ULTRASOUND-ASSISTED EXRACTION OF PHYTOCHEMICALS USING RESPONSE SURFACE METHODOLOGY IN COMMERCIAL *Moringa oleifera Lam* LEAVES

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A dissertation submitted in partial fulfilment of the
requirements for the award of the degree of
Master of Science

Chemistry Department
Faculty of Science
Universiti Teknologi Malaysia

NOVEMBER 2019

DEDICATION

This thesis is dedicated to my father, who taught me that the best kind of knowledge to have is that which is learned for its own sake. It is also dedicated to my mother, who taught me that even the largest task can be accomplished if it is done one step at a time.

ACKNOWLEDGEMENT

Alhamdulillah, praise is to Allah for giving me enough time to complete this dissertation. First and foremost, I would like to express my thanks to God of His love and strength that He has given to me to finish this research. I do thank for His blessings to my daily life, good health, healthy mind and good ideas.

This dissertation is important to fulfil part of the criteria that is a requirement to fulfil the Master of Science (Chemistry) at UTM. I know that there are still many lacks of arranging this research even when doing my lab work and I thank those who have supported me to perform the project up to carry out this dissertation.

Furthermore, I would like to express my gratitude to my supervisor, Dr. Norazah binti Basar and Dr. Susilawati binti Toemen for their excellence in supervision and devotion to inspire during the research. Without their untiring assistance, direction, encouragement, comments, suggestions, continuous guidance, support, ideas and constructive criticism throughout this research, this research could not be well.

Last but not least, I want to give my special thanks to lab assistants because this project will not be accomplished without cooperation and involvement of them and also special thanks to all my friends for sharing their experiences, time and commitment especially during finishing this project. I am grateful because I have a lot of friends were help and support me throughout completing the project.

ABSTRACT

Moringa oleifera Lam is a rich source of phenolic and flavonoid compounds that act as antioxidant, antibacterial and anti-inflammatory agents. *M. oleifera* is one of the species belong to *Moringa* genus from the Moringaceae family. It also well-known as a "pokok kelor". This research was carried out to optimize the ultrasound-assisted extraction (UAE) of the extract from commercial sample of *M. oleifera* leaves by using response surface methodology (RSM). Box-Behnken design (BBD) was used to design the 17 experiments using three variables which were extraction temperature (40, 50, 60°C), time (25, 30, 35 min) and methanol composition as solvent (60, 70, 80%). The effect of parameters on the responses of extraction yield, quantification of quercetin (**2**) and chlorogenic acid (**23**) as well as DPPH (2,2-diphenyl-1-picrylhydrazyl) assay for antioxidant were determined. Quantification of quercetin (**2**) and chlorogenic acid (**23**) in the extracts was done using reversed-phase high-performance liquid chromatography coupled with a diode array detector. It was discovered that both compounds were present in the optimized extracts. The highest percentage of yield (48.99%) and the concentration of chlorogenic acid (**23**) (31.07% w/w) were found at the extraction temperature of 50°C, time of 30 minutes and 70% of methanol. The highest antioxidant activity was also found in this condition with the IC_{50} value of 31.77 μg/ml. However, highest concentration of quercetin (**2**) (5.40% w/w) was discovered when the extraction was carried out at 50°C for 35 minutes and 80% of methanol concentration. The experimental values were closed agreed with the predicted values. The determination coefficient (R^2) of extraction yield, quercetin (**2**), chlorogenic acids (**23**) and DPPH assay were 0.993, 0.9094, 0.9098 and 0.9849, respectively. The analysis of variance (ANOVA) showed a good statistical and model fitting of the quadratic model. The numerical data indicated the success of the RSM method for optimizing the parameter for extraction of phytochemicals from *M. oleifera* leaves. This research recommended that the ultrasound-assisted extraction method under specific parameters has favourable potential to be used in the extraction process. Therefore, this extraction process can improve the effect of efficiency on yield, quantification on phytochemicals and antioxidant activities.

