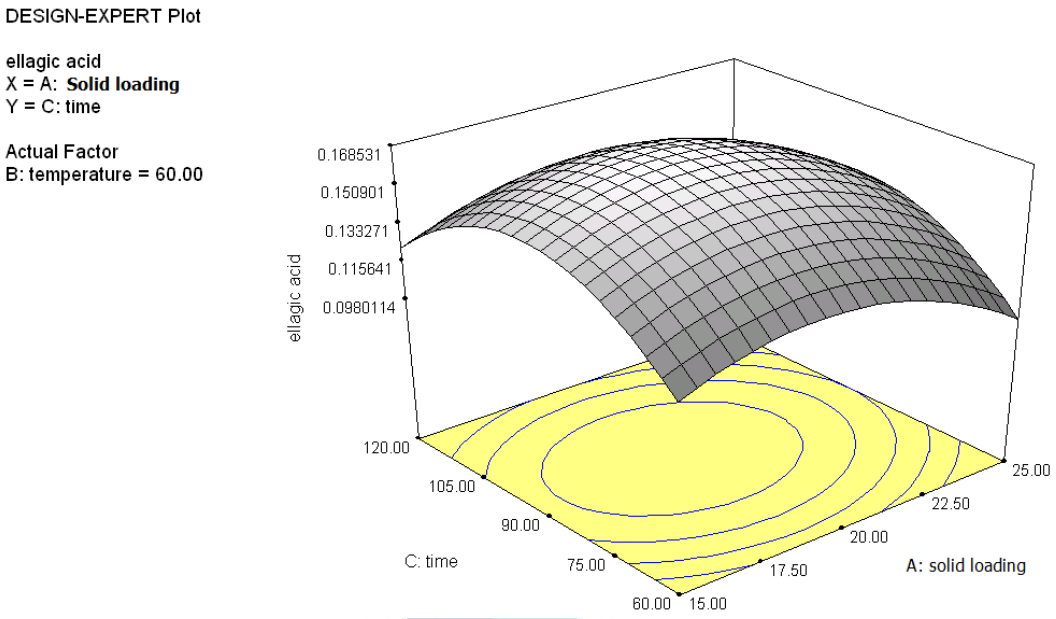


Figure 4.20: 3D surface graph of ellagic acid with respect to the solid loading and extraction temperature

In the model graph shown in Figure 4.20 shows the 3D surface graph of ellagic acid with varying solid loading and extraction temperatures. The factor which influenced in the ellagic acid analysis was the solid loading (A) which indicated that this effect was significant. It was obvious that as the ellagic acid yield initially increased, there was also an

increase in the solid loading. Further increase in the concentration of the extract would result in decrease in the yield of ellagic acid.

The following equations were the final empirical models in term of coded factors and actual factors for ellagic acid respectively. These equations were generated by the Design Expert 6.0.4 software after the transformation had been carried out.

Final equation in terms of coded factors:

$$\text{Ellagic acid} = +0.1675 - 0.0079*A + 0.0003*B + 0.0040*C - 0.0225*A^2 - 0.0267*B^2 - 0.0342*C^2 - (0.0045*A*B) + (0.0011*A*C) + (0.0057*B*C) \quad (4.4)$$

Final equation in terms of actual factors:

$$\text{Ellagic acid} = -1.43687 + (0.039253*\text{Concentration}) + (0.032152*\text{Temp}) + (5.68803.10^{-3}*\text{time}) - (9.01773.10^{-4}*\text{Conc}^2) - (2.66818.10^{-4}*\text{Temperature}^2) - (3.80215.10^{-5}*\text{time}^2) - (9.05000.10^{-5}*\text{Concentration}*\text{Temperature}) + (7.50000.10^{-6}*\text{Concentration}*\text{time}) + (1.90000.10^{-5}*\text{Temperature}*\text{time}) \quad (4.5)$$

4.3.2.3 Response Surface Analysis of Total Phenol

ANOVA (analysis of variance) is a statistically based, objective decision making tool for detecting any differences in the average performance of parameters tested and also to summarize the experimental results. The result from the analysis of variance and case statistics were studied for further analysis and interpretation. Table 4.7 shows the ANOVA table for total phenol analysis of MML extract after transformation as recommended by Box-Cox plot (State-Ease, Inc., 2000) using power (lambda = 1.69) (Appendix E3). The model is significant with insignificant "Lack of Fit" (Prob > *F* = 0.0627) which denoted that the model was desirably fit, whereby A and C had the significant effect in this model term. A and C in the ANOVA table had the values of 'Prob>F' less than 0.05 which indicated the model was significant at a 95% confidence level. The R^2 value was 0.9976 wich was high and almost reaching the value of 1, thus indicating that it was desirable. The

difference between adjusted R^2 and predicted R^2 was supposed to be lower than 0.2 whereby the result for this experiment was 0.012 which was acceptable. Adequate precision also indicates an adequate signal, where the ratio obtained was 57.250 which were greater than 4. The normal probability plot of residuals, plot of predicted versus actual, plot of residual vs. predicted, and outlier T plot were performed and these plots is shown in Appendix E2.

Table 4.7: ANOVA for the regression model and respective model terms for total phenol

Source	Sum of squares	Degree of freedom	Mean of square	F-value	Prob > F[a]
Model	18713.16	9	2079.24	461.82	< 0.0001
A	282.85	1	282.85	62.82	< 0.0001
B	14.92	1	14.92	3.31	0.0987
C	327.67	1	327.67	72.78	< 0.0001
A^2	5876.52	1	5876.52	1305.23	< 0.0001
B^2	11078.80	1	11078.80	2460.70	< 0.0001
C^2	8536.59	1	8536.59	1896.05	< 0.0001
AB	1.39	1	1.39	0.3084	0.5909
AC	2.92	1	2.92	0.6486	0.4393
BC	4.10	1	4.10	0.9111	0.3623
Residual	45.02	10	4.50		
Lack of Fit	36.81	5	7.36	4.48	0.0627
Pure Error	8.21	5	1.64		
Cor Total	18758.19	19			
Std. Dev.	2.12		R^2		0.9976
Mean	52.70		Adj R^2		0.9954
Adeq Precision	57.250		Pred R^2		0.9834

[a]Prob>F-value less than 0.05 is significant

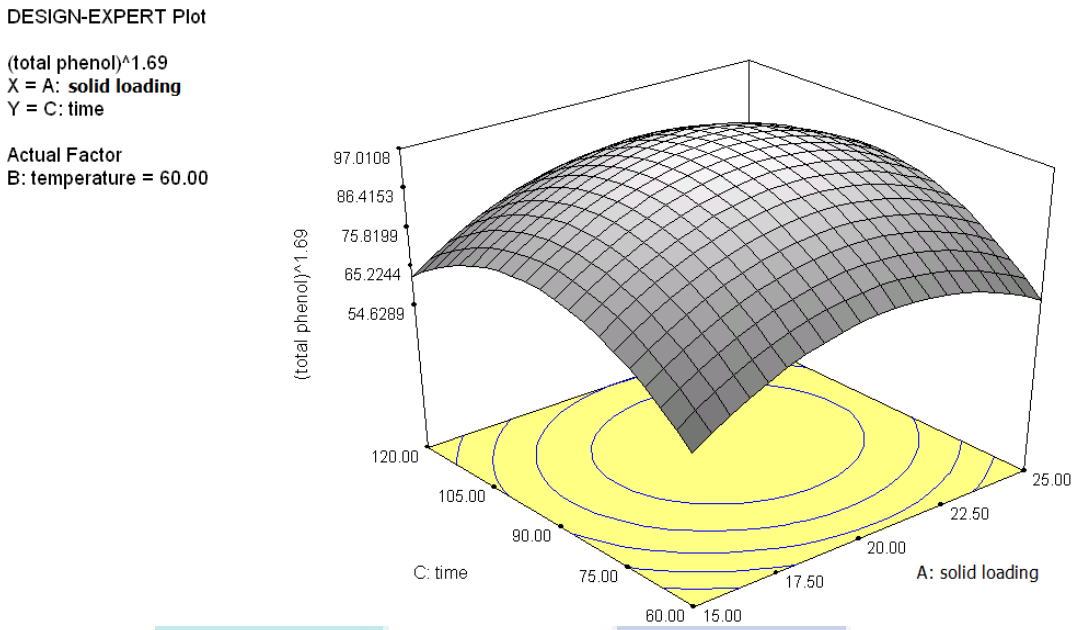


Figure 4.21: 3D response surface of total phenol with respect to the extraction time and solid loading

In the model graph shown in Figure 4.21 showed the 3D surface graph of total phenol with varying solid loading and extraction temperature. The factors which influenced in the total phenol analysis were extraction temperature (A) and extraction time (C), indicating that there effects were significant but there was no significant interactions between A and C. It was obvious that the total phenol yield significantly increased when there was an increase in solid loading and extraction time. A future increase in extraction time and solid loading would result in a decrease in the yield of total phenol.

The following equations were the final empirical models in term of coded factors and actual factors for total phenol respectively. These equations were generated by the Design Expert 6.0.4 software after the transformation had been carried out.

Final equation in terms of coded factors:

$$(\text{Total phenol})^{1.69} = +96.4690 + 4.2045*A - 0.9655*B + 4.5254*C - 15.2881*A^2 - 20.9918*B^2 - 18.4262*C^2 - (0.4166*A*B) + (0.6042*A*C) + (0.7161*B*C) \quad (4.6)$$

Final equation in terms of actual factors:

$$(\text{Total phenol})^{1.69} = -1084.12289 + (25.43928*\text{Concentration}) + (25.04482*\text{Temperature}) + (3.61231*\text{time}) - (0.61152*\text{Concentration}^2) - (0.20991*\text{Temperature}^2) - (0.020474*\text{time}^2) - (8.33250.10^{-3}*\text{Concentration}*\text{Temperature}) + (4.02782.10^{-3}*\text{Concentration}*\text{time}) + (2.38688.10^{-3}*\text{Temperature}*\text{time}) \quad (4.7)$$

4.3.2.4 Validation of Empirical Model Adequacy

Finding the solutions in optimizing the phenolic compound (gallic acid, ellagic acid and total phenol) accuracy was generated by the Design Expert software. The setting for this optimum solution would maximize the goals. In order to verify the adequacy of the models that were developed (Equations 4.3, 4.5 and 4.7), four confirmation runs were performed. Using the point prediction tool of the software, the gallic acid, ellagic acid and total phenol of selected experiments were predicted from the confirmation runs when compared by calculating the residuals and percentage of error.

Table 4.8 shows an example of the output by using the point prediction tool based on the models that were developed by the software. The predicted and the actual values from confirmation runs were compared by calculating the residuals and percentage of error. These values are presented in Tables 4.9, 4.10 and 4.11.

Based on confirmation trials it could be suggested that the empirical models for gallic acid (Table 4.9), ellagic acid (Table 4.10) and total phenol (Table 4.11) that were developed were reasonably accurate and was acceptable. This was proven when all the actual values for the confirmation runs were within 95% prediction interval (PI). The percentage error between actual and predicted value ranged from 1.66 to 4.35%, 2.70 to 4.55% and 2.08 to 4.65% for gallic acid, ellagic acid and total phenol. Since the differences percentage error between actual and predicted response were always less than 5%, thus provided its validity (Zularisam et al., 2008).

