CHEMICAL CHARACTERIZATION AND BIOLOGICAL STUDIES OF NEEM (*Azadirachta indica*) EXTRACTS

JESSINTA A/P SANDANASAMY

MASTER OF SCIENCE (INDUSTRIAL CHEMISTRY)
UNIVERSITI MALAYSIA PAHANG

UNIVERSITI MALAYSIA PAHANG

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JESSINTA A/P SANDANASAMY

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SUPERVISOR'S DECLARATION

We hereby declare that we have checked this thesis and in our opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Master of Science in Industrial Chemistry.

Signature

Name of Supervisor : DR. AZHARI HAMID NOUR BIN ABDUELRAHMAN

Position : SENIOR LECTURER
FACULTY OF INDUSTRIAL SCIENCES & TECHNOLOGY, UNIVERSITI MALAYSIA PAHANG

Date : 28 NOVEMBER 2014

Signature

Name of Co-supervisor : ASSOC. PROF. DR. SAIFUL NIZAM BIN TAJUDDIN

Position : ASSOCIATE PROFESSOR
FACULTY OF INDUSTRIAL SCIENCES & TECHNOLOGY, UNIVERSITI MALAYSIA PAHANG

Date : 28 NOVEMBER 2014

STUDENT'S DECLARATION

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Signature

Name : JESSINTA A/P SANDANASAMY

ID Number : MKD 12004

Date : 28 NOVEMBER 2014

Special dedication of this grateful feeling to my beloved parents;

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ABSTRACT

Neem (*Azadirachta indica*) (Family: *Meliaceae*), also known as 'Pokok Mambu' in Malaysia is widely known to contain variety of bioactive compounds that had been proven for the cure of various infections and diseases related to toxicity and bacteria. The extractions of the plant constituents are mainly dependent on the extraction methods, conditions and solvents. This study aims on the extraction of the chemical constituents' and identification of the volatile constituents of *A. indica* extracts. The cytotoxicity effect was studied for the extracts that lead towards the isolation of a bioactive compound. The oil of the plant was studied for its physicochemical properties and antibacterial activity. The crude extracts (barks, leaves and roots) were extracted via solvent extraction (acetone, chloroform, maceration and refluxed in ethanol), while the fractions (hexane, chloroform, ethyl acetate and aqueous) were produced from partitioning of 80% methanol crude. Seed oil was extracted via Soxhlet with hexane for six hours. Volatile compound analysis via GC-MS was performed for all crude and fractions and cytotoxicity test against brine shrimp, *Artemia salina* for determination of LC_{50} after 24 h. The isolation and identification of bioactive compound from the most active fraction of cytotoxicity were performed via preparative-HPLC, UV-Vis, IR, MS and NMR. Physicochemical properties of oil were studied according to standard methods. Antibacterial activity of oil was determined against *B. subtilis*, *E. coli* and *S. aureus* via well diffusion method. Yields of the extracts were varied among different parts due to polarity of solvents and extraction conditions. Highest and lowest yields of crude extracts were leaf and bark reflux in ethanol with 5.46% and 0.13%, respectively. Minimum and maximum yield was obtained for fractions by root ethyl acetate (0.06%) and leaf chloroform (1.15%). The n-hexadecanoic acid was detected in all samples with seven similar compounds in both crude and fractions as the most abundant volatile compound. Cytotoxicity proves that root acetone (457.09 ± 0.88 ppm) and leaf ethyl acetate extract (1.35 ± 0.40 ppm) are the most toxic. All the fractions and only root acetone crude extract falls under toxic level (LC_{50} values < 500 ppm). The partitioning to different fractions separates the complex plant constituents according to polarity that influences the cytotoxicity. The isolation of quercetin-3-*O*-β-D-glucopyranoside from leaf ethyl acetate proves the cytotoxic effect. Major characteristics of the oils are; density: 0.95 g/cm^3; refractive index: 70.90; acid value: 4.80 mg KOH/g; free fatty acid (oleic): 4.75 %; iodine value: 93.09 gI_2/100g; pH: 4; peroxide value: 8.49 meq O_2/kg; moisture & volatile matter: 0.83 % and unsaponifiable matter: 1.84 %. The major fatty acid chains in the oil are; linoleic-: 34.69 %; oleic-: 20.46 %, stearic-: 20.42 % and palmitic acid: 18.66 %. Inhibition zone for antibacterial study with 20, 40, 60 and 80% of oil lies between 1.23 ± 0.03 to 1.70 cm, 1.33 ± 0.06 to 1.6 ± 1.57x10^{-16} cm and 1.4 ± 0.03 to 1.63 ± 0.03 cm for *B. subtilis*, *S. aureus* and *E. coli*, respectively. The MIC was 0.63± 0.0002, 2.50± 0.0010 and 5.00± 0.006% for *B. subtilis*, *E. coli* and *S. aureus*, respectively and *S. aureus* is more resistant. Results obtained supports that *A. indica* plant has high advantage to be used as drug in chemical and pharmaceutical industries. The study should be further continued through direct study with insect and human cell line to confirm the effect of the drug.

ABSTRAK

Neem (*Azadirachta indica*) (Keluarga: *Meliaceae*), dikenali sebagai Pokok Mambu di Malaysia mengandungi pelbagai juzuk kimia yang terbukti berkesan untuk mengubati pelbagai jenis penyakit berhubung dengan ketoksikan dan bakteria. Pengekstrakan juzuk kimia daripada tumbuhan bergantung pada cara dan situasi pengekestrakan serta pelarut kimia. Kajian ini bertujuan untuk mengekstrak juzuk kimia dan pengenalpastian juzuk kimia yang senang meruap daripada ekstrak *A. indica.* Fitokimia yang boleh meruap ditentukan dalam ekstrak pecahan kulit kayu, daun dan akar dan pengajian sitotoksiti menyumbang kepada pengasingan juzuk aktif. Ciri fisikokimia minyak dikaji mengikut kaedah piawai. Aktiviti anti-bakteria minyak ditentukan terhadap *B. subtilis, E. coli* dan *S. aureus* melalui kaedah penyebaran. Ekstrak mentah (kulit kayu, daun dan akar) diekstrak melalui pengekstrakan pelarut (aseton, kloroform, rendaman pada suhu bilik dan refluks dalam etanol), manakala ekstrak pecahan (heksana, kloroform, etil asetat dan air) dihasilkan daripada pembahagian sebanyak 80% ekstrak metanol mentah. Minyak biji diekstrak melalui soxhlet dengan heksana selama enam jam. Analisis sebatian meruap melalui GC-MS telah dilaksanakan ke atas semua ekstrak mentah dan pecahan dan ujian sitotoksiti terhadap udang air garam, *Artemia salina* melalui penentuan LC_{50} selepas 24 jam. Pengasingan dan pengenalpastian sebatian bioaktif daripada pecahan yang paling aktif sitotoksiti telah dijalankan melalui HPLC, UV-Vis, IR, MS dan NMR. Sifat-sifat fiziko-kimia dikaji mengikut kaedah piawai dan penilaian aktiviti anti-bakteria daripada minyak biji ke atas *B. subtilis, E. coli* and *S. aureus* dengan kaedah penyebaran. Hasil ekstrak berubah dalam kalangan bahagian yang berbeza kerana kekutuban pelarut dan syarat pengekstrakan. Hasil tertinggi dan terendah ekstrak mentah diperolehi daripada daun dan kulit kayu pengrefluksan etanol dengan 5.46% dan 0.13% masing-masing. Hasil minimum dan maksimum telah diperolehi bagi pecahan akar etil asetat (0.06%) dan daun kloroform (1.15%). Asid n-hexadecanoic dikesan dalam semua sampel dengan tujuh sebatian yang sama dalam kedua-dua mentah dan pecahan. Sitotoksiti membuktikan bahawa ekstrak aseton akar (457.09 ± 0.88 ppm) dan etil asetat daun (1.35 ± 0.40 ppm) adalah yang paling toksik. Semua pecahan dan hanya akar aseton ekstrak mentah dikategorikan dalam paras toksik (nilai LC_{50} <500 ppm). Pembahagian untuk pecahan yang berbeza memisahkan juzuk tumbuhan kompleks mengikut kekutuban yang mempengaruhi sitotoksiti. Pengasingan quercetin 3-*O*-β-D-glucopiranosida dari pecahan asetat etil daun menyokong data sitotoksiti. Ciri-ciri utama minyak adalah; ketumpatan: 0.95 g/cm^3; indeks biasan: 70.90; nilai asid: 4.80 mgKOH/g; asid lemak bebas (oleik): 4.75%; nilai iodin: 93.09 gI_2/100g; pH: 4; nilai peroksida: 8.49 meqO_2/kg; kelembapan & bahan meruap: 0.83% dan bahan tidak larut: 1.84 %. Rantai asid lemak utama dalam minyak ini adalah; asid linoleic: 34.69%; oleic: 20.46%, stearik: 20.42% dan palmitik: 18.66%. Zon perencatan untuk kajian antibakteria dengan 20, 40, 60 dan 80% daripada minyak terletak di antara 1.23 ± 0.03-1.70 cm, 1.33 ± 0.06-1.6 ± 1.57x10^{-16} cm dan 1.4 ± 0.03-1.63 ± 0.03 cm bagi *B. subtilis, S. aureus* dan *E. coli* masing-masing. MIC adalah 0.63 ± 0.0002, 2.50 ± 0.0010 dan 5.00 ± 0.006% bagi *B. subtilis, E. coli* dan *S. aureus* masing-masing dan *S. aureus* lebih menahan impak ekstrak. Keputusan yang diperolehi menyokong bahawa *A. indica* mempunyai kelebihan yang tinggi untuk digunakan sebagai dadah dalam industri kimia dan farmaseutikal. Kajian perlu diteruskan secara langsung dengan sel serangga dan manusia untuk mengesahkan kesan dadah.

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LIST OF SYMBOLS

-	Subtract
%	Percentage
~	Tilde
+	Add
<	Less-than
=	Equals
>	Greater-than
±	Plus-minus
≤	Less than or equal to
≥	More than or equal to
°C	Degree celsius
°C/min	Degree celsius per minute
µg/mL	Micrograms per milliliter
µL/mL	Microliter per milliliter
µm	Micrometer
a	Statistical alpha
bar	Unit of pressure
CFU/mL	Colony forming unit per mililiter
cm	Centimeter
eV	Electron volt
g	Gram
g/cm^3	Gram per cubic centimeter
g/g	Gram per gram

g/kg	Gram per kilogram
g/L	Gram per liter
g/mol	Gram per mol
GHz	Gigahertz
h	Hour
Hz	Hertz
KHz	Kilohertz
KV	Kilovolt
L	Liter
lbs	Pounds
Lx	Lux
m	Meter
M	Molarity
m/z	Mass-to-charge ratio
meq/kg	Milliequivalents per kilogram
mg	Miligram
mg/g	Miligram per gram
mg/kg	Miligram per kilogram
mg/mL	Miligram per mililiter
MHz	Megahertz
min	Minutes
mL	Mililiter
mL/kg	Mililiter per kilogram
mL/min	Mililiter per minute
mm	Milimeter

mTorr	Militorr
N	Normality
nm	Nanometer
ppm	Parts per million
ppt	Parts per trillion
psi	Pound-forceper square inch
s	Seconds
Tcm^{-2}	Torr per cubic centimeter
V	Volt
v/v	Volume per volume
W	Watt
w/v	Mass per volume ratio
wt%	Weight percentage
x	Multiple
δ	Delta
λ	Gamma
λ_{max}	Lamda maximum
µL	Microliter
π	Pi
π*	Pi star
cm^{-1}	Reciprocal centimeter

LIST OF ABBREVATIONS

^{13}C-NMR	Carbon Nuclear Magnetic Resonance
1D-NMR	One dimensional Nuclear Magnetic Resonance
^{1}H-NMR	Proton Nuclear Magnetic Resonance
2D-NMR	Two dimensional Nuclear Magnetic Resonance
A. indica	*Azadirachta indica*
A. salina	*Artemia salina*
AOAC	Association of Analytical Chemists
AOCS	American Oil Chemists' Society
AED	Atom emission detector
AIDS	Acquired immunodeficiency syndrome
ANOVA	One way analysis of variance
ATCC	American type culture collection
B. subtilis	*Bacillus subtilis*
CO_2	Carbon dioxide
COSY	Correlated spectroscopy
DAD	Diode array detector
DEPT	Distortionless enhancement by polarization transfer
DMAE	Diffused microwave multi-mode cavity system
DMSO	Dimethyl sulfoxide
E. coli	*Escherichia coli*
ECD	Electron capture detector
EI-MS	Electron Ionization-Mass Spectrometry
ELSD	Evaporate light scanning detector

FFA	Free fatty acid
FID	Flame ionization detector
FMAE	Focused microwave single-mode cavity system
FTIR	Fourier Transform Infrared
FTIRD	Fourier Transformed Infrared Detector
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectroscopy
H	Hydrogen
HETCOR	Heteronuclear correlation spectroscopy
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum correlation
HPLC	High Performance Liquid Chromatography
HUS	Hemolytic uremic syndrome
IR	Infrared
LC	Liquid chromatography
LD_{50}	Median lethal dose
LSD	Least significant difference
MAE	Microwave-assisted extraction
MH	Muller-Hinton
MIC	Minimum inhibitory concentration
MS	Mass Spectrometry
MSD	Mass selective detector
N	Nitrogen
NA	Nutrient agar
NIST	National Institute of Standards and Technology

NMR	Nuclear Magnetic Resonance
NPD	Nitrogen phosphorus detector
O	Oxygen
PV	Peroxide value
S	Sulfur
S. aureus	*Staphylococcus aureus*
SFE	Supercritical fluid extraction
Soxhlet	Solvent semi-continuous extraction
SPSS	Statistical Package for the Social Sciences
TCD	Thermal conductivity detector
TLC	Thin Layer Chromatography
TSA	Trypticase soy agar
TSP	Thermal separation probe
UNICEF	United Nations Children's Fund
USA	United States of America
USDA	United States Department of Agriculture
UV	Ultraviolet Spectroscopy
UV-A	Ultraviolet A
UV-B	Ultraviolet B
UV-C	Ultraviolet C
UV-Vis	Ultraviolet–Visible Spectroscopy
WHO	World Health Organization
XRC	X-ray crystallography

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

The aim of presenting this chapter was to present the motivation and problem statement, objectives and scope of the research together with the significance and contribution of the study. All these aspects would be a foundation in order to further discover the research.

1.2 BACKGROUND AND PROBLEM STATEMENT

Neem (*Azadirachta indica*) also known as 'Pokok Mambu' in Malaysia is an omnipotent tree that was classified under the mahogany family of *Meliaceae*. This plant had been used since prehistoric times as a traditional medicine for the treatment of chicken pox, fever, skin infections, oral care, as a tonic for ulcers, high blood pressure, diabetes, cholesterol and as hair care (Kumar and Navaratnam, 2013). The recent and modern usages of *A. indica* were also expanded to various fields such as for the replacement of petroleum with biodiesel, production of polyurethane coatings, as a modulator in rumen fermentation and the encapsulation of the seed oil for nano-emulsion (Ali et al., 2013 and Dhar et al., 2012). The native of *A. indica* plant was India and now the distribution in Malaysia is also wide. Several reported studies had been conducted on *A. indica* plant from Malaysia such as for the production of product for worm control, effect on c-Myc Oncogene expression in breast cancer cells and stimulation of Glucosamine (Chandrawathani et al., 2013; Kumar et al., 2011; Othman et al., 2011). Almost all the reported study in Malaysia was on the leaves extracts, but

none of it includes the roots of the plant. Therefore, this present study reports the cytotoxic effect of *A. indica* bark, leaves and roots extracts specifically from Malaysia.

The modern (non-conventional) extraction techniques were being recently used for various studies, but in certain cases the traditional (conventional) methods were still effective according to the pros and cons reviewed in Table 2.2. Previous researchers, Carneiro et al. (2012), had reported that the extraction of ethanolic crude; and hexane, chloroform, dichloromethane and aqueous fractions of leaves of *A. indica* exhibit cytotoxic and antileishmanial activity in a single study. But, there were no any records on the comparison of both crude and fraction extracts on the bark and roots of *A. indica*. Therefore, a combined study of both techniques of crude and fractions was employed as the main motif in this single cytotoxic study under the same condition of the bioassay.

The extraction of plant metabolites includes the aid of the solvents (Azmir et al., 2013 and Gupta et al., 2012). The solvent polarity plays a role in producing extracts of different class of phytochemicals and resulted in various amounts of yield. High polar solvents were more prone in extracting high polar compounds and vice versa. Several scientific studies had been conducted for all three parts (bark, leaves and roots) of the plant with different solvents. But specifically for cytotoxic study of crude extracts of *A. indica* against brine shrimp, *Artemia salina*, only leaves and stem bark had been reported. Alcohols (methanol and ethanol) and aqueous cytotoxic study were reported for crude extract of the leaves and stem bark of the plant (Al-Emran, 2011 and Kirira et al., 2006). Whereas for the fractions; wide range of solvents had been reported such as aqueous, butanol, chloroform, ethyl acetate, hexane, methanol and petroleum ether on leaves (Al-Samarrai, 2013; Islam et al., 2012 and Kutama, 2008). Therefore, in this work, a combined effect of the crude acetone extract and fractions with other non-reported roots part were assayed against *A. salina* to evaluate the toxicity level.

The occurrence and the concentration of secondary metabolites in plants were restricted according to the plant taxonomy. Basically, these metabolites were not related to any primary metabolism, but they present to exhibit several biological activity or defense. Among these secondary metabolites, certain amounts were classified to be volatile compounds (Holopainen and Blande, 2012). As per the study conducted by

Hossain et al. (2013), the compounds present in *A. indica* was of different classes of hydrocarbon, terpenoids, phenolic, alkaloids, and their derivatives whereby certain were reported to reveal as biologically active molecules. The volatile compounds in *A. indica* leaves was reported for the butanol, chloroform, dichloromethane, ethyl acetate, hexane, methanol and petroleum ether extracts (Akpuaka et al., 2013; Helmy et al., 2007; Hossain et al., 2013; Moorthy and Boominathan, 2011 and Nand et al., 2012). One of the study reports the volatile compounds in bark extracts of dichloromethane, methanol and petroleum ether (Nand et al., 2012) and no study were reported for the roots extracts. Due to limited literature, this study has also focused on the volatile compound identification in both crude and fractions in order to report the possible activity of the plant and to support the cytotoxicity data.

One of the effective methods to evaluate the cytotoxicity of a substance was via shrimp lethality assay that was proposed since 1956 by Michael et al. The concept of this assay was to kill a laboratory-cultured invertebrate animal model, *A. salina*. This method was developed to predict the acute toxicity by avoiding the direct usage of laboratory animals that were being demanded by certain organizations and as these shrimp proven to correlate like a mice (Molina-Salinas et al., 2006). Moreover, it was recognized to be simple, fast, cheap, effective and reproducible for various assessments of toxicity for example in the detection of fungal and cyanobacteria toxins, natural products, pesticides and heavy metals (Carballo et al., 2002). This assay was vital in determining the responses of human normal and cancer cells as a preclinical assessment for drugs and was also widely used for *A. indica* extracts. In detail, the aim of this study also expands towards looking forward for bioactive compounds guided by the lethality bioassay of *A. salina*. Various compounds from *A. indica* had been reported to exhibit a cytotoxic effect, namely nimonol, nimbandiol, nimbolide, 2', 3'-dihydronimbolide and 28-deoxonimbolide (Takagi et al., 2014 and Wu et al., 2014). Therefore, isolation of a compound from the most cytotoxic extract of *A. indica* considered to be a scope of this work.