ABSTRAK

Moringa oleifera Lam adalah sumber yang kaya dengan sebatian fenolik dan flavonoid yang berfungsi sebagai antioksidan, sifat antibakteria dan anti-radang. *M. oleifera* adalah salah satu spesies yang tergolong dalam genus *Moringa* daripada keluarga Moringaceae. Ia juga dikenali sebagai pokok kelor. Kajian ini dijalankan untuk mengoptimumkan kaedah pengesktrakan dengan bantuan ultrasonik (UAE) bagi ekstrak daripada sampel komersial daun *M. oleifera* dengan menggunakan metodologi permukaan gerak balas (RSM). Rekabentuk Box-Behnken (BBD) telah digunakan untuk merangka 17 eksperimen dengan tiga pemboleh ubah iaitu suhu pengekstrakan (40, 50, 60°C), masa (25, 30, 35 min) dan komposisi metanol sebagai pelarut (60, 70, 80%). Kesan parameter terhadap tindak balas hasil pengeluaran, kuantitatif dari sebatian fenolik dan flavonoid serta ujian DPPH untuk antioksidan telah ditentukan. Kuantiti quercetin (**2**) dan asid klorogenik (**23**) dalam ekstrak mentah telah ditentukan dengan menggunakan kromatografi cecair prestasi tinggi yang berganding dengan pengesan susunan diod. Kajian mendapati bahawa kedua-dua sebatian ini terkandung dalam ekstrak yang telah dioptimumkan. Peratusan tertinggi hasil (48.99%) dan kepekatan asid klorogenik (**23**) (31.07% dalam % w/w) didapati pada suhu pengekstrakan 50°C, masa 30 minit dan 70% kepekatan metanol. Aktiviti antioksidan tertinggi juga didapati pada keadaan ini dengan nilai IC_{50} 31.77 μg/ml. Walau bagaimanapun, kepekatan quercetin (**2**) (5.40% dalam %w/w) telah dicerap pada suhu pengekstrakan 50°C, masa 35 minit dan 80% kepekatan metanol. Hasil pekali penentuan (R^2) untuk hasil pengeluaran, quercetin (**2**), asid klorogenik (**23**) dan ujian esei radikal DPPH adalah masing-masing, 0.993, 0.9094, 0.9098 dan 0.9849. Analisis varians (ANOVA) ini menunjukkan model statistik yang bagus dan model kuadratik yang bersesuaian. Data kajian ini menunjukkan kaedah RSM berupaya mengoptimumkan parameter bagi pengekstrakan sebatian fitokimia dari daun *M. oleifera*. Kajian ini mencadangkan bahawa kaedah pengekstrakan bantuan ultrasonik pada parameter tertentu mempunyai potensi yang baik untuk digunakan dalam proses pengekstrakan. Oleh itu, proses pengekstrakan ini dapat meningkatkan kesan kecekapan untuk hasil pengeluaran, kuantifikasi sebatian fitokimia dan aktiviti antioksidan.

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LIST OF ABBREVIATIONS AND SYMBOLS

AA	-	Ascorbic acid
AAE	-	Ascorbic acid equivalent
AC	-	Anticancer
ANOVA	-	Analysis variance
APVMA	-	Australian Pesticides and Veterinary Medicines Authority
ABTS	-	2,2'-azinobis(3-ethylbenzothiozoline-6-sulfonic acid)
BBD	-	Box-Behnken design
BDS	-	Base deactived silica
BHA	-	Butylated hydroxyanisole
BHT	-	Butylated hydroxytoluene
C	-	y-intercept
CF_3COOH	-	Trifluoroacetic acid
cm	-	centimeter
CCD	-	Central Composite Design
CE	-	Catechin equivalent
°C	-	Degree celcius
dm	-	Dry mass
DPPH	-	2,2-diphenyl-1-picrylhydrazyl
EDTA	-	Ethylenediamine tetraacetic aicd
EE	-	EDTA equivalent
FRAP	-	Ferric reducing antioxidant power
F-value	-	Fisher value
GAE	-	Gallic acid equivalent
Hz	-	Hertz
IC_{50}	-	Concentration of substrate required to scavenge 50% of inhibition
ICH	-	The International Conference on Harmonisation of Technical Requirements for Pharmaceuticals for

