ISOLATION AND QUANTIFICATION OF FLAVONOIDS FROM LEAVES OF *Moringa oleifera Lam* AND ITS ANTIOXIDANT ACTIVITY

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Special dedication to

My parents,

Mohd Rozi Bin. Hj. Ab. Malek

Andek Siti Aichah Binti Hj. Yakkob

My brother, Ahmad Shahir Bin Mohd Rozi

For all your patience and prayers.

Thank you.

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ABSTRACT

Moringa oleifera Lam. (*M. oleifera*) which is also called "*pokok kelor*" is known to be a rich source of flavonoids. Three flavonoids known as isoquercetin, quercetin and kaempferol were isolated from the ethanolic extract of *M. oleifera* leaves. The compounds were elucidated using spectroscopic methods such as ^{1}H NMR, ^{13}C NMR and FTIR. In a separate study, the different extraction methods of ethanolic extract including cold maceration, soxhlet, ultrasound-assisted by water bath and ultrasound-assisted by probe and solid phase extraction were done. The extracts were subjected to qualitative and quantitative analysis to determine the phytochemicals present in *M. oleifera* leaf. Qualitative analysis on the extracts showed that *M. oleifera* contains flavonoid, tannin, alkaloid, phenol, steroid, quinone and coumarin. The result of quantitative analysis was obtained by screening the ethanolic extract for antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl assay, 2,2'azino bis(3-ethylbenzothiozoline-6-sulfonic acid) and ferric reducing antioxidant power assay. Extract from Fraction 2 of solid phase exhibited highest antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl, 2,2'-azinobis(3-ethylbenzothiozoline-6-sulfonic acid) and ferric reducing antioxidant power assay with IC_{50} of 35.81 µg/mL, IC_{50} of 41.58 µg/mL and FRAP equivalent of 91.36 mM, respectively. Moreover, the extracts were further analysed using a reversed phase high performance liquid chromatography to quantify the contents of isoquercetin, quercetin and kaempferol. Quantification of isoquercetin, quercetin and kaempferol were found higher using solid phase extraction with 7.98%, 0.86% and 1.11% in w/w%, respectively. The validated HPLC method was effective and practical for quantification of isoquercetin, quercetin and kaempferol in *M. oleifera* leaf extracts.

ABSTRAK

Moringa oleifera Lam. (*M. oleifera*) turut dikenali sebagai "*pokok kelor*" terkenal dengan kekayaan sumber flavonoid. Tiga flavonoid iaitu isoquercetin, quercertin dan kaempferol telah diasingkan daripada ekstrak etanol daun *M. oleifera*. Semua sebatian telah dianalisis menggunakan kaedah spektroskopi seperti ^{1}H RMN, ^{13}C RMN dan FTIR. Dalam kajian berasingan, kaedah pengekstrakan etanol yang berbeza termasuk rendaman, soxhlet, pengekstrakan berbantukan ultrabunyi oleh rendaman air, pengekstrakan berbantukan ultrabunyi oleh *probe* dan pengekstrakan fasa pepejal telah dilakukan. Analisis kualitatif dan kuantitatif telah dijalankan ke atas ekstrak bagi menentukan fitokimia yang terdapat dalam daun *M. oleifera*. Analisis kualitatif ke atas ekstrak menunjukkan bahawa *M. oleifera* mengandungi flavonoid, tanin, alkaloid, fenol, steroid, kuinon dan kumarin. Hasil analisis kuantitatif diperoleh melalui penyaringan ekstrak etanol untuk aktiviti antioksidan menggunakan ujian 2,2-difenil-1-pikrilhidrazil, asid 2,2'-azinobis(3-etilbenzotiozolin-6-sulfonik) dan potensi antioksidan penurunan ferik. Ekstrak daripada Fraksi 2 pengekstrakan fasa pepejal menunjukkan aktiviti antioksidan tertinggi melalui ujian 2,2-difenil-1-pikrilhidrazil, asid 2,2'-azinobis(3-etilbenzotiozolin-6-sulfonik) dan potensi antioksidan penurunan ferik dengan nilai setiap satu IC_{50} 35.81 µg/mL, IC_{50} 41.58 µg/mL dan nilai FRAP 91.36 mM. Selain itu, ekstrak telah dianalisis dengan menggunakan kromatografi cecair prestatsi tinggi fasa terbalik untuk mengukur kandungan isoquercetin, quercetin dan kaempferol. Kuantiti isoquercetin, quercetin dan kaempferol didapati lebih tinggi menggunakan kaedah pengekstrakan fasa pepejal dengan nilai 7.98%, 0.86% dan 1.11% dalam w/w%. Pengesahan kaedah HPLC adalah berkesan dan praktikal untuk pengukuran kandungan isoquercetin, quercetin dan kaempferol dalam ekstrak daun *M. oleifera*.