Table 4.8: An example output from the prediction tool

Factor	Name	Level	Low Level	High Level	Std. Dev.
A	Solid loading	20.00	15.00	25.00	0.0000
B	Temperature	60.00	50.00	70.00	0.0000
C	Time	90.00	60.00	120.00	0.0000

	Prediction	SE Mean	95% CI low	95% CI high	SE Pred	95% PI low	95% PI high
Gallic Acid	1.71742	0.012	1.69	1.74	0.031	1.65	1.79
Ellagic Acid	0.167511	3.58E-03	0.16	0.18	9.67E-03	0.15	0.19
Total Phenol	14.9346		14.76	15.11		14.46	15.4

Table 4.9: Analysis of confirmation experiment for gallic acid

No	Sample (g)	T (°C)	Time (min)	Predicted (mg/g)	Actual[a] (mg/g)	Residual	% Error
1	25	60	120	1.36	1.30 ± 0.06	-0.06	4.35
2	15	60	120	1.36	1.40 ± 0.05	+0.04	2.86
3	20	70	90	1.43	1.41 ± 0.04	-0.02	1.66
4	20	50	60	1.23	1.18 ± 0.06	-0.05	4.24

[a]response are means ± SD (n=3)

Table 4.10: Analysis of confirmation experiment for ellagic acid

No	Sample (g)	T (°C)	Time (min)	Predicted (mg/g)	Actual[a] (mg/g)	Residual	% Error
1	25	60	120	0.11	0.11 ± 0.01	+0.00	3.12
2	15	60	120	0.12	0.12 ± 0.02	+0.00	2.7
3	20	70	90	0.14	0.15 ± 0.02	+0.01	4.55
4	20	50	60	0.11	0.11 ± 0.02	+0.00	3.12

[a]response are means ± SD (n=3)

Table 4.11: Analysis of confirmation experiment for total phenol

No	Sample (g)	T (°C)	Time (min)	Predicted (mg/g)	Actual[a] (mg/g)	Residual	% Error
1	25	60	120	12.57	12.92 ± 0.78	+0.35	2.73
2	15	60	120	11.55	12.11 ± 0.10	+0.56	4.65
3	20	70	90	12.82	13.40 ± 1.54	+0.58	4.35
4	20	50	60	10.62	10.40 ± 0.62	-0.22	2.08

[a]response are means ± SD (n=3)

4.3.2.5 Confirmation Run of The Predicted Optimization Conditions

Optimization procedure had been conducted for *MML* extract and the prediction results of the empirical model were tabulated in Table 4.12. The solid loading, extraction temperature and extraction time were set to range within the levels defined previously while gallic acid (GA), ellagic acid (EA) and total phenol (TP) were fixed to a maximum value. Results had shown optimum solid loading, extraction temperature and extraction time for optimal gallic acid, ellagic acid and total phenol were determined to be 20.07 g, 59.96°C and 92.55min, respectively with total desirability value of 0.95 was obtained on a scale of 0 to 1, where 0 represented a completely undesirable response and 1 represented the most desirable response. Under these proposed optimized conditions, the maximum value of gallic acid, ellagic acid and total phenol predicted from the model were 1.72, 0.17, 14.96 mg/g. In order to confirm the predicted optimization conditions, experimental confirmation runs were performed by employing the suggested model conditions. Apparently the optimal values of 1.79, 0.16 and 15.10 mg/g were obtained, respectively. It is worth to note results of the experimentals carried out here adequately implied that the proposed mathematical models suggested were reasonably accurate and reliable as most of the actual values for the confirmation runs were well within the 95% prediction interval. Percentages error between 0.93 to 4.68 %, suggested that the model adequacy was reasonably within the 95% of prediction interval. By this, further analysis with regards to ideal operational process for optimal phenolic compound (gallic acid, ellagic acid and total phenol) from *M. malabathricum* L. would be based on this model. Table 5.2 showed the results between

before and after optimization. In comparision to before optimalisation, gallic acid increased by 4.07% and total phenol increased by 3.45%. While before and after optimalisation ellagic acid had the same value.

Table 4.12: Results of optimum operational conditions for *MML* extract

	Proposed optimal conditions	Predicted (mg/g)			Confirmation run (mg/g)		
		GA	EA	TP	GA	EA	TP
Solid loading (g)	20.07	1.72	0.17	14.96	1.79±0.05 (4.15%)	0.16±0.02 (4.68%)	15.10±0.06 (0.93%)
Temperature (°C)	59.96						
Time (min)	92.55						

Number in parenthesis means percentage of error

Table 4.13: Summary of optimization for *MML* extract

	Before optimization	After optimization
Parameter:		
Solid loading (g)	20	20.07
Extraction temperature (°C)	60	59.96
Extraction time (min)	90	92.55
Response:		
Gallic Acid (mg/g)		
a) Predicted	-	1.72
b) Actual	1.72 ± 0.09	1.79 ± 0.05
Ellagic Acid (mg/g)		
a) Predicted	-	0.17
b) Actual	0.16 ± 0.04	0.16 ± 0.02
Total Phenol (mg GAE/g)		
a) Predicted	-	14.96
b) Actual	14.78 ± 0.37	15.10 0.06

CHAPTER 5

CONCLUSIONS AND RECOMMLNDATIONS

5.1 Conclusions

Generally, the study was carried out to determine the effects of ultrasonic-assisted extraction method of gallic acid, ellagic acid and total phenol from MML Thus this study compared the extraction methods using ultrasonic waterbath, homogenizer and incubated in waterbath as a control. The ultrasonic-assisted extraction method was selected for the best extraction method, thus it is suggested that in future study conducted, only the ultrasonic-assisted extraction method is to be implemented. Three factor of extraction processs that had been considered in the preliminary study in production of phenolic compounds; there are solid loading, extraction temperature and extraction time. The purpose of preliminary study was to identification the parameter range of extraction process to conducting an experimental design approach using Design Expert software. This experimental design was carried out using Central Composite Design in order to determine the best setting for the experimental factors that would produce the optimum phenolic compound in MML extract.

The main conclusions that could be drawn from this study are summarized as followed:

1. Extraction of MML extract

   Extraction techniques were identified as one of the important factors influencing the phenolic compound yield. The ultrasonic-assisted extraction method exhibited a significant ($p<0.05$) higher gallic acid and total phenols compounds compared to the other methods. It was followed by the homogenizer and incubated in waterbath as a control. Where the result of ellagic acid from ultrasonic-assisted extraction showed no

significant differences ($p>0.05$) compared to the homogenizer method but there were significant differences when compared to the control. In comparison to control (incubated), ultrasonic extraction gave 54.46% increase in gallic acid, 38.00% increase in ellagic acid, 57.02% increase in total phenol, while 11.61% gallic acid, 29.37% ellagic acid and 47.22% total phenol increase was achieved by homogenizer. In post-treatment extraction, acid-hydrolysis method was found of the best method for determine gallic acid, ellagic acid and total phenol of MML extract.

2. Characterization of MML extract
   a. Nutrient Composition

      MME extract contain small amount of ash, protein, fat, carbohydrate, energy, fiber and cholesterol. In mineral content identification part, pottasium was found as a major components followed by phosphor and sodium. Selenium as a toxic mineral was not detected in MME extract. Thus indicated that MML extract was safe to be added in beverages or food for preservative.

   b. Chemical Properties

      The FTIR spectrum of MML extract have similar spectrum with gallic acid and ellagic acid standard. The analysed by FTIR showed that MML extract have functional groups of hydroxyl, aromatic and hydrocarbon. That indicated that MML extract contains gallic acid and ellagic acid.

   c. Antimicrobial Activity

      Extract of MML showed inhibitory activity in microorganism. The maximum zone inhibition of MML extract as following *Bacillus cereus* > *Bacillus subtillis* > *Salmonella typhi* > *Eschericia coli*.

3. Optimization of MML extract

   The optimum conditions were determined and the optimum performance at these conditions was predicted based on the analysis carried out by the RSM. Optimal conditions were identified as solid loading of 20.07 g, extraction temperature at 59.96°C and extraction time with in the range of 92.55 min. Under this condition, the yield of gallic acid, ellagic acid and total phenol compounds was 1.79 mg/g, 0.16 mg/g

and 15.10 mg GAE/g. Optimalisation gave 4.07% increase in gallic acid and 3.45% increase in total phenol. While before and after optimization, ellagic acid had the same value. The effects of solid loading (A) were found significant ($p<0.05$) on phenolic compound, namely gallic acid, ellagic acid and total phenol yield. The effect of extraction time (C) had a significant ($p<0.05$) effect on the yield of gallic acid and total phenol. The effect of extraction temperature (B) was not a significant ($p>0.05$) factor in phenolic compound. The interaction between extraction temperature and extraction time (BC) in gallic acid had a significant effect.

5.2 Recommendations

Recommendations are made to suggest for future work which could be performed to give better understanding and improvement on the phenolic compounds extraction from *MML* Below are some recoMMLndations for future work:

1. The influence of ultrasonic frequency and intensity on ultrasonic performance is suggested to be studied. Higher ultrasound frequencies produce smaller cavitation bubbles. The change in the cavitation activities is expected to influence the overall performance of the extraction process. In another aspect, ultrasonic intensity is expected to influence the degree of cavitation vigorousity during treatment, which will directly affect the extraction yield.

2. The maximum yield of gallic acid, ellagic acid and total phenols in the *MML* extract with condition: 20.07 g sample (dry weight) with extraction temperature at 59.96 °C and extraction time about 92.55 min. Beyond the maximum condition, there will be a decrease of the yield of gallic acid, ellagic acid and total phenols. The decline that occurred in this case needs to be studied further, whether this is caused by the degradation of the phenolic compound or the polymerization process.

3. Study others factor in antimicrobial testing such as type of microorganism and mechanism action of inhibitation.

4. Isolation and purification for gallic acid and ellagic acid from *MML* may be developed to antimicrobial testing. The testing should be done to confirm that these two phenolic compounds (gallic acid and ellagic acid) possess antimicrobial activity.

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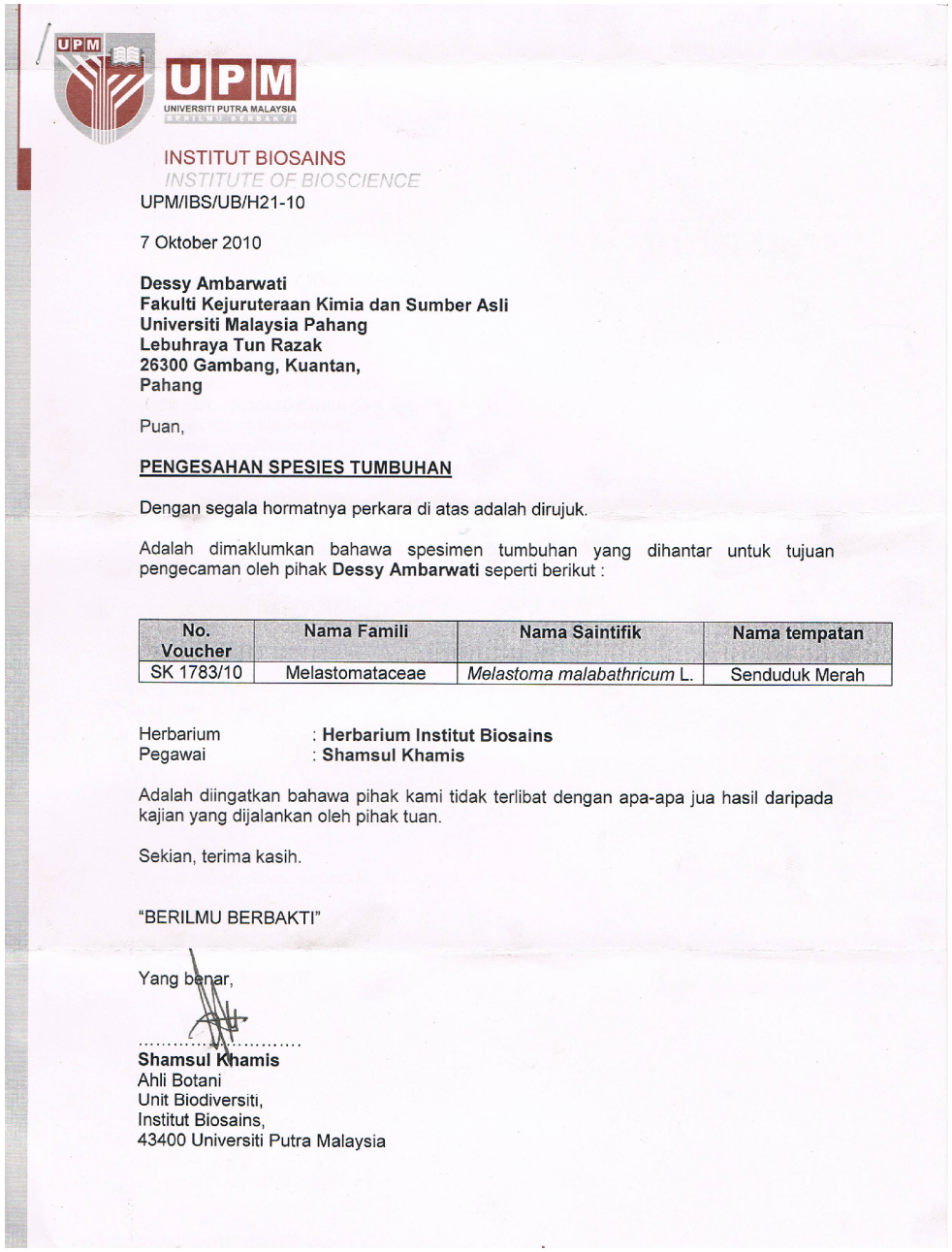
APPENDIX A