The seed oil of *A. indica* was another economic product since the oil has potential industrial uses and advantage in Indian medicine for many years (Gandhi et

al., 1988). The fixed oil obtained from these seeds was the fatty oil that contains more fatty substances and denser compared to the essential oil that were volatile. Oils were a form of triglycerides that were non-polar and preferred to be extracted by non-polar solvent such as hexane (Ferreira-Dias et al., 2003). They were also different from extracts. Extracts were the crude mixtures as in the bark, leaves and roots of *A. indica.* The various fatty acids were reported to be present in *A. indica* seed oil with correlation of numerous biological activities as well as raw materials and feedstock's in industry (Atabani et al., 2013; Biswas et al., 2002; Dhar et al., 2012; Djenontin et al., 2012 and Kumar et al., 2010). However, other parameters on the physicochemical properties of the oil were also testified and proven to be influencing the choice of the industry, for example in the production of skin protector (Teressa et al., 2004) and biodiesel (Ali et al., 2013). The geographical variation, environmental condition and storage were being one of the key factors that impact the physicochemical properties of the plant oil (Dhar et al., 2012 and Goja, 2013). However, Malaysian *A. indica* oil has so far not been investigated to determine its physicochemical properties. This subsequently classifies the oil to suit world market requirements, especially if it is to be commercially produced in Malaysia as potential sources for export. Therefore, the soxhlet (solvent semi-continuous extraction) extraction of *A. indica* seed oil via hexane together with the study on the physicochemical and antibacterial properties would be one of the aims of this work to increase the economic feasibility for future commercial cultivation of this tree.

1.3 OBJECTIVES OF THE RESEARCH

The objectives of this present study were;

i. To investigate the different extraction techniques, conditions and solvents on the extraction of *A. indica* plant parts.
ii. To analyze and determine the volatile compounds of *A. indica* extracts.
iii. To study the cytotoxic effect of the extracts, isolate and identify compound from the most cytotoxic fraction of *A. indica*.
iv. To study the physicochemical and antibacterial properties of *A. indica* seed oil.

1.4 SCOPE OF THE RESEARCH

As a way to accomplish the objective of this study, the scope of this research focuses on the extraction of the crude of three different parts of *A. indica* by using three different solvents. Extractions for the solvent (ethanol) were performed at the different extraction condition of maceration and reflux. The same parts were also extracted with 80% of aqueous methanol and were further partitioned to four different fractions by using four different solvents. They were labelled as Fraction 1, 2, 3 and 4, respectively. Seed oil of *A. indica* was extracted via soxhlet method. All the extraction was conducted at room temperature except for soxhlet (mild heat) and reflux method at 60 °C.

The volatile compounds in the crude and fractions were analyzed and determined by performing chromatography and spectroscopy technique via Gas Chromatography-Mass Spectroscopy (GC-MS). All the injected liquid samples were prepared at similar concentration and solid samples at similar weight.

Brine shrimp larvae, *Artemia salina* was used to study the cytotoxic activities of both crude and fractions at different test concentrations. The results were analyzed to calculate the lethal concentrations of 50% of the sample population and the data's were evaluated in terms of probit analysis, further validated with Tukey's test and one way analysis of variance (ANOVA). Isolation of the compound was performed from the most cytotoxic fraction via preparative-High Performance Liquid-Chromatography (prep-HPLC) and the identification via absorption, functional groups, mass fragments and structure by using various spectroscopy methods.

The physicochemical properties of seed oil were studied according to standard methods proposed by American Oil Chemists' Society (AOCS), Association of Official Analytical Chemists (AOAC) and some reported literature. Besides that, to support the study, the seed oil alone was tested against three different pathogenic bacteria (*Bacillus subtilis, Escherichia coli* and *Staphylococcus aureus*) to prove the antibacterial properties of the oil at four different concentrations (20, 40, 60 and 80%). In order to prove the effectiveness of the oil in fighting against the bacteria, the minimum

inhibitory concentration of the oil was also determined through a broth tube dilution method. The data's were endorsed with Tukey's test and ANOVA.

1.5 SIGNIFICANCE AND CONTRIBUTION OF THE RESEARCH

One of the aims of the study was to differentiate the varying solvent polarity, ability and efficiency in producing the highest yield via different extraction techniques. The usage of different class of solvents in one similar study gives a better comparison on the extraction of the different plant parts that had never been studied before. Thus, the study points out the most suitable solvent and condition for the extraction of high yield *A. indica* extracts with complete utilization of all the major parts of the plant. The combined study of cytotoxicity of both crude and fractions of all plant parts in one study gives a better comparison on the toxic level presented by the plant.

The study of the plant roots of *A. indica* species from Malaysia for the first time and limited report concerning on the cytotoxic effect proves the capability of the plant growing in different geographical variation. The extract of the roots shows positive cytotoxic activity, whereby two of the roots crude extracts; acetone and chloroform extract present to be toxic (LC_{50} < 500 ppm) and less toxic (≥ 500 ≤ 1000 ppm), respectively. Whereas all the fractions; hexane, chloroform, ethyl acetate and aqueous were toxic. Therefore, the present work on cytotoxicity was able to proof the activity of *A. indica* plant roots.

The volatile compound identification of roots acetone crude extracts presented in this study contributes towards a significant study since there was no existence of a previous report. The identified volatile compound of all the extracts gives a clearer image on the abundance of the bioactive compounds in each extract.

1.6 OVERVIEW OF THE THESIS

In detail, the Chapter 1 reveals the research motivation with the support of facts and figures from reporting studies. The objectives of the research were pointed out with

an expended study scope. The brief significance or the contributions of the study were performed together with the necessity to contribute towards a research.

The Chapter 2 discusses the review of the literature or the background on the plant material *A. indica*, previous methods for the extraction, chromatography and spectroscopy techniques adapted to achieve the aim of the study. Several different methods were studied and were correlated with the current study. This method addresses some of the fundamental and practical methods that were suitable for the progress of the research.

As for Chapter 3, detail explanation of the material and methods were written according to the way of the analysis being conducted. The brief descriptions of the study were as drawn in the methodology flowchart whereby it represents the two different methods adapted.

Chapter 4 was allocated for results and discussion of the obtained data according to the objective whereby the yield of the extraction methods was discussed. The data in the analysis of the volatiles in the extracts were listed and reviewed according to different plant parts. The data on the cytotoxicity study were evaluated to provide scientific supporting data with the concentration level of sample that was required to behave as an aid in proving toxic effects. Lastly, the isolation and identification of a compound, quercetin-3-*O*-β-D-glucopyranoside was included from the extract that exhibits highest toxicity. The physicochemical and antibacterial studies of the seed oil reveal the capability of the plants grown it different geographical area.

The Chapter 5 represents the concluding chapter of the overall work and it summarizes the research work and point out the result in accordance with the objective of the study. Some recommendations were also suggested to expand the study in several terms.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

The purpose or the aim of presenting this chapter was to review and analyze the critical points of current knowledge as well as theoretical and methodological relation via specific subtopics whereby it could be an aid in the current study. The main topics discussed were on the plant material and extraction, chromatography and spectroscopy techniques and physicochemical properties.

2.2 PLANT *Azadirachta indica*

Natural products mainly flora (plants) had been widely studied and reported to contain biologically active compounds for development as medicinal or curative agents (El-Mahmood et al., 2010). One of the plants, *A. indica* native of India, was a tropical tree at bout of 25 m height and 3 m girth. The physical image and agronomy of the plant was shown in Figure 2.1 and Table 2.1. This tree could produce flowers and seeds upon maturity period of 3-5 years. They were now having wide distribution in the entire world. The two species of *Azadirachta* were the *Azadirachta indica* A. Juss, natural to the Indian subcontinent and *Azadirachta excelsa* Kack, limited to the Philippines and Indonesia. Nowadays, these trees were often found in Bangladesh, Burma, Pakistan, Sri Lanka, Malaysia, Thailand and Indonesia. The distribution was as in Figure 2.2. It could grow in dry area with less amount of water supply and in very high temperature as high as 49 °C (Girish and Shankara Bhat, 2008). This plant were being planted in terms of large scale because it helps to combat desertification, deforestation, soil erosion, reduces excessive global temperature and produces more oxygen.

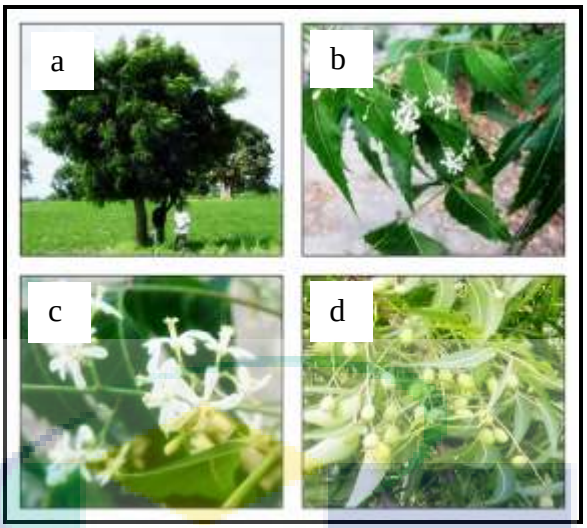


Figure 2.1: An illustration of *A. indica* plant (a: tree; b: leaves, c: flowers, d: seeds)

Source: USDA (2011)

Table 2.1: Plant classification of *A. indica*

Kingdom	**Plantae (Plants)**
Division	Magnoliophyta (Flowering plants)
Class	Magnoliopsida (Dicotyledons)
Order	Sapindales
Family	Meliaceae (Mahogany family)
Genus	*Azadirachta* A. Juss. (*Azadirachta*)
Species	*Azadirachta indica* A. Juss. (Neem)

Source: USDA (2011)

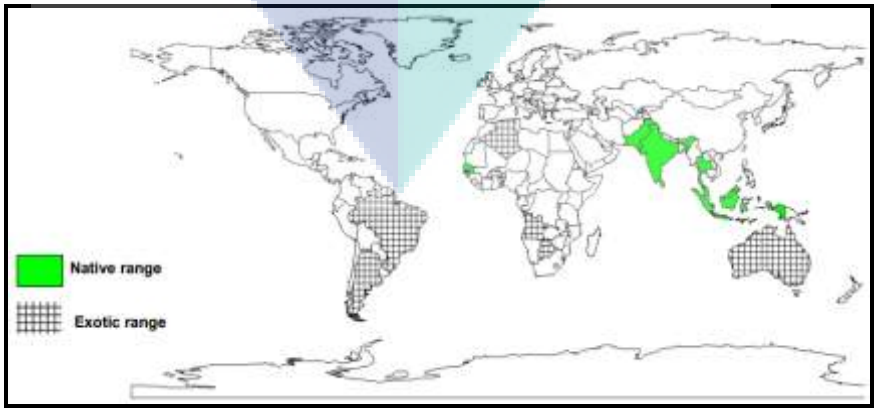


Figure 2.2: World distribution map of *A. indica*

Source: USDA (2011)

The *A. indica*'s medicinal property has been known for many years in the field of Ayurvedic (Girish and Shankara Bhat, 2008). In the traditional method, burned smoke of leaves of *A. indica*'s could repel mosquito as high as 70%. The oil of this plant gives better synergistic combination with coconut oil to provide skin protection for the duration of 12 h from mosquito and the *A. indica* oil alone prevents the breeding of *Anopheles stephensi* and *Aedes aegypti* (Atawodi and Atawodi, 2009). In accordance with developing technology, various products have been produced as per reported positive and promising results with this plant. Almost all the parts of the plants had proven biological activity whereby the methanolic bark extracts could produce positive effects towards various snail species. Toxicity effect on mice could produce LD_{50} of 13 g/kg, while for the antiulcer activity of rats produce toxicity with 2 g/kg body weight.

The seeds of this plant and its kernels extract produced a toxic effect on the Tilapia fish (*Oreochromis niloticus)* whereby 60% mortality achieved in a day with LC_{50} of 1124.6 ppm. Spermatogenesis retardation was observed in the rats that were fed with seed cake whereby the same feed for calves results in depression and reduction of blood hemoglobin content. The oil results toxicity in rats and rabbits whereby the lungs get affected and the LD_{50} values were 14 and 24 mL/kg, respectively. Due to presence of toxic compounds in this oil, it does also produce toxic effects in humans by causing diarrhea, nausea and vomiting. The methanol extract of *A. indica* causes toxicity in mice through the signs of illness and discomfort and finally results in the death of those mice.

Another study on mice with the leaves extract produces an antifertility effect. Guinea pigs died when they were exposed to aqueous extract of leaves with dosage level of 40 mg/kg body. The death in them was caused by toxic manifestation. Higher dosage (5-200 mg/kg) of the same aqueous extract of leaves in those pigs reduces heart rate and thus increased the arterial pulse (Biswas et al., 2002).

The components of nimbin and azadirachtin were the most active insecticidal ingredient and they were present in huge amount in the seeds, leaves and other parts of the *A. indica* (Mondali et al., 2009). Allelochemicals like azadirachtin, nimbin, nimbidin, nimbolides, nimolic acid, salannin, melianttriol and azadirachtol present affect the biochemical and physiological processes of insect system and thus controls

the problems (Dua et al., 2009). Other active compounds could be divided into several different classes such as the isoterpenoids (triterpenoids and diterpenoids), nonisoterpinoids (proteins and carbohydrates sulfurous) and polyphenolics (flavonoids, glycosides dihydrochalcone, coumarin and tannins). Among many compounds isolated and studied, azadirachtin was known to be very effective as reported by its repellent activity towards some insects at low concentrations in parts per million. The tetranortriterpenes (nimbin, nimbinin, nimbidinin, nimbolide and nimbidic acid) possess pesticide, repellent and medicinal properties as well. The nimbidin, a major crude bitter extract from oil of seeds kernels shows antifungal activity by inhibiting the growth of *Tinea rubrum* and anti-inflammatory activity against carrageenin induced acute paw oedema in rats and formalin-induced arthritis, whereas nimbolide shows antibacterial activity against *S. aureus*. The gedunin from oil proves antifungal, antimalarial and antibacterial action against some strains of human pathogenic bacteria. Tricyclic diterpenoids (margolone, margolonone and isomargolonone) from bark shows antibacterial activity against *Klebsiella, Staphylococcus* and *Serratia* species. A sulfur compound isolated from matured leaves was effective as the antifungal agent against *Trichophyton mentagrophytes* (Biswas et al., 2002).

2.3 EXTRACTION TECHNIQUES

The term of extraction is known to be solid-liquid extraction of natural products from plants or microorganisms (Kaufmann and Christen, 2002). In the field of natural products such as pharmaceutical industry, defines the separation of medicinally active constituents of the plant from other inert constituents through selective procedures involving various solvents (Meghna and Minal, 2013). The extraction was also classified into different ideas by Sticher (2008), whereby it was desorption and diffusion of components towards the matrix, solubility of compounds in solvent and finally the collection of extracts.

For the extraction, various conventional methods had been used for many decades but now the modern methods (non-conventional methods) were also being widely used due to some drawbacks that occur in the conventional methods. It would be due to the longer extraction duration, higher solvent volumes, degradation of thermo

labile components (Gupta et al., 2012; Meghna and Minal, 2013 and Sticher, 2008), higher energy, inconsistent extraction, poor safety (Gupta et al., 2012) and requirement for labor (Sticher, 2008).

Therefore, the modern methods plays and important role in enhancing those limitations besides increasing extraction yield, enhancing the quality and efficiency of samples (Gupta et al., 2012 and Meghna and Minal, 2013), elimination of undesirable and insoluble components of plant and reduction in effort of sample cleanup (Gupta et al., 2012). Chemat et al. (2012), worked on the 'Green Extraction of Natural Products' with the definition of providing an extraction procedure with ideal consumption of raw materials, improving and optimization of current methods, using non-dedicated equipment and discovering alternative solvents.

Azmir et al. (2013) and Gupta et al. (2012), pointed that solvents was the major parameter that influences the extraction procedure. The selection of solvents should be according to the policy of 'like dissolves like' whereby the polar compounds have a higher probability in extracting polar constituents and vice versa. The mixture of solvents such as the hydro alcoholic (water and alcohol) was reported to produce higher yields of extraction (Gupta et al., 2012). The polarity, toxicity towards living things, cost, efficiency of molecular interaction energies between solute and solvent and mass transfer was the key factor in choosing the suitable solvent (Azmir et al., 2013). From the view of various extraction methods, extraction temperature, pressure and duration also have the possibilities in contributing towards the difference in the properties and yield of the extracts.

2.3.1 Conventional Extraction Technique

The conventional extraction method includes the decoction, hydrodistillation, maceration, percolation and soxhlet extraction (Kaufmann and Christen et al., 2002). Each of the technique was described according to its principle and mechanism together with operating procedures. One of the parameters that differentiate the various conventional methods was the temperature.

A) Decoction

Decoction, a hot aqueous based extraction was conducted through the application of heat whereby this extraction was suitable for water soluble and thermally stable constituents (Handa et al., 2008).

The extraction was conducted in an open-type cylindrical vessel usually made up of stainless steel. The bottom of the reactor vessel was fitted with filter cloth and discharge valve whereas the outer part was wrapped with steam jacket. The plant material was boiled under steam heat supplied into the jacket in a fixed volume of water usually around 1:4, 1:8 or 1:16 w/v of sample and water for a specific duration. The ratio was determined according to the physical feature of the material whereby it was soft, moderately hard or very hard. High volume of water was needed as the hardness increases. Boiling was done by the volume reduced to one-fourth and the mixtures were then cooled and filtered from the marc (extracted plant material) into the holding tank. In order to remove fine impurities, the extract was passed through sparkler filter and concentrated with spray drying to obtain a dry and powdered extract (Handa et al., 2008).

B) Hydrodistillation

The mechanism of hydrodistillation involves certain physicochemical processes that were the hydrodiffusion, hydrolysis and decomposition by heat. Hydrodiffusion in terms of extraction were the flow of solvent and solute across the plant membranes and the rate of this diffusion were influenced by the solubility of the matrix constituents in the solvent; whereas the concept of hydrolysis was, the chemical reaction between the constituents and solvent. Heat was also applied in the reaction to accelerate the reaction. All the physicochemical process described gives simultaneous effect in the process whereby the high heat causes higher solubility of constituents through higher diffusion rate across the membrane of matrix (Azmir et al., 2013 and Handa et al., 2008).

Some of the examples of hydrodistillation were water, water and steam and direct steam distillation. To perform the extraction, the plant material was filled in the still compartment with water and heat supplied to boil it. In the case of steam, direct steam was flown into the sample container and cooling was provided to condense steam vapor, water and constituents. The mixtures were collected and water was removed to obtain the extract (Azmir et al., 2013). The removal of water could be done by adding anhydrous sodium sulfate (Na_2SO_4) to absorb the moisture (Yamini et al., 2008) and the schematic diagram on the extraction was as in Figure 2.3.