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LIST OF ABBREVIATIONS/SYMBOLS

AA	-	Ascorbic acid
ABTS	-	2,2'-azinobis(3-ethylbenzothiozoline-6-sulfonic acid)
ASE	-	Accelerated solvent extraction
c	-	y-intercept
°C	-	Degree celcius
^{13}C NMR	-	Carbon Nuclear Magnetic Resonance
cm^{-1}	-	Per centimeter
δ	-	Chemical shift
d	-	Doublet
dd	-	Doublet of doublet
DPPH	-	2,2-diphenyl-1-picrylhydrazyl
EBV-EA	-	Epstein-Barr Virus
FRAP	-	Ferric reducing antioxidant power
FTIR	-	Fourier Transform Infrared Spectroscopy
g	-	Gram
^{1}H NMR	-	Proton Nuclear Magnetic Resonance
H^-	-	Hydride
H_2SO_4	-	Sulphuric acid
HCl	-	Hydrochloric acid
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
J	-	Coupling constant

kHz	-	Kilo Hertz
L	-	Liter
Lit	-	Literature
LOD	-	Limit of detection
LOQ	-	Limit of quantification
MAC	-	Maceration
MAE	-	Microwave assisted extraction
MeOH	-	Methanol
Methanol-d_4	-	Deuterated methanol
MHz	-	Mega Hertz
µg	-	Microgram
µL	-	Microliter
µm	-	Micrometer
mg	-	Milligram
mL	-	Milliliter
mm	-	Millimeter
mM	-	Millimolar
mins	-	Minutes
m	-	Multiplet
NaOH	-	Sodium hydroxide
nm	-	Nanometer
-OH	-	Hydroxyl
PLE	-	Pressurized liquid extraction
ppm	-	Part per million
R^2	-	Correlation coefficient
R_f	-	Retention factor
RP HPLC-DAD	-	Reversed phase High Performance Liquid Chromatography with diode array detector
RSD	-	Relative standard deviation
RSM	-	Response surface methodology
SD	-	Standard deviation
Sephadex LH-20	-	Silica gel

SFE	-	Supercritical fluid extraction
σ	-	Standard deviation of response
SOXH	-	Soxhlet
SPE	-	Solid phase extraction
t	-	Triplet
TFA	-	Trifluoroacetic acid
TLC	-	Thin layer chromatography
TPTZ	-	2,4,6-Tri(2-pyridyl)-s-triazine
UAE	-	Ulrasound-assisted extraction
v/v	-	Volume per volume
w/w	-	Weight per weight
w/v	-	Weight per volume
x	-	Concentration
y	-	Peak area

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CHAPTER 1

INTRODUCTION

1.1 Background of study

Modern chemistry has opened a new era of the uses of natural products. In conjunction with the plentiful amount of biologically active compound found for therapeutic uses, Malaysia that prolific in plant diversity has been enrolled actively in the correlative research project. Therefore, herbal plants are still trustworthy as one of alternative way in medicinal field [1]. One of the notable medicinal plant is *Moringa oleifera Lam*. The *M. oleifera* is widely known as '*pokok kelor*' in Malaysia belonging to the *Moringaceae* family.