IDENTIFICATION OF *MML* LEAVES



UNIVERSITI PUTRA MALAYSIA
BERILMU BERBAKTI

INSTITUT BIOSAINS
INSTITUTE OF BIOSCIENCE

UPM/IBS/UB/H21-10

7 Oktober 2010

Dessy Ambarwati
Fakulti Kejuruteraan Kimia dan Sumber Asli
Universiti Malaysia Pahang
Lebuhraya Tun Razak
26300 Gambang, Kuantan,
Pahang

Puan,

PENGESAHAN SPESIES TUMBUHAN

Dengan segala hormatnya perkara di atas adalah dirujuk.

Adalah dimaklumkan bahawa spesimen tumbuhan yang dihantar untuk tujuan pengecaman oleh pihak **Dessy Ambarwati** seperti berikut :

No. Voucher	Nama Famili	Nama Saintifik	Nama tempatan
SK 1783/10	Melastomataceae	*Melastoma malabathricum* L.	Senduduk Merah

Herbarium : **Herbarium Institut Biosains**
Pegawai : **Shamsul Khamis**

Adalah diingatkan bahawa pihak kami tidak terlibat dengan apa-apa jua hasil daripada kajian yang dijalankan oleh pihak tuan.

Sekian, terima kasih.

"BERILMU BERBAKTI"

Yang benar,

.....
Shamsul Khamis
Ahli Botani
Unit Biodiversiti,
Institut Biosains,
43400 Universiti Putra Malaysia

Figure A: Identification of *MML* leaves

APPENDIX B

PICTURE OF SAMPLE

Fresh leaves of MML

Cut leaves

Ground powder after drying using oven

Figure B: Sample (*MML* leaves)

APPENDIX C1

ANOVA ANALYSIS FOR TYPE OF EXTRACTION PROCESS

Table C.1.1: ANOVA table for type of extraction on gallic acid

ANOVA

GA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.612	2	.306	324.082	.000
Within Groups	.006	6	.001		
Total	.618	8			

Multiple Comparisons

Dependent Variable: GA
Tukey HSD

(I) EXTR	(J) EXTR	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	.4767(*)	.02509	.000	.3997	.5537
	3	.6067(*)	.02509	.000	.5297	.6837
2	1	-.4767(*)	.02509	.000	-.5537	-.3997
	3	.1300(*)	.02509	.005	.0530	.2070
3	1	-.6067(*)	.02509	.000	-.6837	-.5297
	2	-.1300(*)	.02509	.005	-.2070	-.0530

* The mean difference is significant at the .05 level.

Table C.1.2: ANOVA table for type of extraction on ellagic acid

ANOVA

EA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.004	2	.002	32.600	.001
Within Groups	.000	6	.000		
Total	.004	8			

Multiple Comparisons

Dependent Variable: EA
Tukey HSD

(I) EXTR	(J) EXTR	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-.0100	.00609	.300	-.0287	.0087
	3	.0367(*)	.00609	.002	.0180	.0553
2	1	.0100	.00609	.300	-.0087	.0287
	3	.0467(*)	.00609	.001	.0280	.0653
3	1	-.0367(*)	.00609	.002	-.0553	-.0180
	2	-.0467(*)	.00609	.001	-.0653	-.0280

* The mean difference is significant at the .05 level.

Table C.1.3: ANOVA table for type of extraction on total phenol

ANOVA

TP

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	50.841	2	25.421	203.039	.000
Within Groups	.751	6	.125		
Total	51.592	8			

Multiple Comparisons

Dependent Variable: TP
Tukey HSD

(I) EXTR	(J) EXTR	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	.9333(*)	.28891	.041	.0469	1.8198
	3	5.4433(*)	.28891	.000	4.5569	6.3298
2	1	-.9333(*)	.28891	.041	-1.8198	-.0469
	3	4.5100(*)	.28891	.000	3.6236	5.3964
3	1	-5.4433(*)	.28891	.000	-6.3298	-4.5569
	2	-4.5100(*)	.28891	.000	-5.3964	-3.6236

* The mean difference is significant at the .05 level.

APPENDIX C2

ANOVA ANALYSIS FOR EFFECT OF SOLID LOADING

Table C.2.1: ANOVA table for effect of solid loading on gallic acid

ANOVA

GA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.593	4	.648	265.633	.000
Within Groups	.024	10	.002		
Total	2.617	14			

Multiple Comparisons

Dependent Variable: GA

Tukey HSD

(I) SAMPLE	(J) SAMPLE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
5	10	-.3500(*)	.04033	.000	-.4827	-.2173
	15	-.6933(*)	.04033	.000	-.8261	-.5606
	20	-1.1767(*)	.04033	.000	-1.3094	-1.0439
	25	-.9233(*)	.04033	.000	-1.0561	-.7906
10	5	.3500(*)	.04033	.000	.2173	.4827
	15	-.3433(*)	.04033	.000	-.4761	-.2106
	20	-.8267(*)	.04033	.000	-.9594	-.6939
	25	-.5733(*)	.04033	.000	-.7061	-.4406
15	5	.6933(*)	.04033	.000	.5606	.8261
	10	.3433(*)	.04033	.000	.2106	.4761
	20	-.4833(*)	.04033	.000	-.6161	-.3506
	25	-.2300(*)	.04033	.001	-.3627	-.0973
20	5	1.1767(*)	.04033	.000	1.0439	1.3094
	10	.8267(*)	.04033	.000	.6939	.9594
	15	.4833(*)	.04033	.000	.3506	.6161
	25	.2533(*)	.04033	.001	.1206	.3861
25	5	.9233(*)	.04033	.000	.7906	1.0561
	10	.5733(*)	.04033	.000	.4406	.7061
	15	.2300(*)	.04033	.001	.0973	.3627
	20	-.2533(*)	.04033	.001	-.3861	-.1206

* The mean difference is significant at the .05 level.

Table C.2.2: ANOVA table for effect of solid loading on ellagic acid

ANOVA

EA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.021	4	.005	97.876	.000
Within Groups	.001	10	.000		
Total	.022	14			

Multiple Comparisons

Dependent Variable: EA
Tukey HSD

(I) SAMPLE	(J) SAMPLE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
5	10	-.03333(*)	.005989	.002	-.05304	-.01362
	15	-.07000(*)	.005989	.000	-.08971	-.05029
	20	-.09267(*)	.005989	.000	-.11238	-.07296
	25	-.09933(*)	.005989	.000	-.11904	-.07962
10	5	.03333(*)	.005989	.002	.01362	.05304
	15	-.03667(*)	.005989	.001	-.05638	-.01696
	20	-.05933(*)	.005989	.000	-.07904	-.03962
	25	-.06600(*)	.005989	.000	-.08571	-.04629
15	5	.07000(*)	.005989	.000	.05029	.08971
	10	.03667(*)	.005989	.001	.01696	.05638
	20	-.02267(*)	.005989	.023	-.04238	-.00296
	25	-.02933(*)	.005989	.004	-.04904	-.00962
20	5	.09267(*)	.005989	.000	.07296	.11238
	10	.05933(*)	.005989	.000	.03962	.07904
	15	.02267(*)	.005989	.023	.00296	.04238
	25	-.00667	.005989	.796	-.02638	.01304
25	5	.09933(*)	.005989	.000	.07962	.11904
	10	.06600(*)	.005989	.000	.04629	.08571
	15	.02933(*)	.005989	.004	.00962	.04904
	20	.00667	.005989	.796	-.01304	.02638

* The mean difference is significant at the .05 level.

Table C.2.3: ANOVA table for effect of solid loading on total phenol

ANOVA

TP

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	197.463	4	49.366	233.629	.000
Within Groups	2.113	10	.211		
Total	199.576	14			

Multiple Comparisons

Dependent Variable: TP
Tukey HSD

(I) SAMPLE	(J) SAMPLE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
5	10	-2.2167(*)	.37532	.001	-3.4519	-.9815
	15	-6.0700(*)	.37532	.000	-7.3052	-4.8348
	20	-10.0933(*)	.37532	.000	-11.3285	-8.8581
	25	-7.4767(*)	.37532	.000	-8.7119	-6.2415
10	5	2.2167(*)	.37532	.001	.9815	3.4519
	15	-3.8533(*)	.37532	.000	-5.0885	-2.6181
	20	-7.8767(*)	.37532	.000	-9.1119	-6.6415
	25	-5.2600(*)	.37532	.000	-6.4952	-4.0248
15	5	6.0700(*)	.37532	.000	4.8348	7.3052
	10	3.8533(*)	.37532	.000	2.6181	5.0885
	20	-4.0233(*)	.37532	.000	-5.2585	-2.7881
	25	-1.4067(*)	.37532	.025	-2.6419	-.1715
20	5	10.0933(*)	.37532	.000	8.8581	11.3285
	10	7.8767(*)	.37532	.000	6.6415	9.1119
	15	4.0233(*)	.37532	.000	2.7881	5.2585
	25	2.6167(*)	.37532	.000	1.3815	3.8519
25	5	7.4767(*)	.37532	.000	6.2415	8.7119
	10	5.2600(*)	.37532	.000	4.0248	6.4952
	15	1.4067(*)	.37532	.025	.1715	2.6419
	20	-2.6167(*)	.37532	.000	-3.8519	-1.3815

* The mean difference is significant at the .05 level.