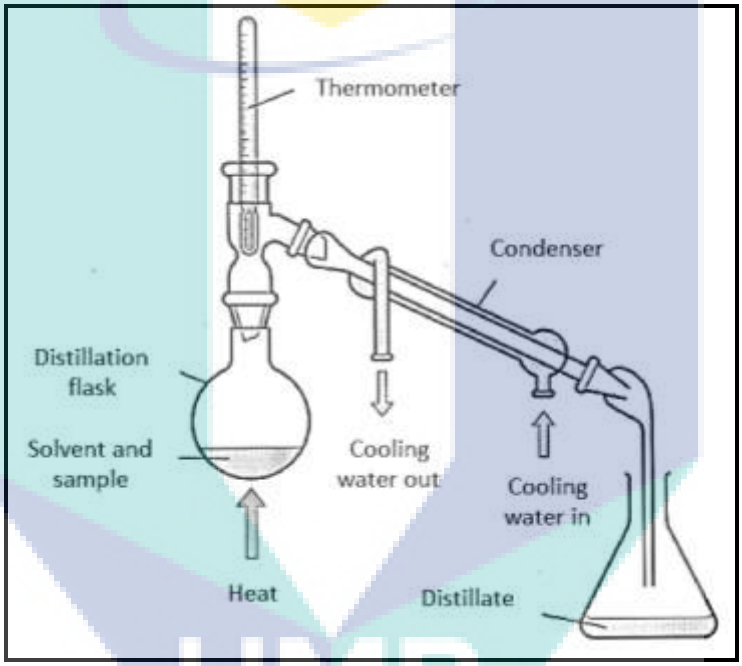


Figure 2.3: Schematic figure of hydrodistillation

Source: Scottish Sensory Centre (2013)

C) Maceration

The principle of maceration involves soaking and agitation of plant material in order to obtain the extract from solid materials through a leaching process (Handa et al., 2008). The soaked plant material cellular wall were softened by the *menstruum* (solvent) and penetrates in, to extract the soluble constituents. The agitation technique facilitates the diffusion of solvents into the samples and replaces the concentrated

solution from the sample with new solvents (Azmir et al., 2013). This process was known to be a batch process (Meghna and Minal, 2013). According to Handa et al. (2008) this process was influenced by several factors; transport rate of menstruum into the sample and out of insoluble material, the solubility rate of active principle and surface area of plant material for mass transfer.

Handa et al. (2008) and Azmir et al. (2013) had described the small scale extraction steps of maceration whereby, the uniformly ground parts of the plant were soaked in selected menstruum in a tightly stoppered container and were exposed to occasional shaking or agitation at room temperature. The liquid was strained off after achieving soaking duration via filtration technique to remove the marc (damp plant residue) and impurities. The marc was pressed during filtration in order to maximize the yield of extraction. The filtrate was concentrated to remove the menstruum leaving behind dry extract.

D) Percolation

Percolation was a batch process that was often used to extract the active components for the extraction of alcoholic and fluid extracts. The principle of this extraction was on the equilibrium concept whereby the sample was macerated in solvent so that the active constituents attain equilibrium by leaching. This technique was also based on the mass transfer principle according to the capacity and utility of the percolator (a narrow, both ends opened, cone shaped vessel). The application of gravity was also involved towards the weight of the column of liquid (Handa et al., 2008).

The extraction image was as in Figure 2.4 whereby it has been conducted in the percolator; whereby just like maceration, the plant samples were soaked in menstruum in a closed container for required duration and were transferred into the percolator. The maceration was further continued in the percolator. The bottom end of the percolator was opened, allowing the percolate (solvent extract) to drip. Required amount of menstruum was added to wash the marc whereby the sample particle has high probability getting exposed to the passing menstruum and the marc was pressed to

remove any solution. The enriched solution was further concentrated by filtration and evaporation to obtain the extracts (Handa et al., 2008).

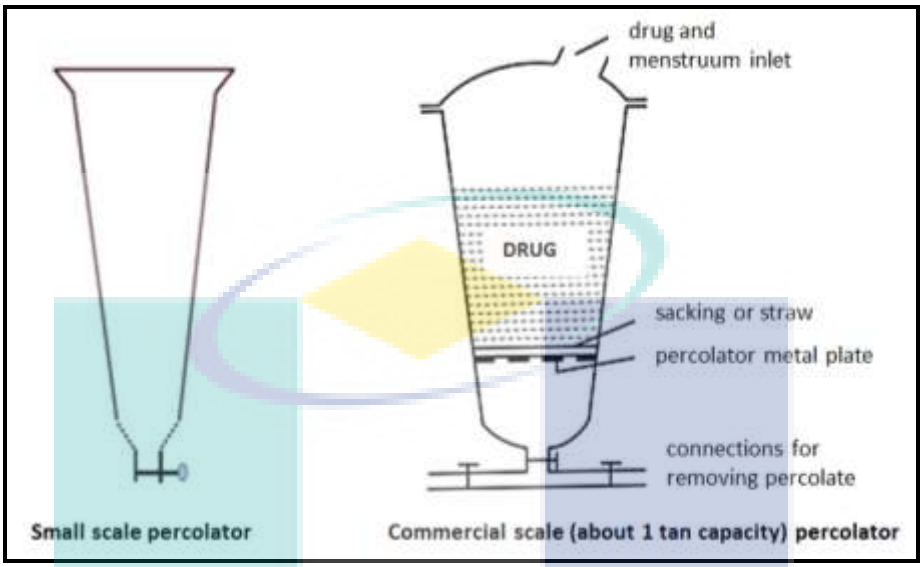


Figure 2.4: Schematic figure of percolator for percolation extraction

Source: Handa et al. (2008)

E) Hot Solvent Semi-Continuous (Soxhlet) Extraction

The principle and mechanism involved in soxhlet extraction were heat and recycling of solvents (Handa et al., 2008). Continuous heat was supplied to the solvents to evaporate, distill and extract the thermo labile components. Azmir et al. (2013) had reported that this extraction was first invented by Franz Ritter Von Soxhlet, a German chemist, in 1897 for the extraction of lipids. But now, the usage of this technique had expanded up to the level of extraction of various constituents.

In laboratory scale, the finely grounded sample was placed into the sample holder, thimble and was placed into the soxhlet chamber. The holder was then attached to the distillation flask and choice of solvent was added in via soxhlet chamber at least two cycles of volumes. The condenser was attached at the top end of the soxhlet and continuous supply of water was ensured. The heat was supplied to the extracting solvent, vapor condenses in the distillation condenser and drips into the soxhlet chamber

washing soluble constituents. The solvent together with the extract flows back into the distillation flask as they reach the siphon level (Azmir et al., 2013 and Handa et al., 2008). Continuous heat evaporates the fresh solvent again to the solid bed of the plant for several cycles leaving behind the solute (extract). The extraction continues cycle up to a ensured time for complete extraction; whereby solution in the siphon tube was completely colorless (Solanki and Nagori, 2012). The final extract was the distillation still that was rich in active constituents to be concentrated. The setup of the apparatus was illustrated as in Figure 2.5.

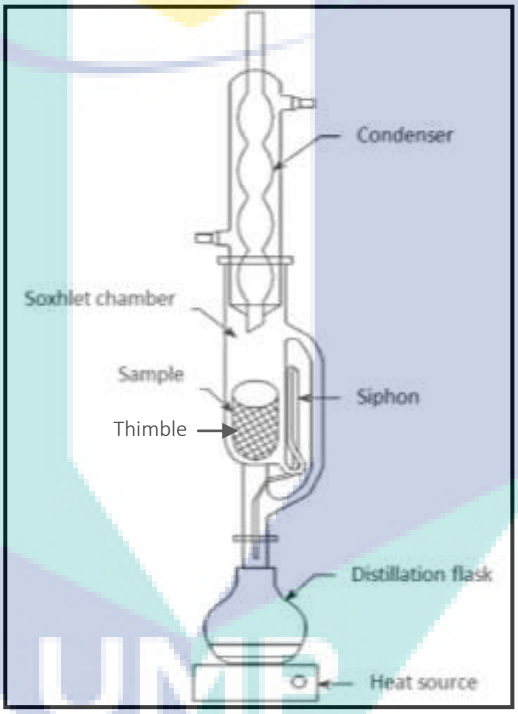


Figure 2.5: Schematic figure of conventional soxhlet extraction

Source: Dutta et al. (2014)

2.3.2 Non-Conventional Extraction Technique

Microwave-assisted, supercritical fluid (Kaufmann and Christen et al., 2002; Sticher, 2008; Gupta et al., 2012) and ultrasound-assisted extraction were the non-conventional technique (Sticher, 2008). The differences between the operating

parameters of these methods were the agitation, magnetic field, pressure, radiation and temperature.

A) Microwave-Assisted Extraction (MAE)

This microwave-assisted extraction (MAE) works based on the principle of heat through microwave energy with the frequency level of 0.3-300 GHz (Kaufmann and Christen, 2002). This energy does possess electric that causes heat and magnetic power; that causes dipolar spin and ionic transmission (Kaufmann and Christen, 2002). Heat was transferred directly to the solvent mixture. The magnetic dipole rotation of the solvent and matrix molecules speeds up the extraction within the time limit of usually less than 30 min (Kaufmann and Christen, 2002 and Sticher, 2008). The heating causes the moisture evaporates from the matrix and breaks the cell wall, releasing constituents into the sample (Meghna and Minal, 2013).

The two different techniques of MAE (Figure 2.6) were the extraction via closed vessels under controlled pressure and temperature (diffused microwave multi-mode cavity system (DMAE)) and open vessels at atmospheric pressure (focused microwave single-mode cavity system (FMAE)). The factor that differentiates these two systems were the boiling point of the solvent and atmospheric pressure; whereby FMAE system was limited to the boiling point of the solvent at atmospheric pressure while PMAE system can be operated above the boiling point at suitable pressure. Thus, it was reported that the PMAE were more efficient due to high extraction speed (Sticher, 2008). Kaufmann and Christen (2002), suggested that the application of the PMAE was for the digestion or acid mineralization or extraction under pressurized conditions above the boiling point of the solvent. For the operation of PMAE system, the samples together with extraction solvent were placed into the extraction teflon vessels located on the rotating carousel. Since there were several single vessels, the solvents may vary. The temperature was set depending on the boiling point and the volume of the solvent. Vessels were cooled to room temperature before opening and filtration of mixture done to obtain the extract. The operation of FMAE uses quartz vessels attached to a vapor condenser for reflux process to take place. The removals of the extract were much

favorable through this system compared to PMAE due to safety handling with application of cooling system.

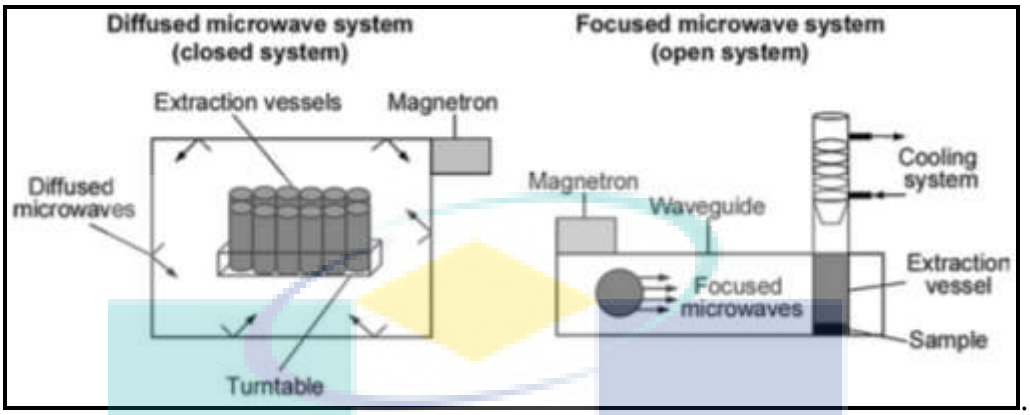


Figure 2.6: Schematic figure of MAE

Source: Sticher (2008)

B) Supercritical Fluid Extraction (SFE)

Supercritical fluid extraction (SFE) was a liquid extraction process whereby the normal liquid solvent phase was replaced by supercritical fluid, a substance above its critical point (Sticher, 2008). The main factor influences SFE were the temperature, pressure, flow rate of CO_2, amount, moisture content, particle size of sample, additional co-solvent and solvent-to-feed-ratio. Generally SFE works on the principle of conducting extraction closer to ambient temperature. Sticher (2008) had reported that carbon dioxide (CO_2) was the most common supercritical fluid being used due to its low critical temperature. Handa et al. (2008), added that CO_2 was favored physical properties, low cost, environmentally safe and easily available. For certain extraction, CO_2 was not suitable due to its polarity limitations and thus the addition of co-solvent or modifier such as organic solvent were needed (Handa et al., 2008).

The SFE extraction can be either in a static mode or dynamic mode whereby in dynamic mode the fresh solvent were continuously supplied into the extraction vessel at higher temperature and pressure (Kaufmann and Christen, 2002). According to Figure

2.7, extraction fluid of preferred pressure supplied through the pump into the extraction vessel held above the critical temperature of the fluid. The pressure was controlled by the restriction device (Kaufmann and Christen, 2002). The supplied fluid flows into the extraction vessel and sample matrix; and flows out through restrictor and ended in the collection vessel (Stricher, 2008). The removal of the fluid from the solute was done through depressurization of supercritical fluid (Azmir et al., 2013).

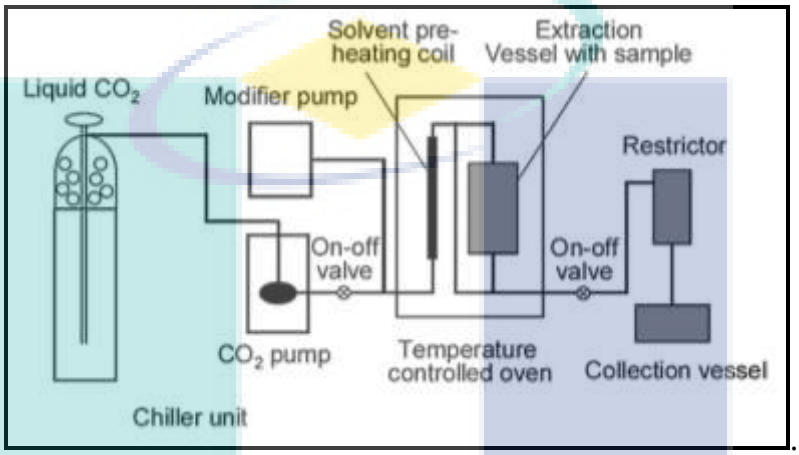


Figure 2.7: Schematic figure of SFE

Source: Sticher (2008)

C) Ultrasound-Assisted Extraction (UAE)

Gupta et al. (2012) reported that the principle and mechanism of ultrasound or sonication work based on high intensity and frequency sound wave interaction with plant material. Azmir et al. (2013) added that the reaction involves the physical phenomena of diffusion of solvent across the cell wall and washing of contents in the matrix. The frequencies of this extraction range from 20-2000 kHz (Handa et al., 2008). This energy wave forms microcavities and microjets that contribute towards rupturing and breaking of plant cell wall whereby the particles of the solid and liquid vibrates and accelerates; and thus enables the constituents to be easily diffused into the solvent. It was also said that, higher energy of ultrasound weakens the intramolecular forces of the molecule creating bubbles that further facilitate the release of compounds and enhance the mass transfer through diffusion of solvent into the sample; by preventing the plant

tissue from getting saturated (Gupta et al., 2012; Azmir et al., 2013 and Meghna and Minal, 2013). Meghna and Minal (2013) added that the movement of the waves causes expansion and compression phase that the energy pulls apart and pushes the molecules together, respectively.

Ultrasonic bath (closed extractor fitted with an ultrasonic horn transducer) and ultrasonic horn transducer were the different type of ultrasound extractor (Meghna and Minal, 2013). The setup image was as in Figure 2.8. The extraction steps involved were the addition of solvent according to the ratio of liquid material into the sample container and the placement of the container into the ultrasonic device. The extraction condition; temperature, duration, and ultrasound power were also set on the device prior to extraction (Samaram et al., 2014).

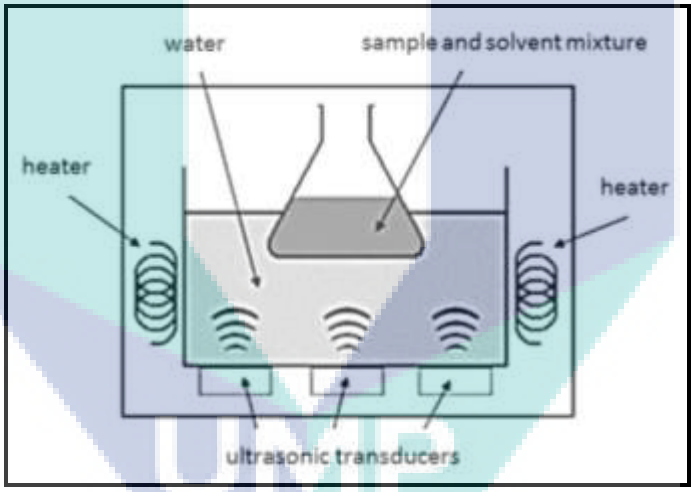


Figure 2.8: Schematic figure of UAE

Source: Samaram et al. (2014)

Even though there were many extraction techniques that could be adapted, each of them has its own pros and cons that play an important role during the selection of proper techniques. Several comparisons had been reviewed and the advantage and disadvantage of those techniques were as being listed in Table 2.2 whereas the Table 2.3 were tabulated to review the types of extraction methods and its conditions that had been used in the extraction of the plant *A. indica.*

Table 2.2: Pros and cons of conventional and non-conventional extraction techniques

Extraction Technique	Advantage	Disadvantage	Reference
Conventional Methods			
Decoction	Suitable for thermally stable and aqueous soluble compounds Suitable for tough and fibrous plant	High energy for heating	Handa et al., 2008
Hydrodistillation	Extraction before hydration of samples	Less suitable for thermo labile compounds	Azmir et al., 2013
Maceration	Favorable for thermo labile components[b] Recovery of solvents[a]	High volume of solvents[b]	Handa et al., 2008[a] andMeghna and Minal, 2013[b]
Percolation	Favorable for thermo labile components and dilute products (tinctures) Recovery of solvents	High volume of solvents Longer extraction duration Energy-consuming	Handa et al., 2008
Soxhlet	Favorable for many natural constituents[b] Promotes fresh solvents for extraction[b] Maintains heat[b] No extract filtration[b] Extraction of large amount of sample[b] Low volume of solvent[a]	No agitation[a] Thermal decomposition of certain compounds[a] Longer extraction duration[a]	Handa et al., 2008[a]; Meghna and Minal, 2013[b]

Superscript (a and b) represents the corresponding reference.

Table 2.2: *Continues*

Extraction Technique	Advantage	Disadvantage	Reference
Non-conventional Methods			
MAE	Shorter extraction duration[b] Low volume of solvent[b]	Hazardous to flammable organic solvents[a] Low reproducibility Excessive temperature and pressure causes solute degradation[c] Filtration or centrifugation of extracts[c] Poor yield when constituents and solvents were non-polar or volatile[c]	Kaufmann and Christen, 2008[a] Meghna and Minal, 2013[b] and Sticher, 2008[c]
SFE	Higher yield due to repeated reflux[a] Easier separation of solute from fluid[a] Recycling of fluid minimizes waste[a] Low temperature and volume of solvent[c] No solvent residues[c] Low cost and toxicity[c] Reduce thermal degradation [c] Favorable for volatile components[c]	Low yield at low temperature[b]	Azmir et al., 2013[a]; Handa et al., 2008[b] and Sticher, 2008[c]
UAE	Shorter extraction duration[b] High extraction efficiency[b] Extraction of organic and inorganic compounds[b] Low volume of solvent and temperature for thermally unstable compounds[c] Suitable for various solvent[c]	Depends on sonication time and power[c] Produces heat[c] High energy converts drug constituents into free radicals[abcd]	Azmir et al., 2013[a]; Gupta et al., 2012[b]; Meghna and Minal, 2013[c] and Handa et al., 2008[d]

Superscript (a,b and c) represents the corresponding reference.