The *M. oleifera* is proved their medicinal properties or traditional uses in human life. Every part of the plant had its own beneficial uses. It can be used as a cure for malnutrition and constipation. The uses of *M. oleifera* not only limited in medicinal field since it also can be used in purification of water [2]. Of all parts of the plant, the leaf of *M. oleifera* has been subject of extensive chemical investigation due to its high benefit values. The flavonoid of *M. oleifera* present remarkable medicinal properties and related with various biological activities.

There are several method can be employed to extract crude from herbs and plants such as percolation, soxhlet extraction, cold maceration and others. However, the current focus of this study is on cold maceration, soxhlet extraction, ultrasound-assisted extraction (by probe and water bath) and solid phase extraction. The work flows of this study involved sample collecting, extraction, isolation, purification, bioactivities and analytical study.

In this study, different extraction method becomes the parameter in optimization of highest flavonoid contents. Phytochemical screening, antioxidant activity and quantification by RP HPLC-DAD were pursued to analyse the flavonoids efficiently. Qualitative analysis of chemical constituents were identified through phytochemical preliminary. The most potent antioxidant activity was determined through antioxidant assay. In order to enhance the optimization, RP HPLC-DAD was carried out to identify more accurately the flavonoid compositions in each of extract quantitatively.

1.2 Problem statement

Nowadays, the concern of people in order to achieve healthy lifestyle increased the demand of plants and herbs rapidly. Malaysia is recognised as one of 12 mega diverse countries around the world that rich in biological resources especially with medicinal plants and herbs. *M. oleifera* is listed among the Malaysian Herbal in National Key Economic Area (NKEA) [3]. Furthermore, this proposed research would produce a scientific analysis which will synchronize with government initiatives in developing herbal industry towards producing surpassing herbal products via amelioration of science and technology.

The extract from *M. oleifera* may contribute to medicinal, skincare uses and Malaysian economy. However, *M. oleifera (pokok kelor)* is one of medicinal plant that

do not fully utilize and explore in Malaysia. This is the reason in choosing *M. oleifera* as our interest in this study. The problem involved in this study was related with the extraction method which is emphasized on cold maceration, soxhlet extraction, ultrasound-assisted extraction (by probe and water bath) and solid phase extraction.

Some researcher stated that the cold maceration and soxhlet extraction gave low percentage yield of extract as compared with solid phase and ultrasound-assisted extraction method. Extract of soxhlet and solid phase extraction gave low antioxidant activities as compared with extract of cold maceration [4]. This study will provide a results on extract that having a high percentage yield, flavonoid compositions and antioxidant activities level.

1.3 Objectives

The purposes of this study were stated as below:

1. To extract the *M. oleifera* leaf using different extraction methods.
2. To identify the presence of constituents through phytochemical screening and evaluate the extracts for antioxidant activities.
3. To isolate and elucidate flavonoids of *M. oleifera* leaf by column chromatography.
4. To quantify the presence of flavonoids in *M. oleifera* extracts by RP HPLC-DAD.

1.4 Scope of study

This study focused on the optimization, quantification of phytochemical constituents, antioxidant activities and isolation of *M. oleifera* using different

extraction methods. Optimization was done using different methods such as cold maceration, soxhlet extraction, solid phase extraction and ultrasound-assisted (by water bath and probe).

The identification of flavonoids and other phytochemical constituents were obtained from phytochemical screening. The antioxidant activities of each extracts of *M. oleifera* were determined and studied. The study on DPPH scavenging assay, ABTS and FRAP of the extracts were carried out to determine the highest antioxidant activities level.

The leaf of *M. oleifera* was extracted by using cold maceration and followed by liquid-liquid partition. The flavonoids of *M. oleifera* were isolated through column chromatography. Characterization of the compounds were done by FTIR, ^{1}H NMR and ^{13}C NMR. Quantification of flavonoids on extracts were done by RP HPLC-DAD.

1.5 Significance of study

Application of various type of extraction methods which are applied in this study will assist to identify the highest antioxidant activity and flavonoid of extracts. The output from this study could be used as a guidance for further optimization and application of *M. oleifera* leaf in nutraceutical, pharmaceutical and cosmetic production. Other than that, this study will contribute in enhancing efforts of government in elevating herbal industries.

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