APPENDIX C3

ANOVA ANALYSIS FOR EFFECT OF EXTRACTION TIME

Table C.3.1: ANOVA table for effect of extraction time on gallic acid

ANOVA

GA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.566	5	.313	322.104	.000
Within Groups	.012	12	.001		
Total	1.577	17			

Multiple Comparisons

Dependent Variable: GA
Tukey HSD

(I) TIME	(J) TIME	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
30	60	-.37000(*)	.025459	.000	-.45551	-.28449
	90	-.85667(*)	.025459	.000	-.94218	-.77115
	120	-.45333(*)	.025459	.000	-.53885	-.36782
	150	-.11333(*)	.025459	.008	-.19885	-.02782
	180	-.05667	.025459	.294	-.14218	.02885
60	30	.37000(*)	.025459	.000	.28449	.45551
	90	-.48667(*)	.025459	.000	-.57218	-.40115
	120	-.08333	.025459	.058	-.16885	.00218
	150	.25667(*)	.025459	.000	.17115	.34218
	180	.31333(*)	.025459	.000	.22782	.39885
90	30	.85667(*)	.025459	.000	.77115	.94218
	60	.48667(*)	.025459	.000	.40115	.57218
	120	.40333(*)	.025459	.000	.31782	.48885
	150	.74333(*)	.025459	.000	.65782	.82885
	180	.80000(*)	.025459	.000	.71449	.88551
120	30	.45333(*)	.025459	.000	.36782	.53885
	60	.08333	.025459	.058	-.00218	.16885
	90	-.40333(*)	.025459	.000	-.48885	-.31782
	150	.34000(*)	.025459	.000	.25449	.42551
	180	.39667(*)	.025459	.000	.31115	.48218

150	30	.11333(*)	.025459	.008	.02782	.19885
	60	-.25667(*)	.025459	.000	-.34218	-.17115
	90	-.74333(*)	.025459	.000	-.82885	-.65782
	120	-.34000(*)	.025459	.000	-.42551	-.25449
	180	.05667	.025459	.294	-.02885	.14218
180	30	.05667	.025459	.294	-.02885	.14218
	60	-.31333(*)	.025459	.000	-.39885	-.22782
	90	-.80000(*)	.025459	.000	-.88551	-.71449
	120	-.39667(*)	.025459	.000	-.48218	-.31115
	150	-.05667	.025459	.294	-.14218	.02885

* The mean difference is significant at the .05 level.

Table C.3.2: ANOVA table for effect of extraction time on ellagic acid

ANOVA

EA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.048	5	.010	273.821	.000
Within Groups	.000	12	.000		
Total	.048	17			

Multiple Comparisons

Dependent Variable: EA
Tukey HSD

(I) TIME	(J) TIME	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
30	60	-.06067(*)	.004819	.000	-.07685	-.04448
	90	-.14600(*)	.004819	.000	-.16219	-.12981
	120	-.09467(*)	.004819	.000	-.11085	-.07848
	150	-.02300(*)	.004819	.005	-.03919	-.00681
	180	-.01067	.004819	.299	-.02685	.00552
60	30	.06067(*)	.004819	.000	.04448	.07685
	90	-.08533(*)	.004819	.000	-.10152	-.06915
	120	-.03400(*)	.004819	.000	-.05019	-.01781
	150	.03767(*)	.004819	.000	.02148	.05385
	180	.05000(*)	.004819	.000	.03381	.06619
90	30	.14600(*)	.004819	.000	.12981	.16219
	60	.08533(*)	.004819	.000	.06915	.10152

	120	.05133(*)	.004819	.000	.03515	.06752
	150	.12300(*)	.004819	.000	.10681	.13919
	180	.13533(*)	.004819	.000	.11915	.15152
120	30	.09467(*)	.004819	.000	.07848	.11085
	60	.03400(*)	.004819	.000	.01781	.05019
	90	-.05133(*)	.004819	.000	-.06752	-.03515
	150	.07167(*)	.004819	.000	.05548	.08785
	180	.08400(*)	.004819	.000	.06781	.10019
150	30	.02300(*)	.004819	.005	.00681	.03919
	60	-.03767(*)	.004819	.000	-.05385	-.02148
	90	-.12300(*)	.004819	.000	-.13919	-.10681
	120	-.07167(*)	.004819	.000	-.08785	-.05548
	180	.01233	.004819	.182	-.00385	.02852
180	30	.01067	.004819	.299	-.00552	.02685
	60	-.05000(*)	.004819	.000	-.06619	-.03381
	90	-.13533(*)	.004819	.000	-.15152	-.11915
	120	-.08400(*)	.004819	.000	-.10019	-.06781
	150	-.01233	.004819	.182	-.02852	.00385

* The mean difference is significant at the .05 level.

Table C.3.3: ANOVA table for effect of extraction time on total phenol

ANOVA

TP

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	207.926	5	41.585	471.400	.000
Within Groups	1.059	12	.088		
Total	208.985	17			

Multiple Comparisons

Dependent Variable: TP
Tukey HSD

(I) TIME	(J) TIME	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
30	60	-7.82667(*)	.242510	.000	-8.64124	-7.01209
	90	-10.51000(*)	.242510	.000	-11.32457	-9.69543

	120	-5.71333(*)	.242510	.000	-6.52791	-4.89876
	150	-3.50333(*)	.242510	.000	-4.31791	-2.68876
	180	-3.18333(*)	.242510	.000	-3.99791	-2.36876
60	30	7.82667(*)	.242510	.000	7.01209	8.64124
	90	-2.68333(*)	.242510	.000	-3.49791	-1.86876
	120	2.11333(*)	.242510	.000	1.29876	2.92791
	150	4.32333(*)	.242510	.000	3.50876	5.13791
	180	4.64333(*)	.242510	.000	3.82876	5.45791
90	30	10.51000(*)	.242510	.000	9.69543	11.32457
	60	2.68333(*)	.242510	.000	1.86876	3.49791
	120	4.79667(*)	.242510	.000	3.98209	5.61124
	150	7.00667(*)	.242510	.000	6.19209	7.82124
	180	7.32667(*)	.242510	.000	6.51209	8.14124
120	30	5.71333(*)	.242510	.000	4.89876	6.52791
	60	-2.11333(*)	.242510	.000	-2.92791	-1.29876
	90	-4.79667(*)	.242510	.000	-5.61124	-3.98209
	150	2.21000(*)	.242510	.000	1.39543	3.02457
	180	2.53000(*)	.242510	.000	1.71543	3.34457
150	30	3.50333(*)	.242510	.000	2.68876	4.31791
	60	-4.32333(*)	.242510	.000	-5.13791	-3.50876
	90	-7.00667(*)	.242510	.000	-7.82124	-6.19209
	120	-2.21000(*)	.242510	.000	-3.02457	-1.39543
	180	.32000	.242510	.770	-.49457	1.13457
180	30	3.18333(*)	.242510	.000	2.36876	3.99791
	60	-4.64333(*)	.242510	.000	-5.45791	-3.82876
	90	-7.32667(*)	.242510	.000	-8.14124	-6.51209
	120	-2.53000(*)	.242510	.000	-3.34457	-1.71543
	150	-.32000	.242510	.770	-1.13457	.49457

* The mean difference is significant at the .05 level.

APPENDIX C4

ANOVA ANALYSIS FOR EFFECT OF EXTRACTION TEMPERATURE

Table C.4.1: ANOVA table for extraction temperature on gallic acid

ANOVA

GA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.059	6	.843	3613.469	.000
Within Groups	.003	14	.000		
Total	5.062	20			

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Multiple Comparisons

Dependent Variable: GA
Tukey HSD

(I) T	(J) T	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
40	50	-.32000(*)	.012472	.000	-.36259	-.27741
	60	-1.16667(*)	.012472	.000	-1.20925	-1.12408
	70	-1.02333(*)	.012472	.000	-1.06592	-.98075
	80	-.01000	.012472	.981	-.05259	.03259
	90	.02333	.012472	.527	-.01925	.06592
	100	.08333(*)	.012472	.000	.04075	.12592
50	40	.32000(*)	.012472	.000	.27741	.36259
	60	-.84667(*)	.012472	.000	-.88925	-.80408
	70	-.70333(*)	.012472	.000	-.74592	-.66075
	80	.31000(*)	.012472	.000	.26741	.35259
	90	.34333(*)	.012472	.000	.30075	.38592
	100	.40333(*)	.012472	.000	.36075	.44592
60	40	1.16667(*)	.012472	.000	1.12408	1.20925
	50	.84667(*)	.012472	.000	.80408	.88925
	70	.14333(*)	.012472	.000	.10075	.18592
	80	1.15667(*)	.012472	.000	1.11408	1.19925
	90	1.19000(*)	.012472	.000	1.14741	1.23259
	100	1.25000(*)	.012472	.000	1.20741	1.29259

70	40	1.02333(*)	.012472	.000	.98075	1.06592
	50	.70333(*)	.012472	.000	.66075	.74592
	60	-.14333(*)	.012472	.000	-.18592	-.10075
	80	1.01333(*)	.012472	.000	.97075	1.05592
	90	1.04667(*)	.012472	.000	1.00408	1.08925
	100	1.10667(*)	.012472	.000	1.06408	1.14925
80	40	.01000	.012472	.981	-.03259	.05259
	50	-.31000(*)	.012472	.000	-.35259	-.26741
	60	-1.15667(*)	.012472	.000	-1.19925	-1.11408
	70	-1.01333(*)	.012472	.000	-1.05592	-.97075
	90	.03333	.012472	.176	-.00925	.07592
	100	.09333(*)	.012472	.000	.05075	.13592
90	40	-.02333	.012472	.527	-.06592	.01925
	50	-.34333(*)	.012472	.000	-.38592	-.30075
	60	-1.19000(*)	.012472	.000	-1.23259	-1.14741
	70	-1.04667(*)	.012472	.000	-1.08925	-1.00408
	80	-.03333	.012472	.176	-.07592	.00925
	100	.06000(*)	.012472	.004	.01741	.10259
100	40	-.08333(*)	.012472	.000	-.12592	-.04075
	50	-.40333(*)	.012472	.000	-.44592	-.36075
	60	-1.25000(*)	.012472	.000	-1.29259	-1.20741
	70	-1.10667(*)	.012472	.000	-1.14925	-1.06408
	80	-.09333(*)	.012472	.000	-.13592	-.05075
	90	-.06000(*)	.012472	.004	-.10259	-.01741

* The mean difference is significant at the .05 level.

Table C.4.2: ANOVA table for extraction temperature on ellagic acid

ANOVA

EA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.053	6	.009	97.571	.000
Within Groups	.001	14	.000		
Total	.054	20			

Multiple Comparisons

Dependent Variable: EA
Tukey HSD

(I) T	(J) T	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
40	50	-.02367	.007736	.093	-.05008	.00275
	60	-.11233(*)	.007736	.000	-.13875	-.08592
	70	-.05600(*)	.007736	.000	-.08241	-.02959
	80	.00500	.007736	.994	-.02141	.03141
	90	.03133(*)	.007736	.016	.00492	.05775
	100	.04433(*)	.007736	.001	.01792	.07075
50	40	.02367	.007736	.093	-.00275	.05008
	60	-.08867(*)	.007736	.000	-.11508	-.06225
	70	-.03233(*)	.007736	.013	-.05875	-.00592
	80	.02867(*)	.007736	.030	.00225	.05508
	90	.05500(*)	.007736	.000	.02859	.08141
	100	.06800(*)	.007736	.000	.04159	.09441
60	40	.11233(*)	.007736	.000	.08592	.13875
	50	.08867(*)	.007736	.000	.06225	.11508
	70	.05633(*)	.007736	.000	.02992	.08275
	80	.11733(*)	.007736	.000	.09092	.14375
	90	.14367(*)	.007736	.000	.11725	.17008
	100	.15667(*)	.007736	.000	.13025	.18308
70	40	.05600(*)	.007736	.000	.02959	.08241
	50	.03233(*)	.007736	.013	.00592	.05875
	60	-.05633(*)	.007736	.000	-.08275	-.02992
	80	.06100(*)	.007736	.000	.03459	.08741
	90	.08733(*)	.007736	.000	.06092	.11375
	100	.10033(*)	.007736	.000	.07392	.12675
80	40	-.00500	.007736	.994	-.03141	.02141
	50	-.02867(*)	.007736	.030	-.05508	-.00225
	60	-.11733(*)	.007736	.000	-.14375	-.09092
	70	-.06100(*)	.007736	.000	-.08741	-.03459
	90	.02633	.007736	.051	-.00008	.05275
	100	.03933(*)	.007736	.002	.01292	.06575
90	40	-.03133(*)	.007736	.016	-.05775	-.00492
	50	-.05500(*)	.007736	.000	-.08141	-.02859
	60	-.14367(*)	.007736	.000	-.17008	-.11725
	70	-.08733(*)	.007736	.000	-.11375	-.06092
	80	-.02633	.007736	.051	-.05275	.00008
	100	.01300	.007736	.638	-.01341	.03941
100	40	-.04433(*)	.007736	.001	-.07075	-.01792

	50	-.06800(*)	.007736	.000	-.09441	-.04159
	60	-.15667(*)	.007736	.000	-.18308	-.13025
	70	-.10033(*)	.007736	.000	-.12675	-.07392
	80	-.03933(*)	.007736	.002	-.06575	-.01292
	90	-.01300	.007736	.638	-.03941	.01341

* The mean difference is significant at the .05 level.