Table 2.3: Extraction methods adapted for *A. indica*

Extraction technique and solvent	Plant material	Extraction condition	Objective	Reference
Conventional Methods				
Decoction				
Aqueous (Hot)	Flowers	Sample-to-water ratio, 1:20 w/v; 6 h extraction	Antioxidant	Chaisawangwong and Gritsanapan, 2009
Hydrodistillation				
Aqueous	Seed oil	4 h extraction	Nematicidal properties	Kosma et al., 2011
Kneading				
Aqueous	Seed oil	NR	Pre-oviposition treatment	Lale and Abdulrahman, 1999
Maceration				
Acetone	Seed oil	Occasional shaking; 1 h 30 min extraction	Pre-oviposition treatment	Lale and Abdulrahman, 1999
Methanol	Seeds kernels	3 days extraction	Extraction of Azadirachtin	Jadeja et al., 2011
Aqueous	Leaves	NR	Induction of antioxidant defensive	Hanaa et al., 2011
Aqueous, ethanol, hexane	Seeds kernels	Aqueous, 3-4 h; ethanol and hexane, 24 h extraction	Toxic effect	Nathan et al., 2007

Table 2.3: *Continues*

Extraction technique and solvent	Plant material	Extraction condition	Objective	Reference
Aqueous, methanol, acetonitrile, ethyl-acetate, potassium chloride (0.1M), calcium nitrate and chloride	Seeds	30 min extraction	Insecticidal	Boursier et al., 2011
Methanol, aqueous	Seeds	48 h	Nematicidal properties	Kosma et al., 2011
Ethanol (50%)	Flowers	Sample-to-solvent ratio, 1:20 w/v; room temperature; 4 days extraction	Antioxidant	Chaisawangwong and Gritsanapan, 2009
Ethanol (20, 50, 80, 95%)	Leaves	Sample-to-solvent ratio, 1:20 w/v; 25 °C; 7 days extraction	Antioxidant	Sithisarn et al., 2006
Percolation				
Ethanol (50%)	Flowers	Sample-to-solvent ratio, 1:20 w/v; room temperature; flow rate 1 mL/min	Antioxidant	Chaisawangwong and Gritsanapan, 2009
Ethanol (50, 80, 95%)	Leaves	Sample-to-solvent ratio, 1:20 w/v; 24 h extraction	Antioxidant	Sithisarn et al., 2006
Reflux				
Methanol	Seeds kernels and shell, leaves and leaves stem	10 s-20 min extraction	Determination of total Azadirachtin related limonoids	Dai, 1999

Table 2.3: *Continues*

Extraction technique and solvent	Plant material	Extraction condition	Objective	Reference
Soxhlet				
Acetone	Seed oil	1 h 30 min extraction; 56 °C	Pre-oviposition treatment	Lale and Abdulrahman, 1999
Hexane, chloroform, ethyl acetate, methanol (80%)	Leaves	NR	Anthelmintic	Al-Rofaai et al., 2012
Methanol	Leaves	72 h extraction	Antioxidant	Hossain et al., 2013
Hexane	Seed oil	NR	Repellency	Kebede et al., 2010
Ethanol (50%)	Flowers	Sample-to-solvent ratio, 1:50 w/v; 60-80 °C	Antioxidant	Chaisawangwong and Gritsanapan, 2009
Ethanol (80, 95%)	Leaves	Sample-to-solvent ratio, 1:50 w/v;60-80 °C; 6 h extraction	Antioxidant	Sithisarn et al., 2006
Non-Conventional Methods				
MAE				
Aqueous	Flowers	Microwave energy 2450 Hz single phase output 800 W; 70-85 °C, 8 cycles extraction	Antioxidant	Chaisawangwong and Gritsanapan, 2009
Dichloromethane	Seeds kernels	240 W; 1 min on, 30 s off, 1 min on extraction	Determination of total Azadirachtin related limonoids	Dai et al, 1999
Methanol	Seeds kernels, leaves and leaves stem	Microwave energy 2450 Hz, 150 W 10 s-20 min extraction	Determination of total Azadirachtin related limonoids	Dai, 1999

Table 2.3: *Continues*

Extraction technique and solvent	Plant material	Extraction condition	Objective	Reference
CO_2 SFE				
Petroleum ether	Seed oil	NR	Acaricidal	Deng et al., 2012
Chloroform	Seed oil	NR	Acaricidal	Du et al., 2009
Chloroform	Seed oil	NR	Toxic effect	Deng et al., 2013
Methanol	Seed kernels	NR	Extraction of nimbin	Tonthubthimthong et al., 2001
UAE				
Ethanol (50%)	Flowers	Sample-to-water ratio, 1:20 w/v; 30 min extraction	Antioxidant	Chaisawangwong and Gritsanapan, 2009

*NR: Not reported

2.4 CHROMATOGRAPHIC TECHNIQUES

Various chromatographic techniques were used as an aid in the separation and identification of constituents from a group of mixtures.

2.4.1. Gas Chromatography (GC)

The gas chromatography (GC) technique was reported for the identification and characterization of volatile compounds; whereas the non-volatile compounds need to undergo a derivatization process before being analyzed via GC (Tistaert et al., 2011).

GC works based on the principle of vaporizing the compounds by heat without decomposition to separate and analyzed the compounds. The vaporized analyte were carried by carrier gas, mobile phase, passing through the chromatography column, stationary phase. The choice of column was based on the type of samples polarity. As the analyte passes, they separate into its constituents according to characteristic rates and eluted compounds were detected in time sequence. The flow of the analysis was as shown in Figure 2.9.

This chromatography commonly attached with a sensitive detector either thermal conductivity (TCD), flame ionized (FID), mass selective (MSD), electron capture (ECD), nitrogen phosphorus (NPD), fourier transformed infrared, (FTIRD) and atom emission (AED) detector for identification purpose (Tistaert et al., 2011). The obtained data were in the form of qualitative and quantitative whereby various library suggestions could be obtained from the mass detector; and the intensity of the compound in terms of peaks area could be obtained from the percentage area report.

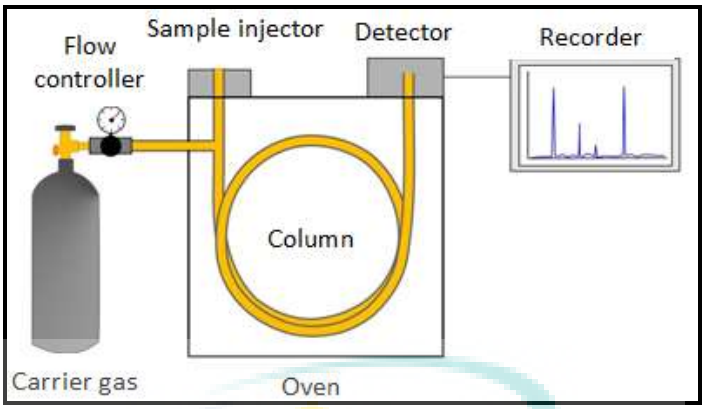


Figure 2.9: Schematic figure of GC

Source: Tistaert et al. (2011)

2.4.2 Thin Layer Chromatography (TLC)

Thin layer chromatography, an adsorption technique, was a chromatography method that was used for screening of compounds present in samples of natural products (Tistaert at al., 2011), stability tests of samples and quality control of final products (Shahid, 2012). This chromatography works based on two parameters that were the mobile and stationary phase whereby the stationary phase behaves as the adsorbent. The principle involved was the adsorptive and/or desorptive capacities that vary for each plant constituents. Ekiert at al. (2010) states that; the composition of the mobile phase influences the separation of the compounds. Capillary action was involved whereby the moving mobile phase carries the constituents according to its polarity towards the mobile phase causing separation of mixtures into single compounds.

In conducting the analysis, the mobile phase was prepared in a specific ratio and was transferred into a closed developing chamber and the atmosphere was allowed to saturate for a few min. The plant mixtures were spotted on the plate and the line was marked as a baseline. The spots were allowed to dry and the plate was transferred into the developing chamber and allowed to develop. The plate was removed and dried when the solvent reaches the solvent front line.

The analysis of the separation of the constituents of the mixture could be visualized under various detection methods. The heating process, ultraviolet light (fluorescent compounds) and detection reagents helps with visibility of certain targeted group of compounds. The visualized compounds were marked and the R_f (retention factor) value was calculated according to the ratio between the distance constituents travelled under standard chromatography conditions.

2.4.3 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) (Figure 2.10) works based on the application flow of mobile phase in high pressure (up to 400 bar) in a packed column. HPLC could be applied in separation, identification and quantitation of constituents present in a sample that could be dissolved by the liquid solvent.

HPLC has high sensitivity as low concentrations (ppt) constituents could be traced. The operation of HPLC starts with the flow of solvent, mobile phase, passing across the high pressure pump to generate a specified flow rate. Auto sampler introduces the sample into the flowing mobile phase and passes through the column, stationary phase containing chromatographic packing material. The eluted chromatogram bands were detected by detector wired into the computer data system. Several detectors could work with HPLC such as the UV absorbance (for UV light active compound), fluorescence (for fluorescent compound), and evaporate light scanning detector (ELSD) (as universal detector) (Waters, 2014).

As per Table 2.4, the chromatography technique had been reviewed and the pros and cons of each technique had been listed.

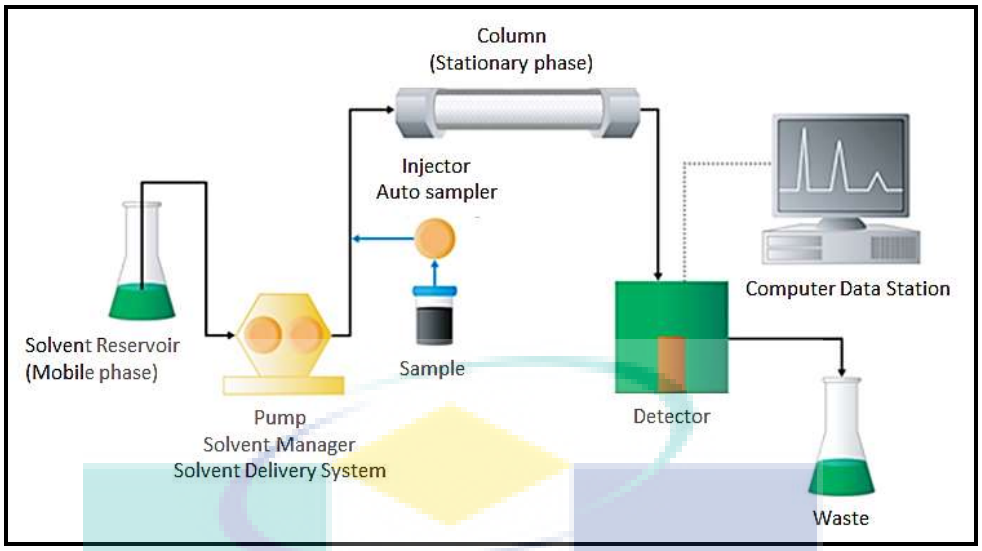


Figure 2.10: Schematic figure of HPLC

Source: Waters (2014)

Table 2.4: Pros and cons of various chromatography techniques

Chromatography Technique	Advantage	Disadvantage	Reference
TLC	Fast[a] Simple[ab] Less costly[ab] Easily optimized parameters (sample application and plate development)[a] Little sample clean-up and equipment[b]	Require specific adsorbent for certain detection reagent[a] Low reproducibility and resolution[a] Require high concentration of constituents in sample[a] Parameters (sample spotting, vapors environment in chamber and unstable colorization of detection reagent) were difficult to be controlled[a]	Tistaert et al., 2011[a] and Shahid, 2012[b]
GC	Shorter analysis time Lower detection limits	Suitable only for volatile and thermo-instable compounds Require derivatization for certain compounds	Tistaert et al., 2011
HPLC	Easy to operate and automatable technique with high resolution, selectivity and sensitivity Able to work with different detectors	Costly instrument Large volumes of environmental friendly solvents	Tistaert et al., 2011

Superscript (a and b) represents the corresponding reference.

2.5 SPECTROSCOPIC TECHNIQUES

Spectroscopic techniques were applied mainly for the identification of the pure constituent. The purity of the constituents plays major role in producing accurate results in identification.

2.5.1 Infrared (IR) Spectroscopy

One of the applications of Infrared spectroscopy (IR) was in Fourier Transform Infrared (FTIR) instrument. This method, specially detects the functional groups that were present through vibrations and bending of bonds in a compound. The molecules were excited to higher energy state when absorbs infrared radiation at selected frequencies (Lampman et al., 2010). The result was presented in spectrum that consists of a bond range from 4000-1600 cm^{-1}. Another region (1550-660 cm^{-1}) that was also present was the fingerprint region that usually gives less information on the functional group (Ngan et al., 2008).

2.5.2 Mass Spectrometry (MS)

Mass Spectrometry (MS) was an analysis to calculate and determine the mass-to-charge ratio (*m/z*) of ions presents in a specific compound; and thus gives an idea of the structure of the compound. The sample of a pure compound was introduced into the high ionization energy source to bombard the parent compound into gas phase ions. It passes through the electron stream; breaks into several fragments based on *m/z* and counted in the detector. The signal was recorded and processed by the data system. This calculation was done from the parent compound through total molecular weight and fragmentation pattern. The detected ion was then resulted in spectrum according to *m/z* in a magnetic field (Lampman et al., 2010 and Ngan et al., 2008).

2.5.3 Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance was known for the purpose of structure elucidation through identification of the detailed chemical structures. There were several sub-

methods that should be adapted in producing a complete result in the structure identification. Some of the methods were the one dimensional technique (1D-NMR) and two dimensional techniques (2D-NMR) (Ngan et al., 2008).

The 1D-NMR includes several analyses that were the proton NMR (^{1}H-NMR) and carbon NMR (^{13}C-NMR). The ^{1}H-NMR data provide information regarding the environment of protons present in a specific structure. It gives a value of chemical shift (usually 0-12 ppm) via various splitting patterns. The splitting pattern relies on the environment of the neighboring proton that could be either vicinal or germinal protons. The overall spectrum show the chemical shift (δ) values of different type of protons and the signal intensities; whereas the ^{13}C-NMR provides information on the types of carbons present and represented in terms of chemical shift (usually 0-230 ppm). The carbon types include the primary, secondary, tertiary and quaternary carbons. In carbon analysis, Distortionless Enhancement by Polarization Transfer (DEPT) analysis provides information on the number of hydrogen attached to a specific carbon (Lampman et al., 2010 and Ngan et al., 2008).

The 2D-NMR involves the Correlated Spectroscopy (COSY) analysis that identify mutually coupled protons involving coupling networks. Besides that, the Heteronuclear Correlation Spectroscopy (HETCOR), Heteronuclear Multiple Quantum Correlation (HMQC) and Heteronuclear Multiple Bond Correlation (HMBC) were also listed in 2D-NMR. The HETCOR analysis gives information relating the specific proton to a specific carbon through direct bonding whereas the HMQC relates the protons with the heteronuclei in terms of heteronuclear scalar coupling. This technique is used to ignore the proton signals that was not coupled to the heteronuclei by providing a spectrum that indicates which protons was attached to which carbon and its nature. The analysis does also provide a spectrum consisting of different spots representing the proton and carbon signals in two different axes, respectively. The HMBC identified the coupling of proton and carbon through two or three bonds away and finally elucidates the overall skeleton of the compound (Lampman et al., 2010 and Ngan et al., 2008).

2.5.4 Ultraviolet (UV) Spectroscopy

The Ultraviolet spectroscopy (UV) was used to identify the conjugated structure of a compound. The concept of energy was used to conduct transitions on the outer core electron. The electron that absorbs energy was promoted from an occupied orbital to an unoccupied orbital of greater potential energy. The class of the compounds was identified via change of wavelength from maxima to higher or lower λ max whereby the value provides prove on identifying the unknown constituent (Lampman et al., 2010 and Ngan et al., 2008).

The transitions include various energy levels according to different single atoms such as the oxygen (O), nitrogen (N) and sulfur (S) and bonded atoms such as the C=O, C=N, N=N, NO_2, -COOR, -COOH. The analysis via UV could be done according to dilution of sample in an appropriate solvent (acetonitrile, chloroform, cyclohexane, ethanol, n-hexane, methanol, isooctane and water). The choice of the solvent was important to avoid absorption at the same range as the sample (Lampman et al., 2010 and Ngan et al., 2008).

The advantage and disadvantage of the spectroscopy technique of IR, MS, NMR and UV were as tabulated in Table 2.5 and the application of these instruments with the combination of chromatography technique adapted in analyzing the compounds and extracts of *A. indica* were as listed in Table 2.6.