		Human Use
LOD	-	Limit of detection
LOQ	-	Limit of quantification
MAE	-	Microwave assisted extraction
MeOH	-	Methanol
M. oleifera	-	*Moringa oleifera*
NA	-	Not available
NPK	-	Nitrogen, phosphorus and potassium
OFAT	-	One factor at a time
OVAT	-	One variable at a time
PRESS	-	Predicted residual sum of squares
µL	-	Microliter
µm	-	Micrometer
mg	-	Milligram
mL	-	Milliliter
mm	-	Millimeter
min	-	Minutes
nm	-	Nanometer
-OH	-	Hydroxyl
PDA	-	Photodiode array
p-value	-	Probability value
ppm	-	Part per million
RE	-	Rutin equivalent
R^2	-	Correlation coefficient
R^2 adj	-	Correlation coefficient adjusted
RP HPLC-DAD	-	Reversed phase High Performance Liquid Chromatography with diode array detector
RSD	-	Relative standard deviation
RSM	-	Response surface methodology
S	-	slope
SD	-	Standard deviation
SFE	-	Supercritical fluid extraction

SPE	-	Solid phase extraction
TE	-	Trolox equivalent
TF	-	Total flavonoids
TFC	-	Total flavonoid content
TP	-	Total polyphenols
TPC	-	Total phenolic content
TT	-	Total tannins
UAE	-	Ulrasound-assisted extraction
w/w	-	Weight per weight
x	-	Concentration
y	-	Peak area
σ	-	Standard deviation
Σ	-	Standard deviation of response
2D	-	Contour plot
2FI	-	Two factorial interactions
3D	-	Response surface plot

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

In recent times, the market segmentation from different industries can improve their demand owing to growing evidence about the antioxidant property from *Moringa oleifera* also known as 'Pokok kelor'. For centuries, this medicinal plant has been used in traditional medicinal and industrial applications. All part of the *Moringa* tree was useful to human from its leaf, stem, seed and flower of the tree (Moodley *et al.*, 2018). *M. oleifera* was one of the medicinal plants known as a magical plant as it was found that every part of the plant can be used as medicine. This growth can be attributed to increasing the demand for organic products, dietary supplements and cosmetics (Anna *et al.*, 2018).

Antioxidant properties were very important as it could provide protection to humans against infectious and disease-causing by element by remove and stop the free radicals (Coz-bolaños *et al.*, 2018). The potential of antioxidants on this species due to the presence of a wide variety of bioactive compounds can be found in this plant. Two of the most important groups of bioactive compounds are flavonoids and phenolic compounds. Phytochemicals protect the system in the human body against free radicals, increase the immune system and reduce the risk of fatal diseases (Raman *et al.*, 2018). Based on the outstanding advantages from phenolic compounds, therefore it is necessary to discover the phenolic compounds in this plant. The discovery of phenolic compounds is the preliminary stage before further investigation about the standardization procedure will be carried out. Therefore, the specific approach is essential to focus on phenolic compounds by optimization of ultrasound-assisted extraction (UAE) using Response Surface Methodology (RSM).

The first phase involved the separation of phytochemical compounds from the plants was the extraction process. Conventional methods like cold maceration consumed a lot of time and a huge amount of solvents to be used. Consequently, novel methodologies such as supercritical fluid extraction (SFE), microwave-assisted extraction (MAE) and UAE are more economical methods, fewer solvents and a short period of extraction. One of these novel methodologies, UAE has been rapidly and continuous globally to extract bioactive compounds from diverse plant sources. UAE was a fast method for standardization product formulation (Barreiro and Ferreira, 2018). The application of UAE significantly speeded up the extraction of the analyte (nicotine from formulations of pharmaceuticals and plants) (Muhammad *et al.*, 2018). In conventional cold extraction techniques, each extraction step takes up to 24 hours, whereas UAE has taken less than 20 minutes to achieve the same extraction efficiency, and environmental harmful organic solvent consumption is also reduced (Muhammad *et al.*, 2018). It has the frequency in the array from 20 kHz to 2000 kHz (Nn, 2015).

All the medicinal herbs have their exclusive properties of chemical constituents. Therefore, it is significant to choose the ideal extraction conditions. Several parameters of extraction such as extraction time, temperature and solvent ratio to samples affect the productivity of UAE. Response surface methodology (RSM) is a plan of mathematical as well as statistical analysis that designs with tentative experimental, statistical modelling and optimization of operating parameters (Sin *et al., 2*014). It used to study the quantitative relation among one or more factors that describing the best condition for a desirable response (Vivek *et al.,* 2016). RSM is broadly used as an optimization procedure for the extraction of bioactive compounds from plants (Pambi and Musonge, 2016). In this study, the goal of optimization of the UAE on phytochemical compounds by RSM is to get the optimum operating parameters. These parameters were extraction time, temperature and methanol concentration. Therefore, this UAE optimization of phytochemicals from *M. oleifera* leaves able to determine the efficient extraction of antioxidant properties and the phytochemical profile of the plant extracts.