Table C.4.3: ANOVA table for extraction temperature on total phenol

ANOVA

TP

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	310.429	6	51.738	273.176	.000
Within Groups	2.652	14	.189		
Total	313.081	20			

Multiple Comparisons

Dependent Variable: TP
Tukey HSD

(I) T	(J) T	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
40	50	-2.0867(*)	.35534	.001	-3.3000	-.8733
	60	-9.9100(*)	.35534	.000	-11.1233	-8.6967
	70	-3.9933(*)	.35534	.000	-5.2067	-2.7800
	80	1.1800	.35534	.059	-.0333	2.3933
	90	1.2233(*)	.35534	.048	.0100	2.4367
	100	1.6067(*)	.35534	.007	.3933	2.8200
50	40	2.0867(*)	.35534	.001	.8733	3.3000
	60	-7.8233(*)	.35534	.000	-9.0367	-6.6100
	70	-1.9067(*)	.35534	.001	-3.1200	-.6933
	80	3.2667(*)	.35534	.000	2.0533	4.4800
	90	3.3100(*)	.35534	.000	2.0967	4.5233
	100	3.6933(*)	.35534	.000	2.4800	4.9067
60	40	9.9100(*)	.35534	.000	8.6967	11.1233
	50	7.8233(*)	.35534	.000	6.6100	9.0367
	70	5.9167(*)	.35534	.000	4.7033	7.1300
	80	11.0900(*)	.35534	.000	9.8767	12.3033

	90	11.1333(*)	.35534	.000	9.9200	12.3467
	100	11.5167(*)	.35534	.000	10.3033	12.7300
70	40	3.9933(*)	.35534	.000	2.7800	5.2067
	50	1.9067(*)	.35534	.001	.6933	3.1200
	60	-5.9167(*)	.35534	.000	-7.1300	-4.7033
	80	5.1733(*)	.35534	.000	3.9600	6.3867
	90	5.2167(*)	.35534	.000	4.0033	6.4300
	100	5.6000(*)	.35534	.000	4.3867	6.8133
80	40	-1.1800	.35534	.059	-2.3933	.0333
	50	-3.2667(*)	.35534	.000	-4.4800	-2.0533
	60	-11.0900(*)	.35534	.000	-12.3033	-9.8767
	70	-5.1733(*)	.35534	.000	-6.3867	-3.9600
	90	.0433	.35534	1.000	-1.1700	1.2567
	100	.4267	.35534	.883	-.7867	1.6400
90	40	-1.2233(*)	.35534	.048	-2.4367	-.0100
	50	-3.3100(*)	.35534	.000	-4.5233	-2.0967
	60	-11.1333(*)	.35534	.000	-12.3467	-9.9200
	70	-5.2167(*)	.35534	.000	-6.4300	-4.0033
	80	-.0433	.35534	1.000	-1.2567	1.1700
	100	.3833	.35534	.924	-.8300	1.5967
100	40	-1.6067(*)	.35534	.007	-2.8200	-.3933
	50	-3.6933(*)	.35534	.000	-4.9067	-2.4800
	60	-11.5167(*)	.35534	.000	-12.7300	-10.3033
	70	-5.6000(*)	.35534	.000	-6.8133	-4.3867
	80	-.4267	.35534	.883	-1.6400	.7867
	90	-.3833	.35534	.924	-1.5967	.8300

* The mean difference is significant at the .05 level.

APPENDIX D1

EXPERIMENTAL RESULTS FOR TYPE OF EXTRACTION PROCESS

Table D.1: Experimental results for type of extraction process on gallic acid, ellagic acid and total phenol

	Replications (mg/g)			Mean			S.D.		
	Gallic Acid	Ellagic Acid	Total Phenol	Gallic Acid	Ellagic Acid	Total Phenol	Gallic Acid	Ellagic Acid	Total Phenol
Ultrasonic waterbath	1.72	0.17	15.05	1.72	0.17	14.99	0.01	0.01	0.08
	1.73	0.16	14.89						
	1.70	0.17	15.02						
Homogenizer	1.22	0.18	13.88	1.24	0.18	14.05	0.04	0.00	0.22
	1.29	0.17	14.30						
	1.21	0.18	13.98						
Incubated in waterbath	1.12	0.13	9.88	1.11	0.13	9.54	0.03	0.01	0.57
	1.08	0.12	9.86						
	1.13	0.14	8.89						

APPENDIX D2

EXPERIMENTAL RESULTS FOR SOLID LOADING

Table D.2.1: Experimental results for sample amout of gallic acid

Amount of Sample (g)	Replications of Gallic Acid Yield (mg/g)			Mean	S.D.
	1	2	3		
5	0.56	0.65	0.52	0.58	0.07
10	0.93	0.90	0.95	0.93	0.03
15	1.23	1.35	1.23	1.27	0.06
20	1.73	1.76	1.77	1.76	0.02
25	1.53	1.45	1.52	1.50	0.04

Table D.2.2: Experimental results for sample amout of ellagic acid

Amount of Sample (g)	Replications of Ellagic Acid Yield (mg/g)			Mean	S.D.
	1	2	3		
5	0.065	0.074	0.064	0.07	0.01
10	0.094	0.101	0.108	0.10	0.01
15	0.145	0.134	0.134	0.14	0.01
20	0.158	0.168	0.155	0.16	0.01
25	0.158	0.165	0.178	0.17	0.01

Table D.2.3: Experimental results for sample amout of total phenol

Amount of Sample (g)	Replications of Total Phenol Yield (mg/g)			Mean	S.D.
	1	2	3		
5	4.32	4.98	4.77	4.69	0.34
10	6.71	6.89	7.12	6.91	0.20
15	10.42	11.32	10.54	10.76	0.49
20	14.89	15.03	14.43	14.78	0.31
25	12.43	12.75	11.32	12.17	0.75

APPENDIX D3

EXPERIMENTAL RESULTS FOR EXTRACTION TEMPERATURE

Table D.3.1: Experimental results for extraction temperature of gallic acid

Temperature (°C)	Replications of Gallic Acid Yield (mg/g)			Mean	S.D.
	1	2	3		
40	0.54	0.55	0.56	0.55	0.01
50	0.87	0.88	0.86	0.87	0.01
60	1.70	1.73	1.72	1.72	0.01
70	1.56	1.59	1.57	1.57	0.02
80	0.56	0.56	0.56	0.56	0.00
90	0.53	0.53	0.52	0.53	0.00
100	0.50	0.46	0.44	0.46	0.03

Table D.3.2: Experimental results for extraction temperature of ellagic acid

Temperature (°C)	Replications of Ellagic Acid Yield (mg/g)			Mean	S.D.
	1	2	3		
40	0.060	0.059	0.044	0.054	0.009
50	0.076	0.078	0.080	0.078	0.002
60	0.168	0.158	0.174	0.167	0.008
70	0.103	0.113	0.115	0.110	0.007
80	0.030	0.065	0.053	0.050	0.018
90	0.019	0.020	0.030	0.023	0.006
100	0.000	0.012	0.018	0.010	0.009

Table D.3.3: Experimental results for extraction temperature of total phenol

Temperature (°C)	Replications of Total Phenol Yield (mg/g)			Mean	S.D.
	1	2	3		
40	5.16	6.03	4.04	5.08	1.00
50	7.18	7.08	7.23	7.16	0.08
60	15.05	14.89	15.02	14.99	0.08
70	9.06	9.08	9.07	9.07	0.01
80	3.55	4.54	3.60	3.90	0.56
90	3.88	3.85	3.83	3.86	0.03
100	3.46	3.55	3.40	3.47	0.08

APPENDIX D4

EXPERIMENTAL RESULTS FOR EXTRACTION TIME

Table D.4.1: Experimental results for extraction time of gallic acid

Time (min)	Replications of Gallic Acid Yield (mg/g)			Mean	S.D.
	1	2	3		
30	0.86	0.86	0.86	0.86	0.00
60	1.25	1.24	1.20	1.23	0.03
90	1.72	1.73	1.70	1.72	0.01
120	1.26	1.29	1.39	1.31	0.07
150	0.97	0.98	0.97	0.97	0.00
180	0.90	0.92	0.93	0.92	0.01

Table D.4.2: Experimental results for extraction time of ellagic acid

Time (min)	Replications of Ellagic Acid Yield (mg/g)			Mean	S.D.
	1	2	3		
30	0.019	0.026	0.017	0.021	0.005
60	0.078	0.090	0.076	0.081	0.008
90	0.168	0.158	0.174	0.167	0.008
120	0.118	0.118	0.110	0.115	0.005
150	0.050	0.044	0.037	0.044	0.007
180	0.031	0.031	0.032	0.031	0.001

Table D.4.3: Experimental results for extraction time of total phenol

Time (min)	Replications of Total Phenol Yield (mg/g)			Mean	S.D.
	1	2	3		
30	4.79	4.35	4.29	4.48	0.27
60	12.04	12.87	12.00	12.30	0.49
90	15.05	14.89	15.02	14.99	0.08
120	10.26	10.19	10.12	10.19	0.07
150	8.09	8.34	7.51	7.98	0.43
180	7.55	7.61	7.82	7.66	0.14

APPENDIX D5

EXPERIMENTAL RESULTS FOR CENTRAL COMPOSITE DESIGN SETTING

Table D.5: Central composite design setting for *MML* extract

Std order	Factors			Response								
	Solid loading (g)	T (°C)	Time (min)	Gallic Acid			Ellagic Acid			Total Phenol		
				Replications (n)	Mean (n=3)	SD	Replications (n)	Mean (n=3)	SD	Replications (n)	Mean (n=3)	SD
1	15	50	60	1.131	0.982	0.144	0.109	0.098	0.012	8.453	8.22	0.691
				0.972			0.085			8.765		
				0.844			0.1			7.443		
2	25	50	60	0.984	1.065	0.071	0.09	0.081	0.012	8.345	9.214	0.785
				1.112			0.086			9.873		
				1.1			0.067			9.424		
3	15	70	60	0.871	0.907	0.309	0.083	0.082	0.006	8.113	8.221	0.104
				1.232			0.087			8.321		
				0.618			0.076			8.228		
4	25	70	60	0.791	0.988	0.213	0.076	0.061	0.017	8.678	8.561	0.185
				1.214			0.065			8.656		
				0.96			0.042			8.348		
5	15	50	120	1.141	1.058	0.12	0.082	0.082	0.006	9.762	9.112	0.594
				1.112			0.088			8.978		
				0.92			0.076			8.596		

Table D.5: Continued

Std order	Factors			Response								
				Gallic Acid			Ellagic Acid			Total Phenol		
	Solid loading (g)	T (°C)	Time (min)	Replications (n)	Mean (n=3)	SD	Replications (n)	Mean (n=3)	SD	Replications (n)	Mean (n=3)	SD
6	25	50	120	0.963	1.062	0.154	0.083	0.083	0.001	9.983	9.934	0.928
				0.984			0.084			8.983		
				1.239			0.082			10.837		
7	15	70	120	1.224	1.079	0.153	0.102	0.102	0.003	8.789	9.054	0.239
				1.094			0.105			9.12		
				0.92			0.099			9.252		
8	25	70	120	1.073	1.09	0.02	0.081	0.072	0.01	9.893	10.079	0.327
				1.084			0.074			10.456		
				1.112			0.061			9.888		
9	10	60	90	0.985	0.932	0.055	0.099	0.094	0.005	7.093	6.711	0.338
				0.936			0.089			6.587		
				0.876			0.094			6.453		
10	30	60	90	1.073	1.02	0.103	0.066	0.064	0.002	10.013	9.661	0.495
				1.085			0.064			9.874		
				0.901			0.062			9.095		
11	20	40	90	0.555	0.562	0.024	0.055	0.054	0.012	4.873	5.076	0.203
				0.542			0.065			5.078		
				0.589			0.042			5.278		