Table 2.5: Pros and cons of various spectroscopy techniques

Chromatography technique	Advantage	Disadvantage	Reference
IR	High speed and sensitivity	Require sample preparation	Ngan et al., 2008
MS	Measurement via qualitative and quantitative High sensitivity High analysis speed Simultaneous detection of multiple analytes Promise of isotope analysis Elucidating chemical structures with fragmentation	High vacuum and power consuming High precision requires often calibration Matrix effect Requiring sample pretreatment	Zhu and Fang, 2013
NMR	Capability of elucidating chemical structures	Poor sensitivity Poor analysis speed Require sample pre-treatment	Zhu and Fang, 2013
UV	Non-destructive of samples Most organic molecules absorbs	Require frequent calibration to maintain accuracy and precision High detection limit Absorption depends on solution conditions	Chester and Winefordner, 1977

Table 2.6: Chromatography and spectroscopy techniques adapted for *A. indica* analysis

Chromatographic technique	Analyte matrix	Objective	Separation remarks			Spectroscopic techniques	Reference
			Mobile phase	**Stationary phase**	**Others**		
GC	Seed oil	Fatty acid composition analysis	Helium gas (Flow rate: 1 mL/min)	HP-INNO Wax capillary column Hewlett Packard (30 m, 0.25 mm i.d. and 0.25 m film thickness)	Injection volume: 1 mL of FAME Split ratio: 1/80 Oven temperature: 150 °C for 3 min, increased at 3 °C/min to 220 °C and kept at 220 °C for 35.3 min. Injector temperature: 250 °C	MS	Djenontin et al., 2012
GC	Seed oil	Sterol content	Helium gas	Supelcowax (25 m, 0.25 mm i.d., 0.25 mm film thickness)	Injector temperature: 280 °C Split ratio: 1/100 Oven temperature: 285 °C FID temperature: 300 °C	FID	Djenontin et al., 2012
HPLC	Seed oil	Tocopherol analysis	Hexane-isopropanol (99/1, v/v) (Flow rate: 1 mL/min)	Luna silica Si 60 column (5 m, 25 cm i.d. and 4.6 mm film thickness)	10 mg of the crude oil was dissolved in 10 mL of hexane	UV DAD (295 nm)	Djenontin et al., 2012

Table 2.6: *Continues*

Chromatographic technique	Analyte matrix	Objective	Separation remarks			Spectroscopic techniques	Reference
			Mobile phase	**Stationary phase**	**Others**		
GC	Leaves extract	Volatile compounds	Helium gas (Flow rate: 1 mL/min)	Polysiloxanes fused capillary silica column (30 m, 0.25 m)	Injection port temperature: 200 °C Oven temperature: 50 °C increase of 8 °C/min to 300 °C and and held for 9 min. Ionization voltage: 70 eV. Splitless mode	MS (45-500 MHz)	Akpuaka et al., 2013
HPLC	Bark, leaves and seeds fraction	Quantitification of Azadirachtin and Nimbin	Water (solvent A) and acetonitrile (solvent B) (Flow rate: 1 mL/min)	C18 column Phenomenex Synergi 4u MA-RP 80A (150 mm, 4.6 mm, 4 µm)	Mobile phase flow: Gradient elution: 0-10 min, 30-40% B; 10-15 min, 40-45% A; 15-20 min, 45-50% B; 20-25 min, 50-60% B; 25-35 min, 60-70%. Injection volume: 10 µL	UV (217 nm)	Ghimeray et al., 2009
NR	Seed oil	Transmission characteristics	(*Commercially available product*)			UV-Vis	Kumar and Viswanathan, 2013

Table 2.6: *Continues*

Chromatographic technique	Analyte matrix	Objective	Separation remarks			Spectroscopic techniques	Reference
			Mobile phase	Stationary phase	Others		
TLC	Leaves extract	Isolation of Citrinin	Toluene-ethyl acetate-formic acid (6:4:0.5, v/v)	Silica gel aluminium plate	NR	UV-Vis (365 nm)	Abou-Zeid, 2012
HPLC	Seeds	Quantification of Azadirachtin	Acidified water (pH 3, 67% H_3PO_4 -33% Acetonitrile (Flow rate: 1 mL/min) 20% water -80% acetonitrile (both containing 0.3% acetic acid) (Flow rate: 0.2 mL/min)	LC18 Column SigmaeAldrich (25 cm, 4.6 mm, 5 mm)	NR	UV (215 nm)	Boursier et al., 2011
LC	Seeds	Quantification of Azadirachtin	Acidified water (pH 3, 67% H_3PO_4 -33% Acetonitrile (Flow rate: 1 mL/min) 20% water -80% acetonitrile (both containing 0.3% acetic acid) (Flow rate: 0.2 mL/min)	Pursuit 5 C18 column (3 mm, 150 mm, 2 mm)	N_2 gas (25 psi) Drying gas temperature and pressure: 250 °C and 25 psi Capillary voltage: 35 kV	MS/MS (Negative ion mode)	Boursier et al., 2011

Abbreviation: NR: Not reported

Table 2.6: *Continues*

Chromatographic technique	Analyte matrix	Objective	Separation remarks			Spectroscopic techniques	Reference
			Mobile phase	Stationary phase	Others		
HPLC	Seeds kernels	Quantification of Azadirachtin	Acetonitrile-water (4:6) (Flow rate: 1 mL/min)	Waters Spherisorb ODS-25 column (4.6 mm, 25 cm i.d., 5 µm)	NR	UV (214 nm)	Dai et al., 1999
CC	Seed oil	Isolation and identification of bioactive compounds	Hexane-ethyl acetate acid (8:2,v/v) mixture	Silica gel G (100– 200 mesh)	NR	IR, EI-MS, DEPT and NMR	Du et al., 2009
GC	Leaves extract	Volatile compounds	Helium gas (Flow rate: 1 mL/min)	Fused silica capillary column (30 m,0.25 i.d., 0.25µm)	Oven temperature: 50 to 150 °C at 3 °C/min, held for 10 min and raised to 300 °C at 10 ◦C/min. Samples dilution: 1/100, v/v. Split ratio: 1:120.	MS	Hossain et al., 2013

Abbreviation: NR: Not reported

Table 2.6: *Continues*

Chromatographic technique	Analyte matrix	Objective	Separation remarks			Spectroscopic techniques	Reference
			Mobile phase	Stationary phase	Others		
GC	Leaves and flowers essential oil	Chemical composition analysis	Helium gas (Flow rate: 1 mL/min)	HP-5MS column (30 m, 0.25 mm, 0.25 μm)	Oven temperature: 3 min to 260 °C at 8 °C/min and held for 10 min Split ratio:60.1 Injector and detector temperature: 225 and 300 °C Ionization mode: 70 eV Electron multiplier: 2500 V Ion source temperature: 250°C	MS (50 to 700 *m/z*)	El-Hawary et al., 2013
CC	Leaves extract	Isolation and identification of quercetin	Chloroform-methanol mixtures	NR	NR	FTIR	Chourasiya et al., 2012
HPLC	Seeds kernels	Quantification of Azadirachtin	Acetonitile-Water (35:65% (v/v)) (Flow rate: 1 mL/min)	Purospher-Star RP 18-e column (250 mm x 4.6 mm i.d, 5µm).	Injection volume: 20 µL. Isocratic elution duration: 25 min	UV-Vis (217 nm)	Jadeja et al., 2011

Abbreviation: NR: Not reported

Table 2.6: *Continues*

Chromatographic technique	Analyte matrix	Objective	Separation remarks			Spectroscopic techniques	Reference
			Mobile phase	Stationary phase	Others		
GC	Leaves extract	Analysis of phthalate isolates	NR	Polysiloxanes fused capillary silica tubing column (30 x 0.25 m)	Injection port temperature: 200 °C and Oven temperature: 50 °C with an increase of 8 °C/min to 300 °C and was held for 9 min. Ionization voltage: 70 ev. Splitless mode. Run time: 36 min	MS (45-500 MHz)	Akpuaka et al., 2013
TLC	Leaves extract	Flavonoid detection	Chloroform-ethyl acetate-methanol-formic acid (7: 2:1: 1 v/v/v/v)	Precoated silica gel 60 GF 254 plate (10 x 10 cm)	NR	UV (366 nm)	Sithisarn et al., 2006
CC	Seed oil	Antibacterial activity	Hexane-acetic ether (8:2, v/v)	Silica gel (60 x 4 cm, id) (400 g, 100–200 mesh)	NR	IR, EI-MS, DEPT, NMR, XRC-1 microscope	Zhang et al., 2010

Abbreviation: NR: Not reported

2.6 PHYSICOCHEMICAL PROPERTIES

The physicochemical properties were related to the physical state and chemistry of a substance or a medium. Specifically for lipids (fats and oils), each of them might vary according to different physicochemical properties and various factors might be influencing them. The examples, physical properties were the physical state, color, odor, density, refractive index (RI) and UV-Vis transmission, whereas the chemical properties were the acid value (AV), free fatty acid (FFA), iodine value (IV), peroxide value (PV), unsaponifiable matter and pH.

The various colors, for example the oil, were attributed by the genetics of the plant according to the family and volatile matter. At times, the moisture level has also played the role in the color pigmentation (Ziyada and Elhussien, 2008). The AV was the number of FFA in a sample as a result of enzymatic activities. The enzymatic activities caused hydrolysis through the removal of the water molecule and the values were expressed in mg of base required to neutralize the acidic constituents per g of oil (Erakhrumen, 2011). The IV analysis was conducted to calculate the number or degree of unsaturation or the number of double bonds occurs in the specific mixtures of fatty acids. The values were reported in the form of number of iodine per 100 g. The usual value permitted for vegetable oils were 120 g/I_2 according to the European Biodiesel standard (Sokoto et al., 2011)

The PV and FFA were to measure the deterioration of lipid during storage. The PV proves the oxidative stabilities or rate that had been undergone by the oil whereby it was actually a value of rancidity index (Goja, 2013). This was influenced by the presence of oil. The higher value indicates the less resistance of the oil on the exposure towards air and thus deteriorated the lipids (Mohammed and Hamza, 2008). The strong, unpleasant smell or taste of the fats or oils shows that the sample had undergone rancidity (Erakhrumen, 2011). The FFA measures the deterioration due to exposure towards moisture or water. Fatty acids were the carboxylic acids attached with long hydrocarbon chains that could be as long as 10-30 carbons and it determines the polarity of the oil. The fatty acid has two different classes that were the saturated and non-saturated and the physicochemical properties were mainly dependent on this (Bachheti

et al., 2012). The fatty acid composition of oil was usually being analyzed for the purpose of product formulation for different purposes, for example, in the aspect of nutrition, industrial usage and pharmaceutical. The various fatty acids had been identified and been proven for many activities in terms of biological and chemical studies (Goja, 2013).

The RI gives a value of the intensity of a light passing through a medium and represented by the degree of unsaturation or conjugation (Arya et al., 1969). Unsaponifiable matter represents the matter that could not be saponifiable with the alkali hydroxides but could dissolve in ordinary fat solvents.

2.7 SUMMARY

This chapter begins with the discussion of plant *A. indica* that describes further on the nature of the plant in terms of its physical, distribution and chemical compounds. It also touches on the biological study of the plant that contributes towards isolation and identification of several bioactive compounds. Extraction technique could also be essential to be studied in order to give an idea of the suitablity and effective technique for the production of better yield. Next were the chromatography and spectroscopy techniques that commonly used in the area of natural products for the plant analysis. The summary of the techniques gives a clear description about the purpose of the study and finally the elaboration on the physicochemical properties gives an image on several factors that might had influenced the properties in general.

CHAPTER 3

MATERIALS AND METHODS

3.1 INTRODUCTION

The overall chapter discusses the process and procedures that were involved in this current study. The chapter was divided into two sub-sections that starts with materials and equipment's used to complete the study and the second parts discusses the experimental procedures and processes practiced. The second part mainly touches on the methods of extraction of the crude and fractions, volatile compound analysis, cytotoxicity test and isolation of compound. It does also include the study on the physicochemical properties and antibacterial test. These two sub-sections were discussed in detail in the following subtopics.

3.2 MATERIALS

The raw plant materials involved in this study were the different parts (bark, leaves, roots and seeds) of *A. indica*. All reagents and solvents used were of analytical and chromatography grade, unless specified. They were obtained from Merck (Germany), Fisher Scientific (USA) and Sigma-Aldrich (USA).

3.3 EQUIPMENT'S

All the instruments and equipment's being used in this current research were being provided by the Laboratory of Faculty of Industrial Sciences & Technology (FIST), Universiti Malaysia Pahang (UMP). Equipment's that were used (UV short and long lamp cabinet (Camag, Switzerland), prep-HPLC 25455 Binary Gradient Module

System (Waters, USA), rotary evaporator (Butchi, Switzerland), drying oven (Binder, Germany), freeze-drier (Labconco, USA) analytical balance (Mettler Toledo, Switzerland), moisture content analyzer (AND MS-70, Japan), melting point apparatus Barnstead electrothermal (Thermo Scientific, USA), Spectrum 100 FT-IR Spectrometer (Perkin Elmer, USA), heating mantle (Mtops, Korea), 1290 Infinity LC system-6250 Accurate-Mass Q-TOF (Agilent, USA), UV 2600 UV-Vis spectrophotometer (Shimadzu, Japan), 7890A GC Systems- MS (Agilent, USA), 500 MHz NMR Spectrometer (Bruker, USA), Refractometer (Atago, Japan), electrical blender (Panasonic, Malaysia), TLC glass reagent sprayer (Analtech, USA), capillary tube (Iso Lab, Germany), TLC plates (Analtech, USA and Merck, Germany), pH indicator strips (Merck, Germany), filter paper (Whatman No.41, USA), soxhlet (Favorit, Malaysia) and etc. were of standard laboratory types. Glassware's that were used for experiments were cleaned with chromic acid and distilled water before drying in an oven.

3.4 SAMPLE PREPARATION AND PROCEDURES

This sample preparation and procedures include the steps in the plant material source, solvent extraction and fractionation, identification of volatile compounds, cytotoxicity test, isolation of compound, determination of physicochemical properties of the seed oil and antibacterial test.

3.4.1 Plant Materials

The plant, *A. indica* used in this research was harvested directly from the tree from Teluk Intan, Perak, Malaysia on 26 August 2012. The taxonomy identification of the plant was done by a botanist of the School of Environmental Sciences and Natural Resources, Universiti Kebangsaan Malaysia, Bangi, Selangor. The freshly plugged samples were separated into four different parts, which were the bark, leaves, roots (1 kg each part) and seeds (30 g) and were placed on empty papers in an open area of a room and left to dry for about one month. Prior to grinding, the percentage moisture content of the plant materials was analyzed via moisture content analyzer and the values were as in Table 3.1.

The dried samples (moisture less than 8%) which were the bark, leaves and roots were ground into coarse powder by using grinder at the uniform size of 0.25 mm sieve. The seeds kernels were removed manually from the dried seeds. Then, the seeds were also analyzed for moisture content and were further crushed by using mortar and pestle to reduce the particle size to a maximum and uniform diameter of 0.25 mm as measured by a sieve. All the samples were then sealed and kept in a desiccator to avoid any fungal activities before it was being extracted.

Table 3.1: Moisture content of dried *A. indica*

Part of plant	Moisture content (%)
Bark	5.896
Leaves	3.240
Roots	7.456
Seeds	5.650

3.4.2 Extraction of Plant Materials

Two different methods, crude extraction via maceration technique and fractionation were adapted to extract the dry extracts from the bark, leaves and the roots. In the case of oil extraction, the seeds were extracted via soxhlet.

A) Solvent Extraction Method

The crude extracts of grounded parts, bark, leaves and roots (each 50 g) were extracted through solvent extraction method by using maceration in acetone, chloroform, ethanol and reflux in ethanol. The maceration process was conducted for 5 days, whereas the reflux method was conducted for minimum extraction of 6 h. Prior to soaking and reflux, the extracts were filtered via filter paper and were concentrated by evaporating the solvents through the rotary evaporator at the temperature of 40 °C and further drying under open air on petri dishes. The dried extracts were scrapped and weighted and the yields were tabulated.

B) Plant Tissues Extraction and Fractionation Method

The fractionation of plant extracts as referred to Hossain et al. (2013) with some modification. Ground parts of the plant (bark, leaves and roots, 200 g each) were soaked separately with 80% methanol (ratio 4:1, methanol: water). The mixture was left to settle for a day. The solution cakes formed was sonicated in an ultrasonic bath for about 30 min and were filtered out. The filtered residue was added with methanol and water (4:1), shaken for 30 min and filtered. This step was repeated for 3 cycles. The filtrates were collected and evaporated by rotary evaporator until one third (1/3) of the extract was left.

The concentrated extract was further transferred into separating funnel and partitioned with different organic solvent (hexane, chloroform and ethyl acetate, respectively) with increasing polarity. Hexane was added and shaken for 30 min. This step was repeated for 3 cycles. The aqueous and organic layer of the plant extract was separated using separating funnel. The organic layer was collected and evaporated using a rotary evaporator until one third 1/3 of the extract was left. The concentrated organic extract was obtained as Fraction 1. Next, the aqueous layer was partitioned with solvent chloroform and ethyl acetate, respectively. The respective layers were separated, evaporated and labeled as Fraction 2 and 3. Finally, the leftover of the aqueous layer was removed from the separating funnel as Fraction 4. All collected fractions were filtered using filter paper to obtain particle free extracts, dried and stored in a chiller for further study.

C) Solvent Semi-Continuous (Soxhlet) Extraction Method

The seed oil was extracted via soxhlet by using n-hexane and mild extraction temperature was chosen to avoid thermal degradation of bioactive compounds in the extracts (Kittiphoom and Sutasinee, 2013). The crushed seeds were placed in the drying oven at 40 °C for 30 min prior to extraction. The seeds were loaded in the thimble of soxhlet apparatus and the bottom part was fitted with 500 mL round bottom flask. Sufficient amount of absolute hexane were added into the flask and the top part of the soxhlet was fitted with a condenser. Constant heat was applied through the heating

mantle and the extraction was conducted for a minimum extraction of 6 h to make sure maximum oil was extracted. After complete extraction and cooling, the obtained oil was filtered through filter paper, evaporated via rotary evaporator and further dried under open air in a dark area. The yield of the oil was calculated and stored in hermetically closed dark bottles and kept in a refrigerator for further physicochemical and bioassay study. The summarized extraction conditions were shown in Table 3.2.

Table 3.2: A brief summary on the experimental conditions of various extraction methods for *A. indica*

	Extraction method			
	Maceration	**Reflux**	**Fractionation**	**Soxhlet**
Plant part	Bark, leaves, roots	Bark, leaves, roots	Bark, leaves, roots	Seeds
Solvent	Acetone, chloroform and ethanol	Ethanol	Extracted with 80% methanol and partitioned with hexane, chloroform, ethyl acetate and aqueous	Hexane
Temperature	25 °C (Room temperature)	60 °C	25 °C (Room temperature)	NR
Duration	5 days	6 h	*Depending on the separation duration*	6 h

Abbrevation; NR: Not related

3.4.3 Determination of Volatile Compounds

The volatile compounds in both crude extracts and fractions of all selected parts (bark, leaves and roots) of the plant were determined through chromatographic method via Agilent Technologies 7890A GC Systems coupled with MS detector. Each of the samples was prepared at the same concentration of 1000 ppm via dilution in respective solvents and was filtered. The particle free diluted extracts were injected into the system. As for comparison, the blank analysis was also being conducted. The details of the chromatography equipment and settings were in Table 3.3. The organic chemical compounds were identified and characterized in various extracts based on GC retention time and the mass spectrum were matched with those of standards available in existing computer library data system (National Institute of Standards and Technology (NIST) Library Chem Station software).

Table 3.3: Chromatographic settings for the analysis of volatile compounds in crude and fractions of *A. indica*

Parameter	Organic sample	Aqueous sample
Chromatograph	Agilent Technologies7890A GC Systems coupled with MS detector	Agilent Technologies7890A GC Systems coupled with MS detector
Sample inlet	GC	GC
Injection source	GC auto sampler	Thermal separation probe (TSP)
Column	Nonpolar capillary DB-1 of 100% dimethyl-polysiloxane (30 m x 0.53 mm id, film thickness 0.25 µm)	Nonpolar capillary DB-1 of 100% dimethyl-polysiloxane (30 m x 0.53 mm id, film thickness 0.25 µm)
Carrier gas	Helium	Helium
Gas flow rate	1mL/min	1mL/min
Injection volume	1 µL	NR
Injection mode	Splitless	Split ratio 1:5
Ionization energy	70 eV	70 eV
Oven equilibrium time	3 min	3 min
Maximum oven temperature	350 °C	350 °C
Oven temperature program	Initial 35 °C, an increase in the rate of 6 °C/min to 180 °C held for 5 min and increase at the rate of 1 °C/min to 230 °C held for 20 min	Initial 35 °C for 2 min, increase in the rate of 2 °C/min to 180 °C held for 5 min and increase at the rate of 6 °C/min to 230 °C held for 30 min
Post run temperature	290 °C	290 °C
Front inlet temperature	250 °C	250 °C
Front inlet pressure	6.78 psi	6.78 psi
Runtime	99.17 min	117.83 min
MS Source	230 °C	230 °C
MS Quad	150 °C	150 °C
Lab data system	NIST Library Chem Station software	NIST Library Chem Station software

*Abbrevation; NR: Not related

3.4.4 Cytotoxicity Test

A) Preparation of Samples

The appropriate amount of each extract was dissolved in artificial sea water with 0.5 mL of dimethyl sulfoxide (DMSO) (Merck, Germany) to prepare 50 mL of extract with final concentrations of 50, 100, 500, and 1000 ppm. Positive and negative controls were used along with test samples for comparison. The positive control was 0.1% of potassium dichromate (Sigma-Aldrich, USA) (Control 1); whereas negative control was 1% DMSO (Control 2).