1.2 Problem Statement

In general, *M. oleifera* has been exploited rapidly in the pharmaceutical industries. However, the low yield of cold maceration technique relatively critical among huge industrial groups. Extraction yields can be significantly improved and the extraction time can be reduced significantly by multiple methods to disrupt cell walls and membranes (Selvamuthukumaran and Shi, 2017). To overcome this circumstance, MAE and UAE can be applied and the results from this extraction verified its efficiency. The problem posed by what the best operating parameters of extraction in terms of extraction time, temperature and solvent concentration. Therefore, observation of extraction on the key parameters is vital to be examined for retaining the maximum yield of extraction at the optimum condition.

Moreover, the increasing demands of natural antioxidant over synthetic antioxidant force researchers to modify the procedure of extraction. This is due to extraction procedure is very significant for the production of natural antioxidant. Therefore, ultrasound-assisted extraction (UAE) has been utilized to extract the phytochemical compounds from natural products. One of the reasons that make UAE so valuable is the ability to decrease the solvent consumption compare to other conventional methods such as soxhlet and maceration. On the other hand, the process endorses for the solvent to be penetrated in the sample by breaking the cell walls undergoes acoustic cavitation (Lemos *et al.*, 2012). In ancient times, the conventional techniques cause the reason loss of nutrient in the extracts and make the process more economical. As a consequence, the conventional methodologies were accredited to have various limitations in terms of solvent consumption, time of reaction and cost. Hence, the UAE approach has focused on several parameters used to optimize the extraction analyzed by response surface methodology (RSM). Therefore, the goal of this study is to examine the prospect of UAE from *M. oleifera* leaves under several parameters of extraction such as time, temperature and methanol concentration. The results of this current works may be helpful further the investigation and then consume the *M. oleifera* leaves as the precious source towards pharmaceuticals application such as cosmetics, dietary supplement and organic products.

1.3 Objectives of Study

In order to realize the goals of this study, these objectives are identified:

(a) To optimize the extraction parameters on ultrasound-assisted extraction (UAE) of the extract from commercial sample of *M. oleifera* leaves by using response surface methodology (RSM).

(b) To investigate the antioxidant property of the extract from commercial sample of *M. oleifera* leaves by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay.

(c) To quantify the presence of quercetin (**2**) and chlorogenic acid (**23**) of the extract from commercial sample of *M. oleifera* leaves by RP HPLC-DAD.

1.4 Scope of Study

This study was directed to find the optimum extraction condition of the phytochemicals from *M. oleifera* leaves by using RSM via Box-Behnken design (BBD) and verified using ANOVA analysis. The monitored parameters were temperature, reaction time and methanol concentration. The antioxidant property from the leaf extracts of *M. oleifera* was determined by using the DPPH scavenging assay. The quantification of quercetin (**2**) and chlorogenic acid (**23**) on extracts was carried out by RP-HPLC with a Diode Array Detector (DAD). The optimization of UAE from *M. oleifera* leaves were responses on the percentage of yields, quantification of quercetin (**2**) and chlorogenic acid (**23**) as well as antioxidant activity.

1.5 Significance of Study

This study provides a simple method about UAE optimization for the extraction of phytochemical compounds using the leaf extract of *M. oleifera.* By using this optimization, it is very helpful to satisfy the demand for the better quality of final products. Furthermore, UAE optimization of the extraction of phytochemical compounds from *M. oleifera* extract using RSM is extensively favored. Besides that, this study will be able to get the optimum results on the percentage of yields, antioxidant properties and phytochemical compounds from *M. oleifera* leaves.

Optimization UAE can reduce less consumption of chemicals over some of the conventional methods. The ideas of the optimization of the extraction process are important given the statement that the growth of this technology can be applied worldwide using any type of plant that has antioxidant potential to act as a reducing agent. For instance, the commercial supplement based on the antioxidant could be done on a large scale of production with an optimum method of extraction.

The optimization of the extraction method enhances the desired outcome for the quality of the final product in terms of percentage of yield, texture and preserves the amount of nutrient content in the plants. Owing to the standardization of extraction able to apply, this cause boosts the production supplement of food from natural resources. Other than, increasing demand from pharmaceutical industrials such as cosmetic and medicines can speed up the production using the standard operating procedure of extraction.

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