Table D.5: Continued

Std order	Factors			Response								
				Gallic Acid			Ellagic Acid			Total Phenol		
	Solid loading (g)	T (°C)	Time (min)	Replications (n)	Mean (n=3)	SD	Replications (n)	Mean (n=3)	SD	Replications (n)	Mean (n=3)	SD
12	20	80	90	0.543	0.591	0.06	0.072	0.07	0.003	3.984	3.898	0.194
				0.654			0.071			4.034		
				0.557			0.067			3.676		
13	20	60	30	0.943	0.896	0.05	0.018	0.021	0.005	4.573	4.477	0.588
				0.844			0.018			5.012		
				0.9			0.027			3.847		
14	20	60	150	0.974	0.971	0.096	0.047	0.044	0.009	7.569	7.982	0.382
				0.873			0.051			8.054		
				1.065			0.034			8.323		
15	20	60	90	1.738	1.707	0.188	0.173	0.185	0.039	15.044	14.923	0.549
				1.877			0.153			14.324		
				1.505			0.229			15.402		
16	20	60	90	1.744	1.732	0.054	0.145	0.158	0.015	14.943	14.892	0.303
				1.778			0.155			14.567		
				1.673			0.174			15.166		
17	20	60	90	1.894	1.702	0.174	0.176	0.163	0.014	15.543	15.018	0.836
				1.658			0.165			15.456		
				1.555			0.148			14.054		

Table D.5: Continued

Std order	Factors			Response								
	Solid loading (g)	T (°C)	Time (min)	Gallic Acid			Ellagic Acid			Total Phenol		
				Replications (n)	Mean (n=3)	SD	Replications (n)	Mean (n=3)	SD	Replications (n)	Mean (n=3)	SD
18	20	60	90	1.776	1.72	0.081	0.177	0.168	0.013	14.894	15.049	0.181
				1.758			0.174			15.004		
				1.627			0.153			15.248		
19	20	60	90	1.676	1.757	0.07	0.167	0.166	0.01	15.033	15.033	0.423
				1.788			0.156			15.456		
				1.806			0.175			14.611		
20	20	60	90	1.774	1.712	0.101	0.172	0.17	0.003	15.093	14.74	0.774
				1.766			0.167			15.274		
				1.596			0.171			13.852		