B) Source of Seawater and Test Organisms

Artificial sea water of 35 ppt was prepared by dissolving 35 g of commercial sea salt in 1 L of distilled water and was filtered to obtain clean solution. The solution was transferred into a cylindroconical tube (hatching system) and a certain amount of 1.5 g/L of *A. salina* eggs (Super Eagle, China) were added and allow for hatching. The eggs were exposed to an optimal incubation period of 24 h at room temperature of 27-29 °C with lateral illumination by a light tube (500-1000 Lux) and gentle aeration from the bottom of the hatching device to maintain the egg in suspension and to ensure the supply of oxygen. Once a while, the solution was swirled to maximize the percentage of hatching cysts by knocking down the eggs that have been stranded on the side of the container above the water-line. After 24 h of incubation, the aeration was stopped to separate the nauplii that settle at the bottom of the container from the hatched shells that floats on the water surface. Collected nauplii were placed into separate containers containing the same concentration of artificial seawater, sufficient light source and gentle aeration. Feeding of nauplii with 15 mL/L of 0.06% yeast solution was done starting from the first day of hatching up to 48 h of bioassay study.

C) Determination of Cytotoxicity

The lethality bioassay was carried out using 48 h old *A. salina* to test the cytotoxicity of the plant extracts as proposed by Meyer et al. (1982) with minor

modification. The test was conducted using prepared test samples (50, 100, 500, and 1000 ppm) as well as positive and negative controls. An amount of 3 mL of each test sample and controls were transferred into different labeled test tubes and a number of 10 actively swimming nauplii were released into them. The test tubes were stored at room temperature at about 27-29 °C. The lethality of the nauplii were observed within its activity range and evaluated after 24 h of exposure. Moribund nauplii within 10 s were counted as dead. The assay was performed in triplicate and values were recorded.

The data on the *A. salina* bioassay were analysed according to lethality. Lethality data on the triplicate reading were calculated through percentage mortality according to Eq. (3.1) and corrected lethality in relation to the negative control were calculated and corrected by applying Abbott's formula as in Eq. (3.2) (Abbott, 1987). The corrected percentage mortality was transferred into a graph whereby the data were evaluated by establishing dose-response curve against the percentage lethality of the nauplii through probit analysis method with the presence of linear functions as described by Finney (1971). Cytotoxicity and lethality were reported as LC_{50} that represents 50% of nauplii moribund or killed within the time interval. The significant difference of the LC_{50} values were also statistically analyzed through ANOVA analysis and the difference between the treatment were determined by Tukey's multiple range test ($P < 0.05$) (Nathan et al., 2005).

$$\% \text{ Mortality} = \frac{\text{Number of moribund or dead nauplii x } 100\%}{\text{Total number of tested nauplii per replicate}} \tag{3.1}$$

$$\text{Corrected \% Mortality} = \left(1 - \frac{\text{Number of nauplii in treated sample after treatment}}{\text{Number of nauplii control after treatment}}\right) \text{x } 100\% \tag{3.2}$$

3.4.5 Isolation of Quercetin-3-*O*-β-D-Glucopyranoside

A) Separation of Quercetin-3-*O*-β-D-Glucopyranoside from Fraction

The separation of the quercetin-3-*O*-β-D-glucopyranoside was performed via chromatography techniques.

i) Analytical and Preparative-High Performance Liquid Chromatography (Prep-HPLC) Analysis

The HPLC separation was performed on the leaves ethyl acetate fraction that was the most active fraction of cytotoxicity against the *A. salina*. The sample was screened for the presence of compounds via analytical method before conducting the preparative technique. The separation of 10 µL mixtures was conducted with prep-HPLC 25455 Binary Gradient Module System with analytical column C18-Zorbax Eclipse XDB (4.6 x 150 mm i.d; particle size 5 µm) by using two chromatographic pumps. The mobile phase was 100% methanol (solvent A) and water (solvent B). Before delivering the solvents into the system, it was filtered through 0.45 µm PTFE filter and degassed via sonication. The analysis was performed via gradient condition (0 min: 100% solvent B; 50 min: 100% solvent A and 60 min: 100% solvent B) at the flow rate of 1 mL/min at room temperature (25 °C). Chromatograms were recorded at 200-360 nm through diode array detector.

Higher volume of sample, 210 µL, was injected into the system and further separation and collection of the targeted compound were done through the preparative column of C18-Zorbax Eclipse XDB (21.2 x 150 mm i.d; particle size 7 µm) and fraction collector. The solvent system was the same as the analytical analysis and the flow rate were increased at 19 mL/min. The pooled compound was re-analyzed by analytical technique to check the purity prior to characterization.

ii) Freeze-Drying Method

The pooled compounds in aqueous condition was frozen overnight at -80 °C and then lyophilized for a period of 36 h via freeze-drier with the temperature and pressure of -84 °C and 50 mbar, respectively. The dried powder of the compounds were then stored in sample bottle for further evaluation.

B) Identification of Quercetin-3-*O*-β-D-Glucopyranoside

The identification of quercetin-3-*O*-β-D-glucopyranoside was performed via several spectroscopy techniques.

i) Appearance, Solubility and Melting Point

The appearance was done by visual analysis in terms of the physical state and color. A small amount of dried compound was dissolved in chloroform, dichloromethane, DMSO, ethanol and methanol; and was visually determined for solubility. The melting point was determined via melting point apparatus. The compound was loaded in one end closed capillary tube to about 1-3 mm thick and was placed into the apparatus. The range of the melting point was recorded from the point the compound starts to melt until complete melting.

ii) Ultraviolet-Visible (UV-Vis) Spectroscopic Analysis

UV-Vis spectrum analysis was performed with Shimadzu UV 2600 UV-Vis spectrophotometer and UV Probe 2.43 software using standard 1.00 cm quartz cell. The sample was dissolved in methanol and was scanned from 200-800 nm. The scanning resolution was 2 nm and the slit width was 1 nm. The measurement was conducted in the mode of absorbance.

iii) Fourier Transform Infrared (FT-IR) Spectroscopy Analysis

Fourier transform IR spectrum was recorded on a FT-IR Spectrometer with Perkin Elmer Spectrum Express software at room temperature of 25 °C. Sample was

prepared in potassium bromide disk with the hydrostatic press force of 5.0 Tcm^{-2} for duration of 15 s. The scanning range was 400-4000 cm^{-1} with resolution and slit width of 4 cm^{-1} and 1 nm. The background analysis was conducted prior to sample and both scanning frequency was 10. The ordinate mode for the spectrum was the percentage transmittance.

iv) Liquid Chromatography-Mass Spectrometry (LC-MS/MS) Analysis

The compound was reconstituted with 1 mL of methanol and was filtered with 0.45 µm, hydrophobic PTFE syringe filter. The analysis was performed on the LC system coupled to mass spectrometer with dual Electrospray ionization (ESI) source. The column used was the Agilent Zorbax SB-C18, Narrow Bore (2.1 x 150 mm id., 3.5 µm) with two different solvent, 0.1% formic acid in water (solvent A) and 100% methanol with 0.1% formic acid (solvent B). The solvent flow was conducted in gradient condition (0 min: 3% solvent B; 0.5 min: 3% solvent B; 15 min: 100% solvent B; 19 min: 100 solvent B) with the flow rate of 0.4 mL/min and sample injection volume of 1 µL. The post and total run time were 4 min and 23 min. The parameters of the MS were negative for ion polarity and the data acquisition ranges from 115-1000 *m/z*. The data was processed with Agilent MassHunter Qualitative Analysis B. 05.00 software.

v) Nuclear Magnetic Resonance (NMR) Analysis

The 1D ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of the compound in methanol-d_4 (CD_3OD) solvent was obtained on a NMR Spectrometer. Chemical shifts were expressed in δ (ppm) downfield from TMS as an internal standard (δ = 0 ppm) and coupling constant were reported in Hz. The 2D analysis of COSY, HMQC and HMBC were performed from the obtained 1D data. Data acquisition, conversion and analysis were controlled under Bruker TopSpin version 1.3.

3.4.6 Physicochemical Characteristics of *A. indica* Seed Oil

The properties of the seed oil were studied according to two main characters that were the physical and chemical properties. Besides, the lipid content was also studied to evaluate the percentage yield that was obtained from the extraction.

A) Lipid Content Determination

The lipid content of the oil was calculated on the basis of dry seed weight that were used in the extraction and expressed in percentage. The mass (g) of the obtained oil was recorded using experimental balance with accuracy of ± 0.001 g and the lipid in the sample was calculated according to Eq. (3.3).

$$\% \text{ Lipid} = \frac{\text{Weight of oil (g)} \times 100\%}{\text{Weight of sample (g)}} \quad (3.3)$$

B) Physical Properties of Seed Oil

The physical characters were studied according to six different aspects as physical state, color, odor, density, reflactive indiex and UV-Vis transmission. The methods of the analysis were explained in details accordingly in the following subtopics.

i) Physical State, Color and Odor Determination

Physical state at room temperature of 25 °C and color of the oil were determined visually whereby odor was determined through sensation through volatilized smell.

ii) Density Determination

The weight of a small empty vial was weighed and was filled with known amount of oil up to the brim. The vial was weighed again and the density was calculated according to Eq. (3.4).

$$\text{Density, } \rho = \frac{\text{(Weight of vial + oil (g)] – [Weight of empty vial (g)]}}{\text{Volume of oil}} \quad (3.4)$$

iii) Refractive Index (RI) Analysis

The RI of the oil was determined by using standard method AOAC Official Method 921.08 (1990). This index was measured at 25 °C via pen Refractometer with resolution and accuracy value of 0.1% and ± 0.2% in 10-60 °C. The pen tip was dipped into the sample and the start key was pressed to obtain the reading. The measurement was repeated in triplicate and the average value was reported.

iv) Ultraviolet-Visible (UV-Vis) Transmission Analysis

A dual beam UV-Vis Spectrophotometer was used to study the transmission characteristics of the oil. The UV transparent quartz cell with the dimension of 10 x 1 mm were loaded with 0.25 mL of sample and scanned at the range of 190-800 nm. The transmission of the blank quartz cell characteristics was taken prior to analysis of each sample. The obtained readings were then transformed into a graph.

C) Chemical Properties of Seed Oil

Various chemical properties were evaluated as it was listed to be the acid value, free fatty acid, iodine value, peroxide value, fatty acid composition, pH and volatile matter analysis.

i) Acid Value (AV) Analysis

The AV was determined through direct titration methods of oil in an alcoholic medium against standard potassium hydroxide (KOH) via AOCS Official Method Cd 3a-63 (2009) with some modification. A mass of 0.5 g of oil was weighed into 250 mL conical flask and 50 mL of freshly neutralized hot ethanol and 1 mL of phenolphthalein indicator solution were added. The mixtures were boiled around 5 min and were titrated

against standardized sodium hydroxide (NaOH) (0.24 M). The AV was then calculated according to Eq. (3.5).

$$AV = \frac{[56.1]\,[\text{Titration of standard (mL)}]\,[\text{Molarity of standard (M)}]}{\text{Weight of sample (g)}} \quad (3.5)$$

ii) Fatty Acid Composition and Percentage of Saturated and Unsaturated Fatty Acid Analysis

The crude oil was analyzed as methyl ester to determine the fatty acid composition. The oil was converted into fatty acid methyl ester through transesterification reaction. A solution of KOH (2 M) was prepared. An amount of 2 mL of oil sample was dissolved in 10 mL of hexane in a test tube. An amount of 1 mL of KOH was added into the same test tube and was vortex. The hexane phase was collected and washed twice with 4 mL of water after 15 min and was further dried over an anhydrous Na_2SO_4. The fatty acid composition analysis was performed on GC-MS. The details of the chromatography equipment and settings were tabulated in Table 3.4.

Table 3.4: Chromatographic settings for the analysis of *A. indica* oil methyl ester

Parameters	**Settings**
Chromatograph	Agilent Technologies 7890A GC Systems coupled with MS detector
Auto-sampler	GC auto sampler
Column	Nonpolar capillary DB-1 of 100% dimethyl-polysiloxane (30 m, 0.25 mm i.d, film thickness 0.25 µm)
Carrier gas	Helium
Gas flow rate	1 mL/min
Injector mode	Splitless mode
Injector temperature	250 °C
Injection volume	1µL/L
Temperature program	60 °C for 3 min, 240 °C at the rate of 3 °C/min and held for 10 min
Runtime	93 min
Lab data system	NIST Library Chem Station software

The individual fatty acid composition was expressed as a percentage. The percentages of saturated and unsaturated fatty acids were calculated by totaling the percentage of fatty acids detected via the analysis of fatty acid composition. The sum percentage of saturated fatty acid was represented as total saturated fatty acid, whereas the sum of all unsaturated (mono- and polyunsaturated) was represented as total unsaturated fatty acid.

iii) Free Fatty Acid (FFA) Analysis

AOCS Official Method Ca 5a-40 (2009) with some modification was adapted to determine the FFA. An amount of 0.2 g of sample was weighted into 250 mL Erlenmeyer flask with the addition of 50 mL of hot neutralized ethanol and 2 mL of phenolphthalein indicator. The solution was swirl to dissolve and titrated with standard NaOH (0.24 M) until the first permanent pink color that persists for 30 s. The volume of titration required for the changes were recorded and the FFA percentage was calculated according to Eq. (3.6).

$$\text{FFA as Oleic (\%)} = \frac{[\text{Titration volume of standard (mL)}]\,[28.2]}{\text{Weight of sample (g)}} \qquad (3.6)$$

iv) Iodine Value (IV) Analysis

The IV was determined through AOAC Official Method 993.20 (1999) via Wij's reagent. An amount sample was filtered through a dry filter paper and 0.35 g of sample was transferred into a clean, dry, 500 mL glass-stoppered flask containing 20 mL of carbon tetrachloride (CCl_4), and 25 mL of Wijs solution were pipeted into the flask. The mixture was swirled and allowed to stand in the dark for 30 min. Potassium iodide (KI) (20 mL) and recently boiled and cooled water (100 mL) was added and the mixture was titrated with sodium thiosulfate ($Na_2S_2O_3$) (0.11 M) until yellow color almost disappears. Starch was added and the titration was continued until blue color disappears entirely. Towards the end of the titration, the stoppered container was shaken violently so that any iodine remaining in solution in the CCl_4 may be taken up by the KI.

Blank determination was conducted in the same manner and condition and the IV was calculated by means of Eq. (3.7).

$$IV = \frac{[\text{Titration of blank} - \text{sample (mL)}]\,[\text{Molarity of standard (M)}]\,(12.69)}{\text{Weight of sample (g)}} \quad (3.7)$$

v) pH Analysis

The pH was analyzed by using pH indicator strips. The universal indicator strip was dipped into the oil and was dried on a dry surface for 60 seconds. The reading was taken by comparing the color changes on the strips with the pH chart.

vi) Peroxide Value (PV) Analysis

AOAC Official Method 965.33 (2002) with some modification was applied to determine the PV. An amount of 0.50 g of sample was weight into 250 mL of stoppered conical flask together with 30 mL of acetic acid-chloroform mixture and was swirl to dissolve. The mixture was then added to 0.5 mL saturated KI and was allowed to stand in dark with occasional shaking for 1 min and 30 mL of water were added. The liberated iodine in the mixture was titrated with $Na_2S_2O_3$ (0.11 M) with vigorous shaking until yellow color was almost gone. Then, 0.5 mL of starch indicator was added and titration was continued until the blue color disappears. The PV was expressed as mili equivalent of peroxide oxygen per kg sample (meq/kg) via Eq. (3.8).

$$PV = \frac{[\text{Titration of standard (mL)}]\,[\text{Molarity of standard (M)}]\,[100]}{\text{Weight of sample (g)}} \quad (3.8)$$

vii) Moisture and Volatile Matter Analysis

Moisture and volatile matter were analyzed according to air-oven method of AOCS Ca 2c-25 (2009) with some modification. About 5 g of oil was weighed on a previously dried and tiered dish. The dish was covered with loose lid and was heated in

the oven at 105±1 °C for 1 h. The dish was removed from oven, cooled in a desiccator and were weighed. The plate was re-heated for the period of 1 h and the cooling and weighing process was repeated. The process was repeated until weight change between two observations less than 1 mg. Eq. (3.9) was used to calculate the observations.

$$\% \text{ Moisture and volatile matter} = \frac{[\text{Loss of material on drying (g)}]\,[100]}{\text{Weight of material taken for test (g)}} \quad (3.9)$$

viii) Unsaponifiable Matter Analysis

The unsaponifiable matter analysis was performed according to AOCS Method Ca 6a-40 (1998). An amount of 50 mL of alcoholic KOH was added into a conical flask containing 5 g of oil sample and were boiled under reflux conditions for 1 h until a transparent medium was formed. The medium was then transferred into a separating funnel and were washed with petroleum ether allowing the layer to separate. The lower layer was collected and the top layer was continued washing for another 3 times with around 50 mL of solvent per wash. The ether extracts were combined and further washed with alcohol and water, 25 mL each. The ether solution was concentrated to 5 mL; then 2 mL of acetone was added with some heat under the water bath to remove the solvent. Further drying at 100 °C for 30 min was done until a constant weight was obtained. The residue was then dissolved in 50 mL of warm nuetralized ethanol with phenolphthalein indicator and titrated with NaOH (0.02 M). The weight of FFA and unsaponifiable matter value was calculated according to the Eq. (3.10) and (3.11) respectively.

$$\text{Weight of FFA in the extract} = [0.282\ \text{Titration of standard (mL)}]\,[\text{Molarity of standard (M)}] \quad (3.10)$$

$$\text{Unsaponifiable matter} = \frac{100\,[(\text{Weight of the residue}) - (\text{Weight of free fatty acid in the extract})]}{\text{Weight of sample}} \quad (3.11)$$

3.4.7 Antibacterial Test

i) Preparation of Samples

A stock solution of 80% was prepared by dissolving 2.4 mL of oil with 0.48 mL of 20% DMSO and diluted up to 3 mL with distilled water. The serial dilution method of the prepared stock solution was used to prepare 1 mL of four different concentrations (20, 40, 60, and 80%) accordingly. Prepared samples were labeled and stored in freezer for further antibacterial test. Standard antibiotic streptomycin at concentration of 50 ppm was used as positive control, whereas 20% DMSO was used as negative control for comparison.

ii) Source of Microorganisms

The organisms used for the antibacterial analysis were one gram negative bacteria, *Escherichia coli* (*E. coli*) ATCC 10536 TSA and two gram positive bacteria *Bacillus subtilis* (*B. subtilis*) ATCC 11774 NA and *Staphylococcus aureus* (*S. aureus*) ATCC BAA 1026 TSA. The strains of microorganisms were identified, characterized and obtained from the bacterial stock of Microbiology Laboratory of FIST, UMP. Subcultures of the organisms were done on separate plates by touching the colonies using a sterilized loop and transferring them into a new medium and were further incubated at 35 ± 2 °C. Subcultures were continued every 24 h until pure colonies were obtained. The preparation of bacterial inoculum was done by taking a loopful of colonies pure colonies and culture them separately into 10 mL Muller-Hinton (MH) broth (Oxoid, Australia) The optical density of bacterial inoculum was adjusted spectrophotometrically via UV-Vis spectrophotometer to give approximately ~1–2 × 10^8 colony forming unit (CFU)/mL at wavelength of 600 nm by adding more colony or sterile saline (Kurekci et al., 2013).

iii) Preparation of Medium

An amount of 18 plates containing Mueller-Hinton (MH) agar was prepared by dissolving 17.1 g of agar in 450 mL of distilled water. The mixture was mixed

thoroughly and heated with frequent agitation and boiled for 1 min. The agar was then autoclaved at 121 °C with a pressure of 15 lbs for 15 min (Elavarasu et al., 2012). The agar was then transferred into each plate at the depth of 5 mm, was allowed to solidify on a flat surface at room temperature and was kept inverted at 4-8 °C for microbial bioassay.

iv) Determination of Antibacterial Activity

The antibacterial activity of the oil was determined via agar well diffusion method. Well with the diameter of 6 mm was made on the agar by using a sterile cork borer. Four wells were made on each agar to locate positive control, negative control and two concentrations of samples. Then, the prepared inoculum that was adjusted spectrometrically was touched with a sterile cotton swab. The swab was streak over the surface of the agar in a back- and-forth motion and by moving across and down the plates to ensure the confluent growth of bacteria. The plates were rotated 60° and streaking was repeated again twice to ensure uniformity. The plates were then allowed to stand at room temperature (25°C) for 3 to 5 min. Control well of positive, standard antibiotic streptomycin (50 ppm) and negative, 20% DMSO were done in each plate for reproducibility and comparative purpose. An amount of 100 µL/L of control and sample were placed into respective wells by using a micropipette. The plate lids were replaced and kept in an incubator at 35 ± 2 °C. Each analysis was done in triplicate against each test bacterium.

At the end of 24 h of incubation period, the plates were examined for the presence of clear zones around the well that indicates the inhibition zones. A measurement of the diameter of the zones (diameter of well plus the diameter of the zone) was measured with a ruler and the mean value was recorded in the unit of centimeter (cm). The growth inhibition zone diameter was classified as intermediate (> 1.2 cm) according to Upadhyay et al. (2010).

v) Determination of Minimum Inhibitory Concentration (MIC) by Tube Dilution Method

The minimum inhibitory concentration (MIC) was studied according to the broth tube dilution method proposed by Pawar et al. (2010); Jain et al. (2013) and Diao et al. (2014). A set of 12 test tubes with nine different concentrations of samples, a blank, positive and negative control were prepared and filled with 9 mL of broth. The sample was dissolved in 20% of DMSO to get the stock solution of 20% of oil and was then serially diluted to two fold with MH broth to obtain nine different concentrations (0.08, 0.17, 0.31, 0.63, 1.25, 2.5, 5, 10 and 20%). An amount of 1 mL of each concentration was then transferred into the respective labeled sample test tube (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256) containing 9 mL of MH broth. Positive and negative controls were included in each run whereby 50 ppm of antibiotic streptomycin and 20% DMSO of 1 mL respectively were placed in the respective test tube. The tube was then vortex to make sure the uniform concentration of the solution. The samples and control tubes were then inoculated with 50 µL of microorganism. The MIC tubes were incubated in an incubator shaker at 37 °C for 24 h at 150 rpm.

The growth was seen to diminish as the concentration of the drug increases and eventually a minimum inhibitory concentration was observed visually at which the growth fails to occur by comparison with positive as well as negative controls (Pawar et al., 2010). Experiments were carried out in triplicate for reproducibility and MICs were determined spectrophotometrically by measuring the bacterial concentration and growth at an absorbance of 600 nm. The mean value of the blank containing only broth was subtracted from readings for the rest of the wells. Each complete round was repeated with different test microorganisms.

Statistical analysis of microbial bioassays was determined through ANOVA, a general linear model and the values (Tukey, $P < 0.05$) were accepted as statistically significant. Differences between the mean values were determined using least significant difference (LSD) test for multiple comparisons. The significance of the difference was determined at a 95% confidence level ($a = 0.5$) (Benavides et al., 2012 and Kurekci et al., 2013).

3.5 SUMMARY

The experimental procedures in the extraction of the extracts of *A. indica* were briefly being discussed via three different methods of extraction, crude extraction through maceration and reflux, fractionation of crude and soxhlet extraction of oil. The extracted oil was further studied for physicochemical properties via described method, whereas the extracts were then being compared with the study of the volatile compounds. Two different biological procedures were explained in detail in the analysis of the antibacterial activity of seed oil and cytotoxicity assay of the crude and fractions of the bark, leaves and roots. The statistical analysis method described as a valid method to justify the obtain result via ANOVA analysis and Tukey's test. The isolation and identification of quercetin-3-*O*-β-D-glucopyranoside were performed via several chromatography and spectroscopy techniques. The summary of all the methods were represented by Figure 3.1.

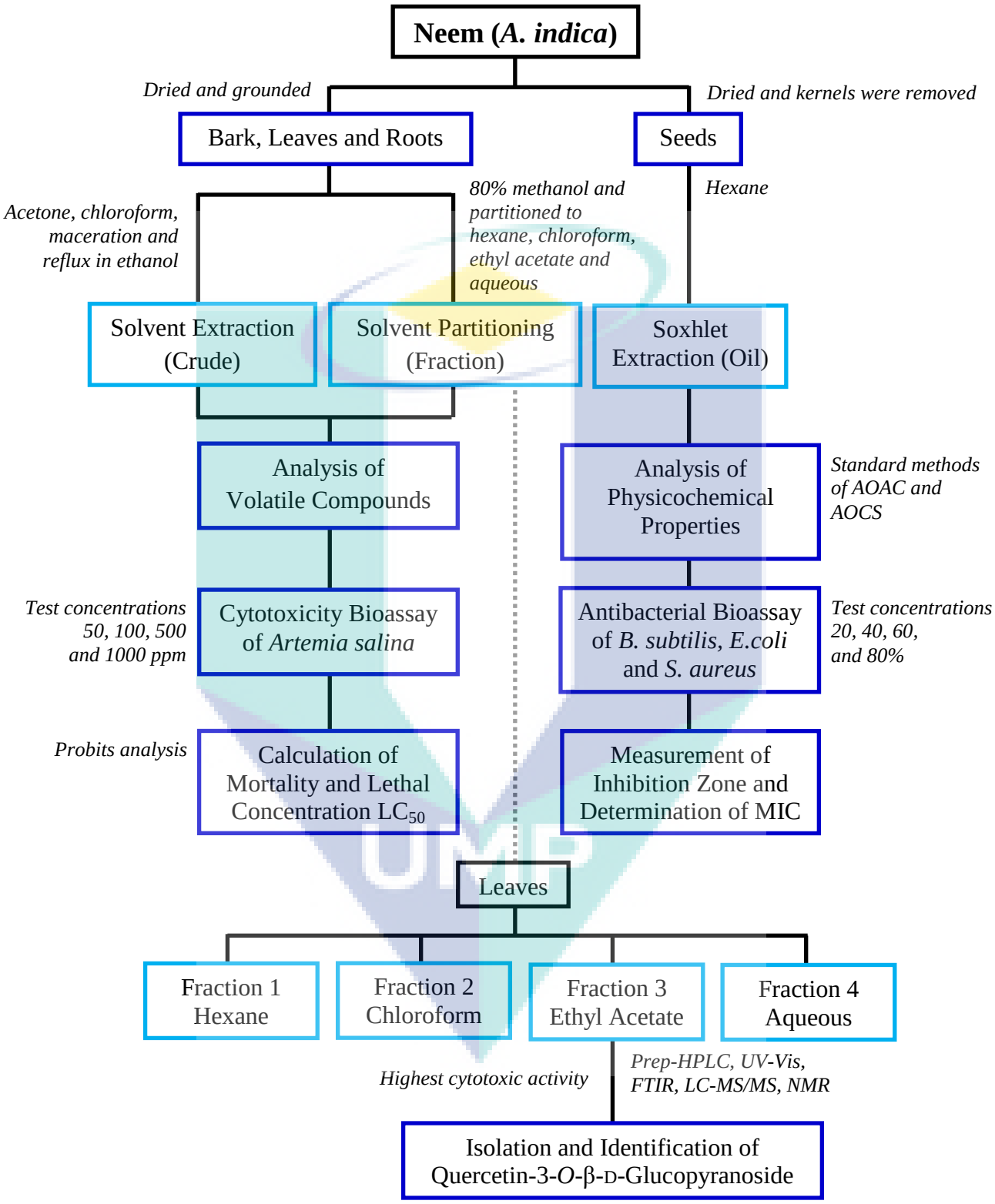


Figure 3.1: Flow chart of research methodology

CHAPTER 4

RESULTS AND DISCUSSION

4.1 INTRODUCTION

This chapter deals with the results and discussion of the current study that was being obtained according to the methods described in the previous chapter, Chapter 3. The extracts of the plant material *A. indica* were extracted via crude extraction and partitioning. The extracts were then further analysed for volatile compounds and the cytotoxic potential of the extracts were determined via brine shrimp, *A. salina* bioassay. A compound was isolated from the most cytotoxic extracts to prove the toxicity of the extract. The seed oil was extracted via soxhlet and was analyzed for physicochemical properties and antibacterial activity against three bacteria's.

4.2 YIELD OF EXTRACTION AND FRACTIONATION OF CRUDE FROM *A. indica*

The extraction technique was a key factor to study the biological activities of the targeted plants whereby it influences the extraction yield and capacity of solvent as extractant (Shahid, 2012). These solvents with varying polarities theoretically extract different plant constituents. The yield of the extraction covers three different extraction methods that were the extraction of crude extracts and fractions (bark, leaves and roots, respectively) and soxhlet extraction of seed oil of *A. indica*.

The crude extracts of different solvents (acetone, chloroform and ethanol) were obtained from dried parts (bark, leaves and roots) and the percentages were shown in Table 4.1. This crude extraction was known as solid-liquid extraction, whereby the plant material was soaked into the solvent through direct contact. The solvent diffuse into the

matrix cell and solubilize the metabolites producing extractant. Specifically for ethanol, two different conditions of extraction were used, maceration at room temperature of 25 ± 2 °C for 5 days and reflux at 60 °C for 6 h.

Table 4.1: Yield of extracts from different parts of *A. indica*

Part of plant	**Crude extract**		**Fraction**	
	Solvent	**Yield (%)**	**Solvent**	**Yield (%)**
Bark	Acetone	0.73	Hexane	1.11
	Chloroform	0.5	Chloroform	0.67
	Maceration in ethanol	1.4	Ethyl acetate	0.26
	Reflux in ethanol	0.13	Aqueous	0.82
Leaves	Acetone	2.58	Hexane	0.37
	Chloroform	2.26	Chloroform	1.15
	Maceration in ethanol	3.64	Ethyl acetate	0.75
	Reflux in ethanol	5.46	Aqueous	1.04
Roots	Acetone	1.48	Hexane	0.15
	Chloroform	0.90	Chloroform	0.26
	Maceration in ethanol	2.73	Ethyl acetate	0.06
	Reflux in ethanol	3.45	Aqueous	0.13
Seeds	Hexane	37.03	ND	ND

ND: Not determined

The extraction yielded in various amounts depending on the type of parts and solvents. The highest amount of yield produced by bark was the extract of maceration in ethanol (1.40%) and followed by acetone (0.73%), chloroform (0.50%) and reflux in ethanol (0.13%). For leaves and roots, the extracts of these two parts have the same trend whereby the highest amount yielded by reflux in ethanol (5.46 and 3.45%) and followed by maceration in ethanol (3.64 and 2.73%), acetone (2.58 and 1.48%) and chloroform 2.26 and 0.90%), respectively. The hot solvents (reflux) penetrate easily into the substance matrix and extract the constituents of the lysed plant cells producing higher yields compared to the room temperature extraction (Khoddami et al., 2013). Therefore, the comparison among the best part that could produce higher yield in terms of the crude extraction was leaves (13.94%) followed by roots (8.56%) and bark (2.76%). The comparison on capabilities of solvent in crude extraction, ethanol extracts present to produce highest amounts (reflux, 9.04% and maceration, 7.77%), then followed by acetone (4.79%) and chloroform (3.66%). Ethanol was the best and it could be supported by a theory; polar alcoholic organic solvent has high permeability to

diffuse across the membrane and facilitates extraction of various polarity compounds (Sarker et al., 2006).

Soxhlet extraction method was used to extract the seed oil and the extraction yielded in 37.03% of oil content. This method was a semi-continuous process as the solvent saturated and solubilizes the constituents. In the past study, El-Mahmood et al. (2010) had reported that the same method of soxhlet and hexane as solvent used for extraction of oil yielded 28.4%, which was lesser than the yield obtained in the current study. The difference in the percentage of yield may be contributed by the origin of the plant, time of fruit harvesting, maturity of the fruit and the drying process.

Besides crude extraction, the same parts of the plant were also extracted via solvent partitioning method through fractionations or also known as liquid-liquid extraction. The partitioning method allows the separation of the plant constituents into fractions of different polarity level with usage of two immiscible solvents of different polarities. The yields of each fraction were as tabulated as in Table 4.1. The bark, leaves and roots that were extracted with 80% methanol were further fractionated to four different fractions namely hexane, chloroform, and ethyl acetate and aqueous respectively. Harborne (1998) stated that alcohol was the best and the most suitable solvent for the extraction; and specifically for the extraction of constituents from the green tissue. The author added that alcohol has capability of removing the chlorophyll leaving the materials free from green color and gives an assumption that all the low molecular weight compounds have been extracted. The addition of water to the solvent behaves as hydro alcoholic solvent mixtures that contribute towards the high yield through the expanded polarity range. The yields obtained from extraction with aqueous methanol were 9.27, 7.92 and 2.14 g for leaves, bark and roots, respectively. From the observation of the yield of different fractions, it can be said that each fraction of each part has different trend according to the highest and lowest yield. For bark, the highest yield was from the hexane with percentage of 1.11% and followed by aqueous (0.82%), chloroform (0.67%) and finally ethyl acetate (0.26%). The yield of the leaves and roots were also arranged in descending order whereby for leaves was chloroform (1.15%), aqueous (1.04%), ethyl acetate (0.75%) and hexane (0.37%). The order for roots was chloroform (0.26%), hexane (0.15%), aqueous (0.13%) and ethyl acetate (0.06%).

As per the crude extracts, again leaves fractions (3.31%) present to be the best part in producing highest yield, but the order of the other two parts changes whereby the bark fractions (2.86%) present to be the second and followed by roots fractions (0.6%). According to the solvents used for partitioning, chloroform fraction (2.08%) present to give higher yield followed by aqueous (1.99%), hexane (1.63%) and ethyl acetate (1.07%). The operation loss of the fraction from the total percentage of the extract due to fractionation of bark, leaves and roots was 2.20, 2.65, and 0.94 g, respectively. This might have been occurred due to the solubility difficulties with the dried residual 80% aqueous methanol fraction that contains insoluble complexes that could not be reconstituted. Factors that might have attributed to the difference in the yield of extraction were the chemical nature of the plant parts, extraction methods, polarity of the solvents and interfering substances.

In this study, the non-polar solvent, hexane was used to partition the non-polar compounds and the phytochemical groups that might be present were the terpenoids or highly methyoxylated phenolics. Acetone, ethyl acetate, ethanol, methanol and water were classified under the medium and high polarity solvents and some of the compounds that could be extracted or partitioned by this solvent were simple phenolics to complex polymeric phenolics (tannins) (Shahid, 2012).

4.3 VOLATILE PHYTOCHEMICALS OF *A. indica*

The volatile phytochemicals analysis detection via GC-MS resulted in identifying a total number of 73 compounds. The compound, n-hexadecanoic acid, a saturated fatty acid chain was the most abundant in the leaves extracts for crude (65.18%) and fractions (44.79%), whereby the other parts; bark and roots yielded 40.14 and 20.55% for crude and 20.1 and 9.14% for fractions, respectively. The volatile compounds in the crude extracts were as in Table 4.2.

Antimicrobial activity had been reported for several detected volatile compounds of *A. indica* and they were the; 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester (Akpuaka et al., 2013); 2-(1H)-phenanthrenone,3,4,4a,9,10,10a-hexahydro-6-hydroxy-1,1,4a-trimethyl-7-(1-methylethyl)-,(4aS-trans)- (Kalaiarasan and

John, 2011); hexadecanoic acid, methyl ester (Akpuaka et al., 2013; Nayan et al., 2013 and Volli and Singh, 2012); n-hexadecanoic acid (Akpuaka et al., 2013; Helmy et al., 2007 and Volli and Singh, 2012); octadecanoic acid, methyl ester (Akpuaka et al., 2013 and Helmy et al., 2007) and phenol, 2,4-bis(1,1-dimethylethyl) (Senthilkumar et al., 2012). Akpuaka et al. (2013) and Helmy et al. (2007) had reported docosane as antibacterial compound; whereas Yogeswari et al. (2012) reported that 2-tetradecene possess antifungal activity. Other researchers, had reported that alpha-cadinol (El-Hawary et al., 2013 and Shivashankar et al., 2012); eicosane (Akpuaka et al., 2013; Helmy et al., 2007 and Nayan et al., 2013) and hentriacontane (Akpuaka et al., 2013 and Hossain et al., 2013) possess both antibacterial and antifungal activities.

Four compounds that correspond towards the antioxidant activity were also being detected and they were the 9-octadecene,(*E*)- (Adeosun et al., 2013); cycloheptasiloxane tetradecamethyl- (Rathinamala et al., 2013); heptacosane (Akpuaka et al., 2013; Helmy et al., 2007; Hossain et al., 2013; Ivanova et al., 2004 and Nand et al., 2012) and hexadecanoic acid, butyl ester (Prakash et al., 2011). Helmy et al. (2007) has studied the antiviral activity of *A. indica* leaves and fruits and reported that the corresponding compounds towards the activity were 9-octadecenoic acid, (*E*)-; ethyl oleate; octacosane and pentadecane. Dichloroacetic acid, heptadecyl ester an antiviral compound were detected and Ara et al. (2012) had also detected the same compound in an herb plant, Datura metel leaves.

As per the study conducted by Nand et al. (2012), the anti-acne compound that were detected to be present in the leaves and bark of *A. indica* extracts were the nimbiol and tetratriacontane. Ferruginol alone had been reported to provide gastroprotective and ulcer healing effect by Rodriguez et al. (2006), whereas toxicity effect had been identified for octacosyl acetate by Robertson et al. (2012). Other compounds, 1-heptadecene; 1-nonadecene (Nayan et al., 2013 and Volli and Singh, 2012); 1-hexadecene; 1-octadecene (Nayan et al., 2013); caryophyllene oxide (Shivashankar et al., 2012); heptadecanoic acid, ethyl ester (Murugesan et al., 2011) and retinoic acid, methyl ester (Kalaivani et al., 2012), had been identified to be a plant source compound but there were no reports of any biological activities.