APPENDIX E1
GALLIC ACID PLOTS

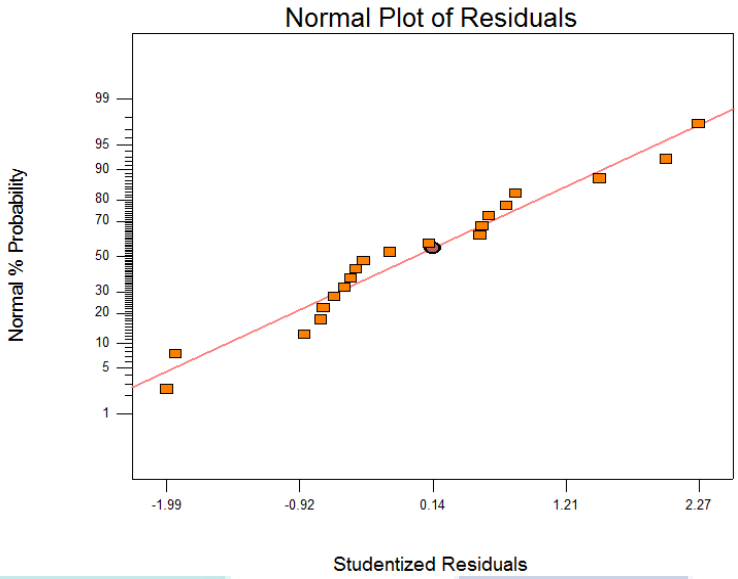


Figure E1.1: Normal probability plot of the residuals for gallic acid

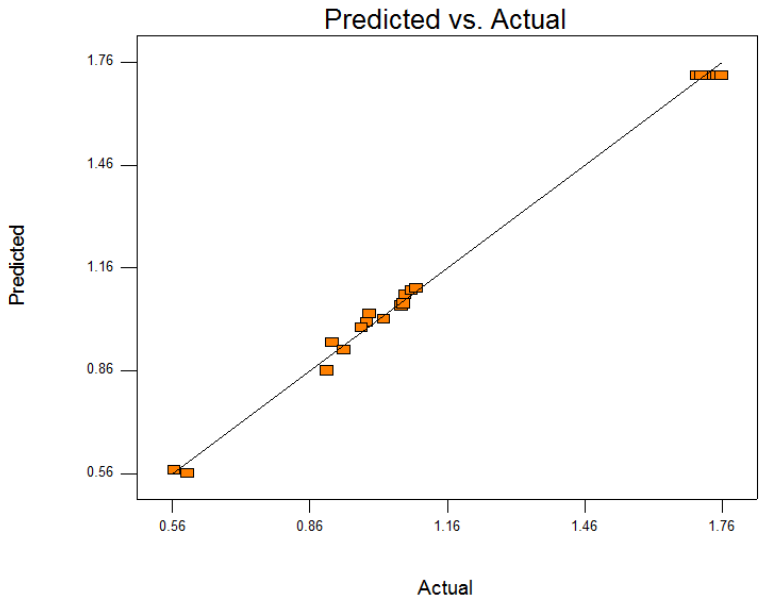


Figure E1.2: Predicted versus actual values for gallic acid

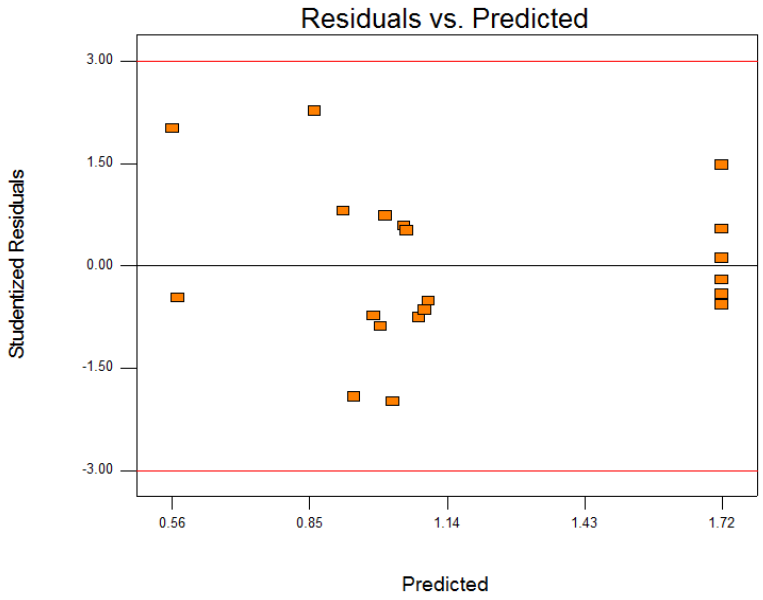


Figure E1.3: Residuals versus predicted values for gallic acid

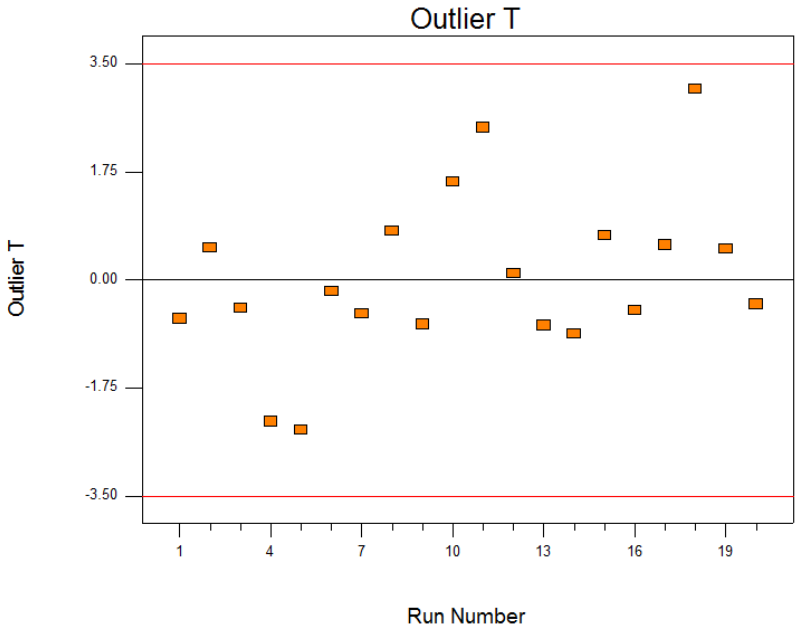


Figure E1.4: Outlier T plot for gallic acid

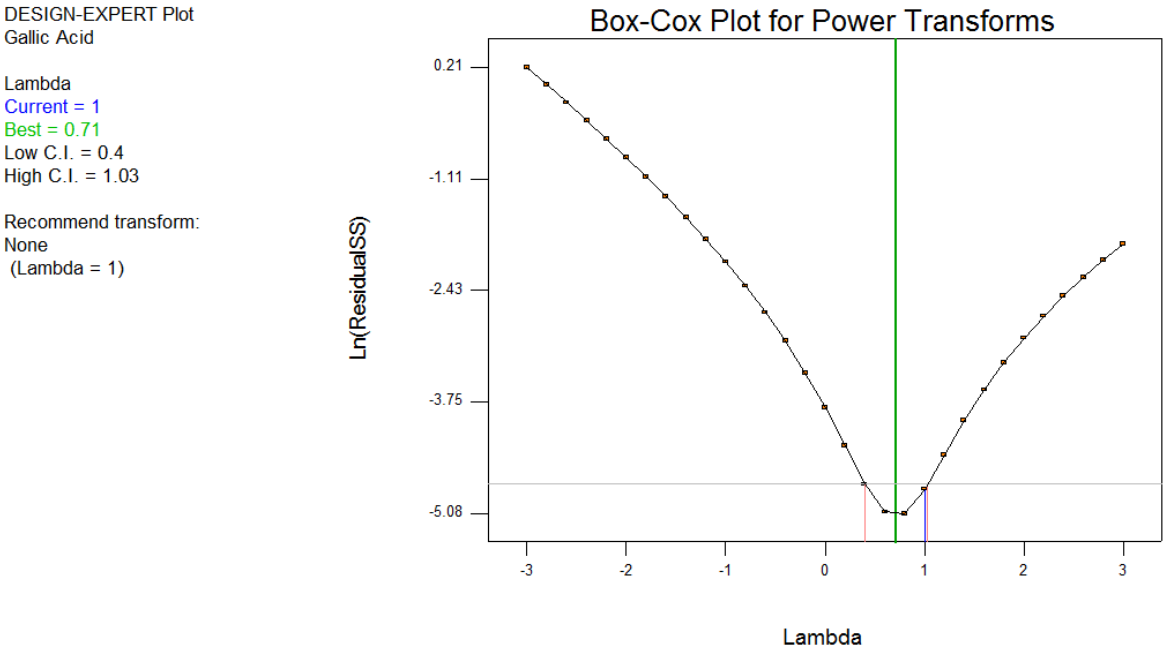


Figure E1.5: Box-cox plot for Gallic Acid

APPENDIX E2
ELLAGIC ACID PLOTS

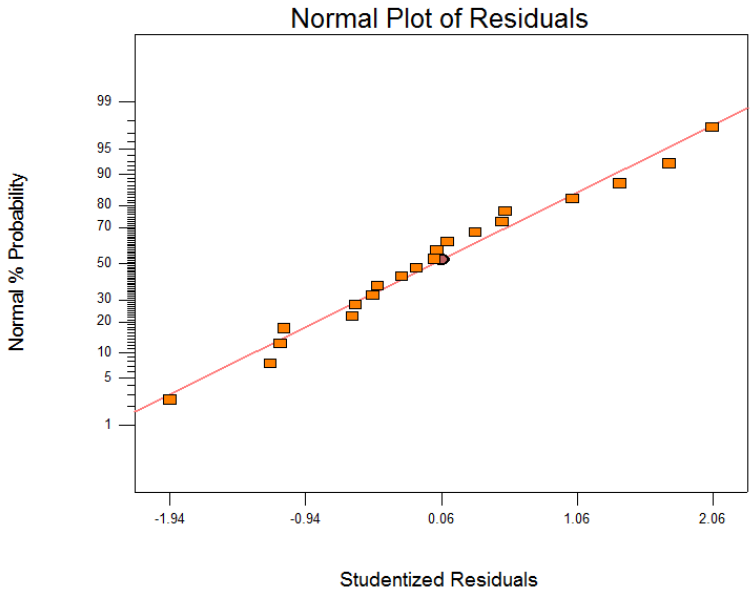


Figure E2.1: Normal probability plot of the residuals for ellagic acid

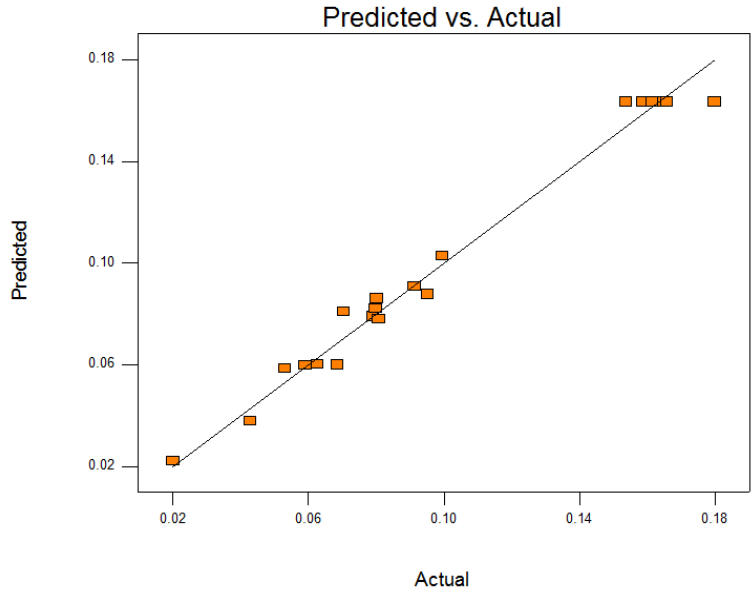


Figure E2.2: Predicted versus actual values for ellagic acid

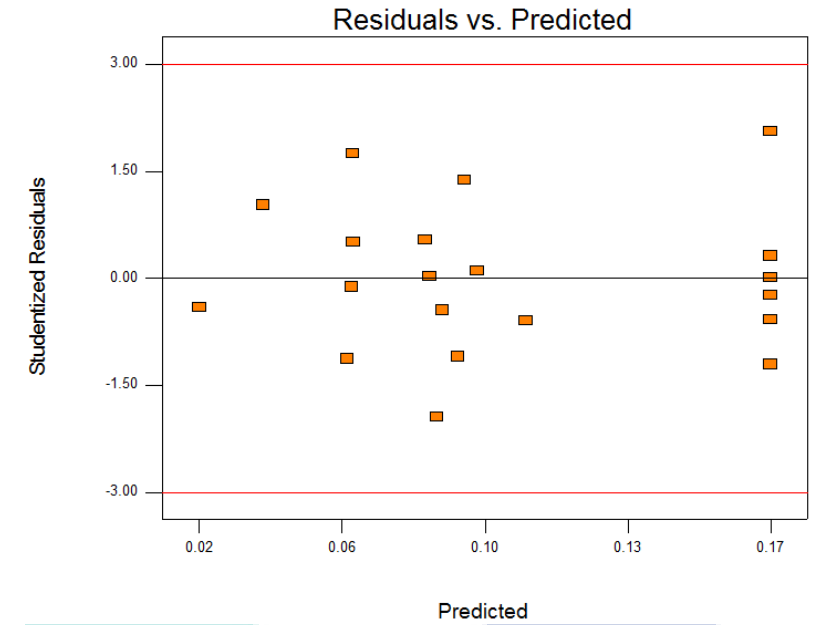


Figure E2.3: Residuals versus predicted values for ellagic acid

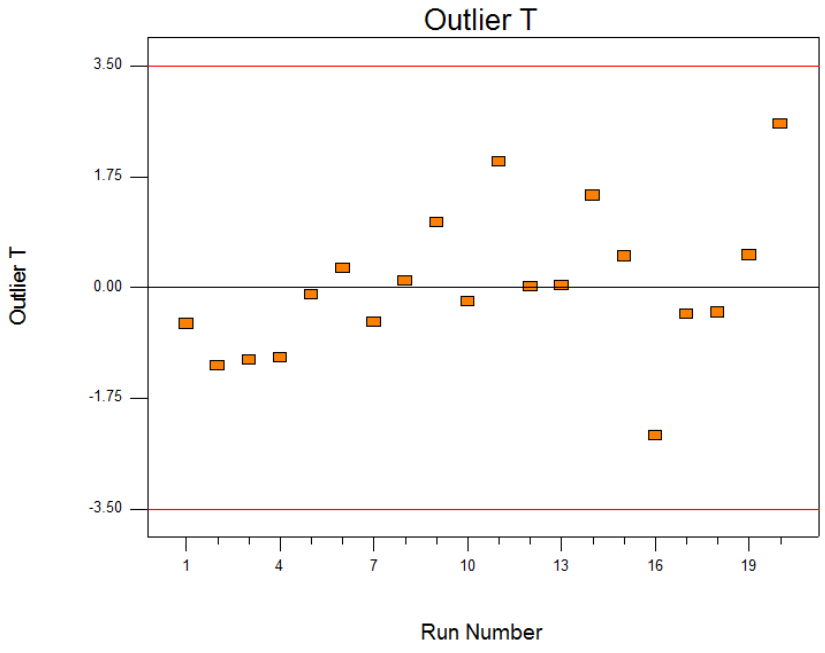


Figure E2.4: Outlier T plot for ellagic acid

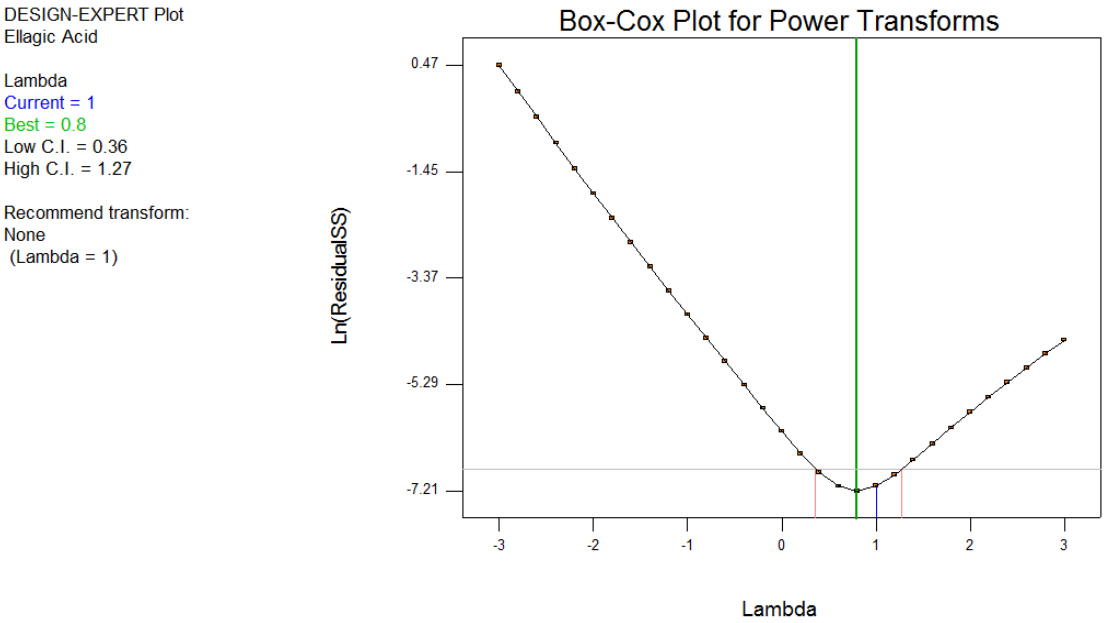


Figure E2.5: Box-Cox plot for Total Phenol

APPENDIX E3
TOTAL PHENOL PLOTS

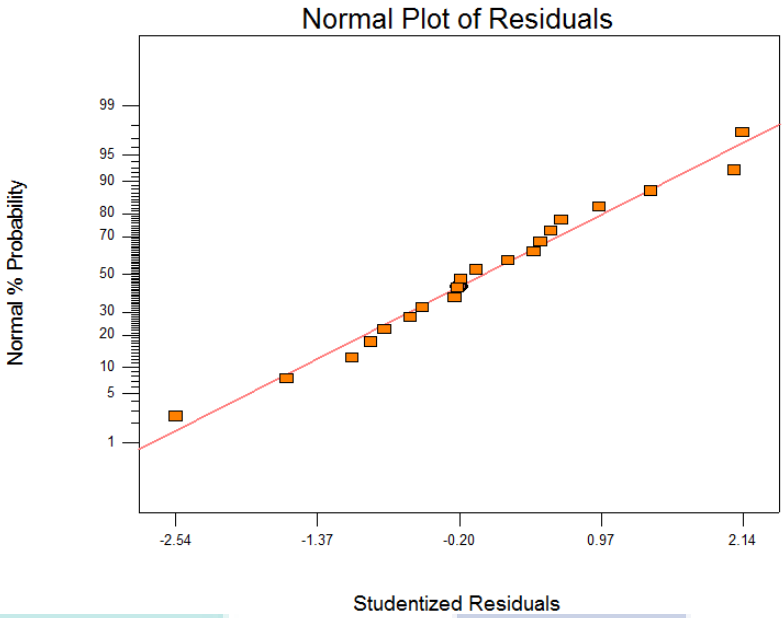


Figure E3.1 : Normal probability plot of the residuals for total phenol

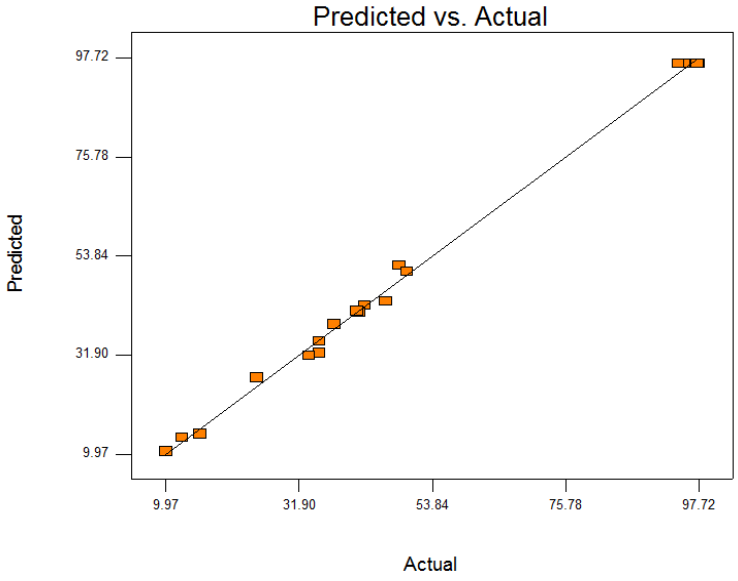


Figure E3.2: Predicted versus actual values for total phenol

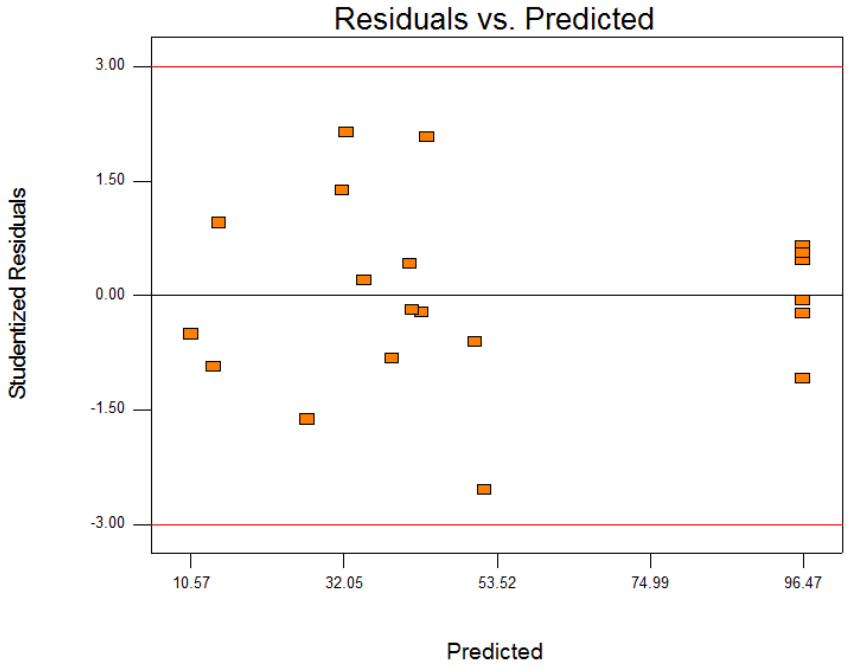


Figure E3.4: Residuals versus predicted values for total phenol

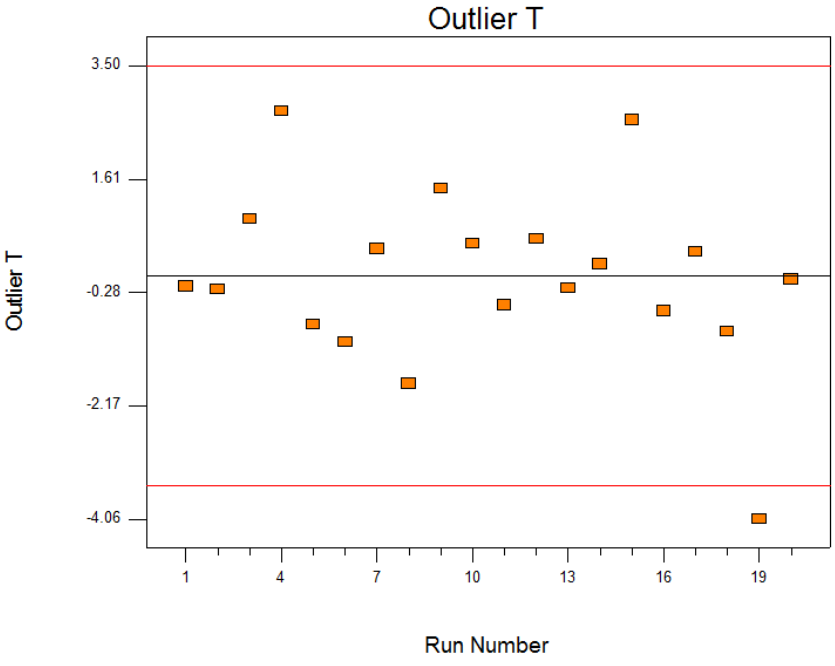


Figure E3.5: Outlier T plot for total phenol

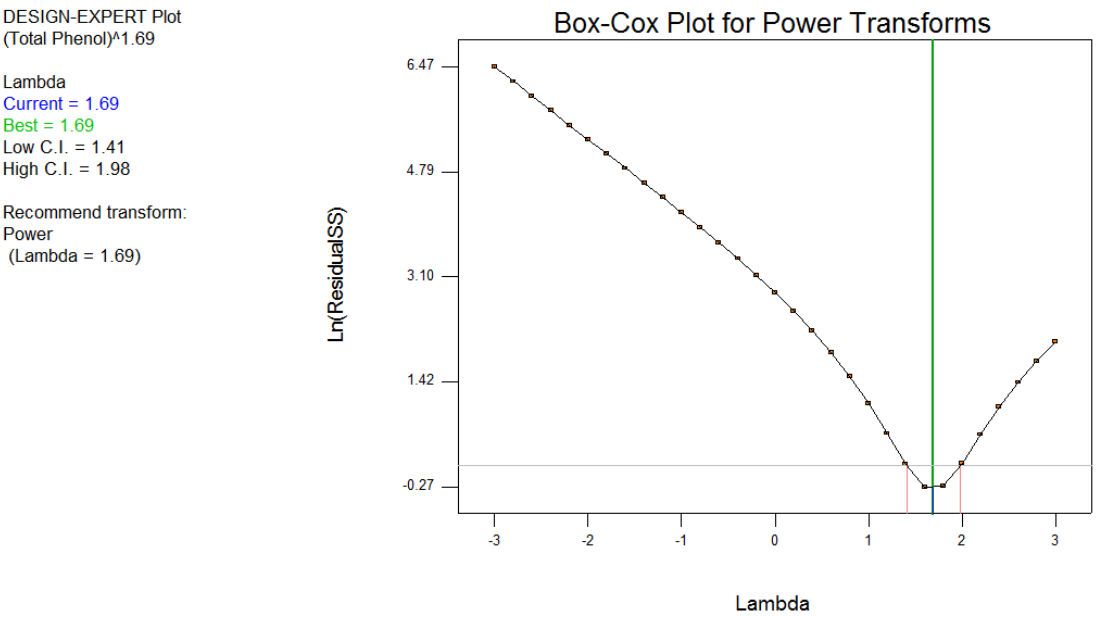
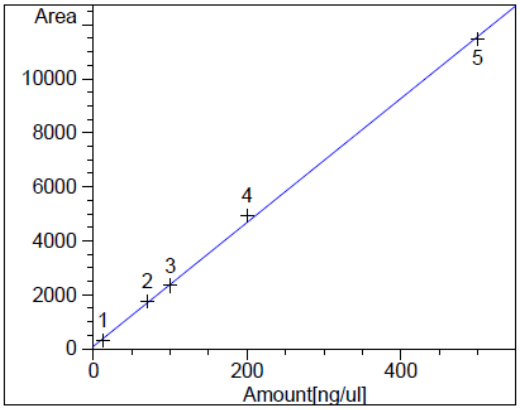


Figure E3.6: Box-Cox plot for Total Phenol

APPENDIX F1

CALIBRATION CURVE FOR GALLIC ACID

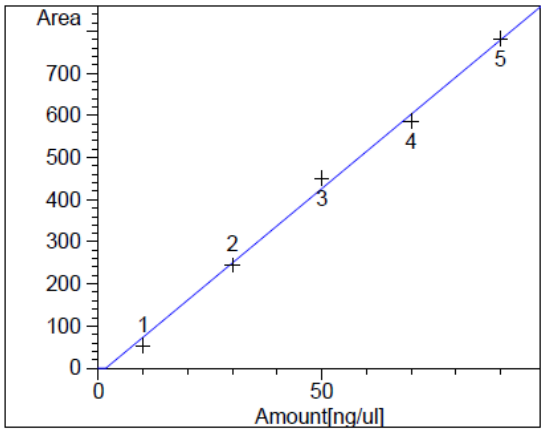



```
Calibration Curves
  at exp. RT: 2.394
DAD1 A, Sig=280,4 Ref=360,100
Correlation:              0.99953
Residual Std. Dev.:     146.60710
Formula: y = mx + b
      m:       22.94495
      b:       92.80953
      x: Amount
      y: Area
```

Figure F.1: Calibration curve for gallic acid

APPENDIX F2

CALIBRATION CURVE FOR ELLAGIC ACID

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Calibration Curves
at exp. RT: 17.931
DAD1 A, Sig=280,4 Ref=360,100
Correlation:            0.99836
Residual Std. Dev.:    19.72847
Formula: y = mx + b
      m:       8.82404
      b:     -14.62655
      x: Amount
      y: Area
```

Figure F.2: Calibration curve for ellagic acid

APPENDIX F3

FOLIN CIOCALTEU GALLIC ACID STANDARD CURVE

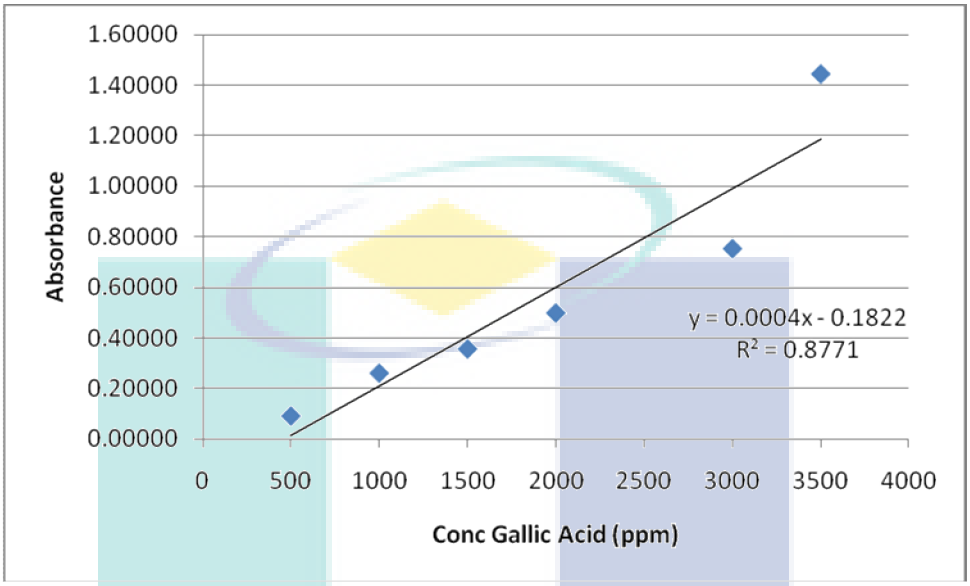

Figure F.3: Folin ciocalteu gallic acid standard curve

APPENDIX G

LIST OF PUBLICATIONS

1. Review: Extraction of Hydrolysable Tannin and Condensed Tannin, Oral presentation - National Conference of Post Graduate Research (NCON-PGR), 1st of October 2009, Universiti Malaysia Pahang, organized by UMP Post Graduate Office and Jabatan Hal Ehwal Akademik dan Antarabangsa (JHEAA).
2. Determination of Gallic Acid and Ellagic Acid in Aqueous Extract from *Melastoma malabathricum* L., Oral presentation – International Conference on Process Engineering and Advanced Materials (ICPEAM), 15th-17th of June 2010, Kuala Lumpur Convention Center, organized by Universiti Teknologi PETRONAS.
3. Effects of Extraction Time and Temperature of Phenols from Aqueous Extract of *Melastoma Malabathricum* L., Accepted in American Journal of Applied Science on 26th of November 2010. ISSN: 1554-3641 (Online).
4. Response Surface Optimization of Phenolic Compounds (Gallic Acid, Ellagic Acid and Total Phenol) From *Melastoma malabathricum* Linn., Submited in Journal of Food Chemistry on 19th of January 2011. ISSN: 0308-8146