Table 4.2: Volatile phytochemicals of *A. indica* crude extracts

No.	Compounds	Molecular formula	Molecular weight (g/mol)	Percentage of compound in crude extract (%)											
				Bark				Leaves				Roots			
				AC	CH	ME	RE	AC	CH	ME	RE	AC	CH	ME	RE
1	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	$C_{16}H_{22}O_4$	278	-	-	-	-	-	-	-	-	1.97	-	-	-
2	1-Heptadecene	$C_{17}H_{34}$	238	2.38	-	-	-	-	-	-	-	-	-	-	-
3	1-Hexadecene	$C_{16}H_{32}$	224	-	-	-	-	-	-	-	-	0.36	-	-	-
4	1-Nonadecene	$C_{19}H_{38}$	266	1.96	-	-	-	-	-	-	-	-	-	-	-
5	1-Octadecene	$C_{18}H_{36}$	252	-	-	-	-	-	-	-	-	0.61	-	-	-
6	2(1H)-Phenanthrenone, 3,4,4a,9,10,10a-hexahydro-6-hydroxy-1,1,4a-trimethyl-7-(1-methylethyl)-, (4aS-trans)-	$C_{20}H_{28}O_2$	300	-	3.24	1.65	-	-	-	-	-	3.99	2.95	1.13	-
7	2-Tetradecene, (*E*)-	$C_{14}H_{28}$	196	-	-	-	-	-	-	-	-	0.24	-	-	-
8	9-Octadecene, (*E*)-	$C_{18}H_{36}$	252	-	-	-	-	-	-	-	3.81	-	-	-	-
9	9-Octadecenoic acid, (*E*)-	$C_{18}H_{34}O_2$	282	-	4.67	10.78	-	-	-	-	-	-	-	-	-
10	alpha-Cadinol	$C_{15}H_{26}O$	222	-	-	-	-	-	-	-	-	-	-	0.25	-
11	Caryophyllene oxide	$C_{15}H_{24}O$	220	-	1.21	-	-	-	-	-	-	-	-	-	-
12	Cycloheptasiloxane, tetradecamethyl-	$C_{14}H_{42}O_7Si_7$	350	-	-	-	-	1.58	-	-	6.84	-	-	-	-
13	Dichloroacetic acid, heptadecyl ester	$C_{19}H_{36}Cl_2$	367	-	-	-	-	-	-	-	-	-	1.57	-	-
14	Docosane	MF: $C_{22}H_{48}$	312	-	-	-	-	-	0.93	-	-	-	-	-	-
15	Eicosane	$C_{20}H_{42}$	282	-	2.26	-	-	-	15.56	-	-	-	-	-	-

Abbreviation: AC: acetone; CH: chloroform; ME: maceration in ethanol; RE: reflux in ethanol

Table 4.2: *Continues*

No.	Compounds	Molecular formula	Molecular weight (g/mol)	Percentage of compound in crude extract (%)											
				Bark				Leaves				Roots			
				AC	CH	ME	RE	AC	CH	ME	RE	AC	CH	ME	RE
16	Ethyl Oleate	$C_{20}H_{38}O_2$	310	-	-	6.44	-	-	-	-	-	-	-	-	-
17	Ferruginol	$C_{20}H_{30}O$	286	-	-	-	-	-	-	-	-	-	6.02	-	-
18	Hentriacontane	$C_{31}H_{64}$	436	-	-	-	-	-	-	-	-	-	0.33	-	-
19	Heptacosane	$C_{27}H_{56}$	380	-	-	-	-	-	2.13	-	-	-	0.45	-	-
20	Heptadecanoic acid, ethyl ester	$C_{19}H_{38}O_2$	298	-	-	5.84	-	-	2.33	-	-	-	-	-	-
21	Hexadecanoic acid, butyl ester	$C_{20}H_{40}O_2$	312	-	-	-	-	-	-	-	-	-	5.64	-	-
22	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	-	-	-	-	-	-	20.62	-	-	-	0.33	-
23	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	5.58	10.78	8.22	15.56	5.12	17.73	16.58	25.75	0.96	1.80	7.92	9.87
24	Nimbiol	$C_{18}H_{24}O_2$	272	-	-	-	-	-	-	-	-	1.11	-	-	-
25	Octacosane	$C_{28}H_{58}$	394	-	0.45	-	-	-	1.6	-	-	-	-	-	-
26	Octacosyl acetate	$C_{30}H_{60}O_2$	452	-	-	-	-	-	3.07	-	-	-	-	-	-
27	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298	-	-	-	-	-	-	10.56	-	-	-	-	-
28	Pentadecane	$C_{15}H_{32}$	212	1.90	0.59	-	-	-	-	-	-	-	-	-	-
29	Phenol, 2,4-bis(1,1-dimethylethyl)	$C_{14}H_{22}O$	206	13.39	-	1.04	-	-	-	-	-	3.15	0.31	0.79	-
30	Retinoic acid, methyl ester	$C_{21}H_{30}O_2$	314	-	6.55	-	-	-	-	-	-	-	-	-	-
31	Tetratriacontane	$C_{34}H_{70}$	478	-	-	-	-	-	1.88	-	-	-	-	-	-

Abbreviation: AC: acetone; CH: chloroform; ME: maceration in ethanol; RE: reflux in ethanol

The analyses of the volatile compounds in the fractions were as shown in Table 4.3. Many of identified constituents had been reported to exhibit certain biological activities. The most abundant compound identified were phytol (diterpene alcohol) from leaves hexane (13.86%) and chloroform (62.25%) fractions; 9,12,15-octadecatrien-1-ol, (*Z,Z,Z*)- (fatty alcohol) from bark hexane (17.04%), leaves hexane (23.35%), bark chloroform (4.54%) and roots chloroform (2.38%); and 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester from all aqueous fractions (bark (7.61%), leaves (9.07%) and roots (5.44%)).

The major compounds in the fractions were the hexadecanoic acid, methyl ester (20.95%), (fatty acid methyl ester) present in maceration in ethanol of leaves and roots; phenol, 2,4-bis(1,1-dimethylethyl) (18.68%), (alkylated phenol) from bark acetone and chloroform; roots acetone, chloroform and maceration in ethanol extracts; eicosane (17.82%) (alkane) from bark and leaves chloroform extracts; 9-octadecenoic acid,(*E*)- (15.45%) (unsaturated fatty acid) identified in bark chloroform and maceration in ethanol extracts and 2(1H)-phenanthrenone, 3,4,4a,9,10,10a-hexahydro-6-hydroxy-1,1,4a-trimethyl-7-(1-methylethyl)-, (4aS-trans)- (12.96%) (phenolic compound) in extracts of bark chloroform and maceration in ethanol and roots acetone, chloroform and maceration in ethanol.

The beta-sitosterol, oleic acid and tetradecanoic acid had been identified in the fractions of *A. indica* and these compounds had been reported for the antiviral activity (Helmy et al., 2007), whereas the hexacosane; methyl stearate and tricosane were reported for both antiviral and anti-acne activities (Helmy et al., 2007 and Nand et al., 2012). El-Hawary et al. (2013) had identified 1-eicosene and caryophyllene in the essential oil of *A. indica* leaves and flowers that correspond towards the antioxidant, antibacterial, antifungal and larvicidal activities.

The antimicrobial compounds that were detected in the fractions of this study were the 2-methoxy-4-vinylphenol, 4,4,8-trimethyltricyclododecane-2,9-diol; phenol, 2,6-dimethoxy (Moorthy and Boominathan, 2011) and cyclohexane, 1-ethenyl-1-methyl-2, 4-bis(1-methylethenyl)-, [1S-(1alpha, 2beta, 4beta)] (Arunkumark and Paridhavi, 2013). Other compounds do also possess antimicrobial activity together with

some other biological activities such as the benzoic acid, 4-hydroxy-3,5-dimethoxy- that do also shows antioxidant properties (Liu et al., 2012); pentadecanoic acid, 14-methyl-, methyl ester that exhibit antifungal, anti-inflammatory and diuretic activities (Akpuaka et al., 2013 and Helmy et al., 2007); phytol (Akpuaka et al., 2013; Hossain et al., 2013 and Nand et al., 2012) and stigmasterol (Helmy et al., 2007; Volli and Singh, 2012 and Nand et al., 2012) that contains anticancer, anti-inflammatory and diuretic properties and p-xylene that have a combination of antioxidant and antifungal activity (Akpuaka et al., 2013).

The antioxidant active compound present in fractions of *A. indica* was 5-octadecene, (*E*) (Conforti et al., 2007). The same antioxidant properties with combination of other activities had been reported for the following compounds; 4-((1*E*)-3-hydroxy-1-propenyl)-2-methoxyphenol that was active for analgesic, anesthetic, antiseptic, antibacterial, antiviral, anticancer and fungicide; 9-octadecenoic acid (*Z*)-, methyl ester as an active compound for rodenticide (Moorthy and Boominathan, 2011); 7-hydroxy-6-methoxy-2H-1-benzopyra for anti-carcinogenic (Akpuaka et al., 2013 and Nayan et al., 2013); gamma-elemene for antifungal (Akpuaka et al., 2013; El-Hawary et al., 2013 and Hossain et al., 2013); gamma-sitosterol for antibacterial and prophylactic activities (Akpuaka et al., 2013 and Volli and Singh, 2012), and squalene for antibacterial, antitumor, cancer preventive, immunostimulant and as pesticide (Akpuaka et al., 2013).

A fatty acid chain, octadecanoic acid had been reported to show antifungal, antitumor and antibacterial activities by Akpuaka et al. (2013) and Volli and Singh (2012), whereas Growther et al. (2012) reported that 9,12,15-octadecatrien-1-ol, (*Z*,*Z*,*Z*)- shows antibacterial activity alone. Other activities such as uterotonic (Sewram, 1997), antidiabetic (Saravanamuttu and Sudarsanam, 2012), wound healing (Pandey et al., 2012), antifungal (Akpuaka et al., 2013) and anticancer (Chen et al., 2013) bioactive compounds were also present in the fractions and they were the 2-propenal, 3-(4-hydroxy-3-methoxy phenyl)-; 3-hydroxy-4-methoxybenzoic acid; alpha-cubebene; benzoic acid and cyclononasiloxane, octadecamethyl-, respectively.

Compounds such as 1-octadecene (Nayan et al., 2013); 2,6,10,14,18,22-tetracosahexaene,2,6,10,15,19,23-hexamethyl-, (all-E)- (Murugesan et al., 2011); 2-propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester (Ivanova et al., 2004); 7-Isopropyl-1,1,4a-trimethyl-1,2,3,4,4a,9,10,10a-octahydrophenanthrene (Hind et al., 2013); 9,12-octadecadienoic acid (*Z*,*Z*)-; catechol; dodecanoic acid; phenol, 2-methoxy (Volli and Singh, 2012); 9,17-octadecadienal, (*Z*)- (Shivashankar et al., 2012); benzene, 1,2,3,4-tetramethyl-; benzene, 1,2,4,5-tetramethyl (Siddiquee et al., 2012); phenol, 2,6-dimethoxy-4-(2-propenyl)- (Nayan et al., 2013) were identified through the analysis and were reported to be plant source compounds, but there were no activities reported for these compounds.

Most of the extracts differ in terms of the components found in different solvent extract and the extraction method of crude and fraction, in which most of them were detected in different percentage level and the nature of the compounds were also differing.

Table 4.3: Volatile phytochemicals of *A. indica* fractions

No	Compound	Molecular formula	Molecular weight (g/mol)	Percentage of compound in fraction (%)									
				Bark*			Leaves				Roots*		
				HE	CH	AQ	HE	CH	EA	AQ	HE	CH	AQ
1	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	$C_{16}H_{22}O_4$	278	-	-	7.61	-	-	-	9.07	-	-	5.44
2	1-Eicosene	$C_{20}H_{40}$	280	-	-	2.13	-	-	-	1.33	-	-	-
3	1-Octadecene	$C_{18}H_{36}$	252	-	-	1.53	-	-	-	-	-	-	-
4	2(1H)-Phenanthrenone, 3,4,4a,9,10,10a-hexahydro-6-hydroxy-1,1,4a-trimethyl-7-(1-methylethyl)-, (4aS-trans)-	$C_{20}H_{28}O_2$	300	-	0.37	-	-	-	-	-	-	-	-
5	2,6,10,14,18,22-Tetracosahexaene,2,6,10,15,19,23-hexamethyl-, (all-*E*)-	$C_{30}H_{50}$	410	-	-	1.31	-	-	-	-	-	-	-
6	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150	-	-	-	-	-	-	-	-	-	3.07
7	2-Propenal, 3-(4-hydroxy-3-methoxy phenyl)	$C_{10}H_{10}O_3$	178	-	-	-	-	-	-	-	-	0.31	
8	2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	$C_{18}H_{26}O_3$	290	-	-	5.76	-	-	-	5.44	-	-	2.00
9	3-Hydroxy-4-methoxybenzoic acid	$C_8H_8O_4$	168	-	-	3.58	-	-	1.20	-	-	-	0.21
10	4-((1*E*)-3-Hydroxy-1-propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$	180	-	0.57	-	-	-	-	-	-	3.44	-
11	4,4,8-Trimethyltricyclododecane-2,9-diol	$C_{16}H_{32}O_2$	238	-	2.51	-	-	-	-	-	-	-	-
12	5-Octadecene, (*E*)	$C_{18}H_{36}$	252	-	-	-	-	-	1.81	1.31	-	-	-
13	7-Hydroxy-6-methoxy-2H-1-benzopyran-2-one	$C_{10}H_8O_4$	192	-	-	1.62	-	-	-	-	-	-	-
14	7-Isopropyl-1,1,4a-trimethyl-1,2,3,4,4a,9,10,10a-octahydrophenanthrene	$C_{20}H_{30}$	270	2.28	-	-	-	-	-	-	-	4.04	-

*No compounds reported for EA fractions of bark and roots. Abbreviation: HE: hexane; CH: chloroform; AQ: aqueous; EA: ethyl acetate.

Table 4.3: *Continues*

No.	Compound	Molecular formula	Molecular weight (g/mol)	Percentage of compound in fraction (%)									
				Bark*			Leaves				Roots*		
				HE	CH	AQ	HE	CH	EA	AQ	HE	CH	AQ
15	9,12,15-Octadecatrien-1-ol, (*Z*,*Z*,*Z*)-	$C_{18}H_{32}O$	264	17.04	4.54	-	23.25	-	-	-	-	2.38	-
16	9,12-Octadecadienoic acid (*Z*,*Z*)	$C_{18}H_{32}O_2$	280	-	-	-	-	-	-	-	9.27	-	-
17	9,17-Octadecadienal, (*Z*)-	$C_{18}H_{32}O$	264	0.78	-	-	-	-	-	-	-	-	-
18	9-Octadecenoic acid (*Z*)-, methyl ester	$C_{19}H_{36}O_2$	296	-	-	-	-	-	-	-	-	1.57	-
19	alpha-Cubebene	$C_{15}H_{24}$	204	-	-	-	0.25	-	-	-	-	-	-
20	Benzene, 1,2,3,4-tetramethyl-	$C_{10}H_{14}$	134	-	-	-	0.67	-	-	-	-	-	-
21	Benzene, 1,2,4,5-tetramethyl	$C_{10}H_{14}$	134	-	-	0.26	-	-	-	-	-	-	-
22	Benzoic acid	$C_7H_6O_2$	122	-	-	2.43	-	-	-	-	-	-	-
23	Benzoic acid, 4-hydroxy-3,5-dimethoxy-	$C_9H_{10}O_5$	198	-	-	0.96	-	-	-	-	-	-	13.42
24	beta-Sitosterol	$C_{29}H_{50}O$	414	-	0.59	-	-	-	-	-	-	1.75	-
25	Caryophyllene	$C_{15}H_{24}$	204	0.43	-	1.15	-	-	-	-	-	-	-
26	Caryophyllene oxide	$C_{15}H_{24}O$	220	0.94	-	1.01	-	-	-	-	-	-	-
27	Catechol	$C_6H_4(OH)_2$	110	-	-	-	-	-	1.28	-	-	-	-
28	Cyclohexane, 1-ethenyl-1-methyl-2, 4-bis (1-methylethenyl)-, [1S-(1alpha, 2beta, 4beta)]	$C_{15}H_{24}$	204	-	-	-	0.20	-	-	-	-	-	-
29	Cyclononasiloxane, octadecamethyl-	$C_{18}H_{54}O_9S$	666	-	-	1.12	-	-	-	-	-	-	-
30	Dodecanoic acid	$C_{12}H_{24}O_2$	200	0.24	0.19	-	-	-	-	-	-	-	-

*No compounds reported for EA fractions of bark and roots. Abbreviation: HE: hexane; CH: chloroform; AQ: aqueous; EA: ethyl acetate.

Table 4.3: *Continues*

No.	Compound	Molecular formula	Molecular weight (g/mol)	Percentage of compound in fraction (%)									
				Bark*			Leaves				Roots*		
				HE	CH	AQ	HE	CH	EA	AQ	HE	CH	AQ
31	Eicosane	$C_{20}H_{42}$	268	-	-	-	2.67	-	-	-	-	-	-
32	gamma-Elemene	$C_{15}H_{24}$	204	-	-	-	7.65	-	-	-	-	-	-
33	gamma-Sitosterol	$C_{29}H_{50}O$	414	-	-	-	-	-	-	-	-	0.32	-
34	Hexacosane	$C_{26}H_{54}$	366	-	-	-	1.56	-	-	-	-	-	-
35	Methyl stearate	$C_{19}H_{38}O_2$	298	-	1.86	-	-	-	-	-	-	3.06	-
36	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	11.73	3.97	4.4	19.06	14.86	6.97	3.90	2.77	2.91	3.46
37	Octadecanoic acid	$C_{18}H_{36}O_2$	284	2.29	0.31	1.43	2.26	-	-	1.93	-	-	-
38	Oleic Acid	$C_{18}H_{34}O_2$	282	8.05	2.32	-	7.32	-	-	-	5.62	1.60	-
39	Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	270	-	16.43	-	-	-	-	-	-	1.09	-
40	Phenol, 2,4-bis(1,1-dimethylethyl)	$C_{14}H_{22}O$	206	-	-	-	-	-	2.50	-	-	-	-
41	Phenol, 2,6-dimethoxy	$C_8H_{10}O_3$	154	-	-	2.40	-	-	-	4.58	-	-	4.97
42	Phenol, 2,6-dimethoxy-4-(2-propenyl)-	$C_{11}H_{14}O_3$	194	-	-	-	-	-	-	-	-	0.18	5.67
43	Phenol, 2-methoxy	C_7H_8O	108	-	-	1.63	-	-	-	-	-	-	-
44	Phytol	$C_{20}H_{40}O$	296	-	-	-	13.86	62.25	-	-	-	-	-
45	p-Xylene	C_8H_{10}	106	-	-	-	-	-	16.12	-	-	-	-
46	Squalene	$C_{30}H_{50}$	410	-	-	-	-	-	-	0.72	-	-	-
47	Stigmasterol	$C_{29}H_{48}O$	412	-	0.47	-	-	-	-	-	-	4.51	-
48	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	0.39	0.29	0.59	-	-	-	0.69	-	-	2.74
49	Tricosane	$C_{23}H_{48}$	324	-	-	0.27	-	-	-	-	-	-	-

***** No compounds reported for EA fractions of bark and roots. Abbreviation: HE: hexane; CH: chloroform; AQ: aqueous; EA: ethyl acetate.

4.4 CYTOTOXICITY OF *A. indica* CRUDE EXTRACTS AND FRACTIONS

The brine shrimp, *A. salina* lethality bioassay was used to indicate the cytotoxicity that involves many different pharmacological effects and diseases; such as pesticide, antitumor and anticancer as this test material corresponds similarly as a mammalian system (Elhardallou, 2011 and Krishnaraju et al., 2005). In this study, the lethality and cytotoxic effect on test organism *A. salina* was used to verify the toxicological effects of extracts from different parts of *A. indica* that were extracted using different solvents (acetone, chloroform, and ethanol (maceration and reflux)) for crude extraction and (hexane, chloroform, ethyl acetate and aqueous) fractionation. The extracts were prepared into four different concentrations (50, 100, 500, and 1000 ppm). The study shows considerable variable potent of toxicological activity of the extracts against *A. salina* at different mortality rate.

The cytotoxic effect on the lethality of *A. salina* due to the exposure towards the crude extracts was represented in Table 4.4. The overall result shows that, the degree of lethality was directly proportional to the concentration of the extracts, whereby the mortality rate of the nauplii was found to be increasing as the concentration of the sample increases. Among the bark extracts; acetone and extracts in ethanol present to achieve highest lethality (97.50%) at the concentration level of 1000 ppm and followed by the chloroform extract (59.80%); whereas for the leaves extracts, acetone, and reflux in ethanol showed 97.50% lethality at the concentration level of 1000 ppm at 24 h of post exposure. This was also followed by the leaves chloroform that achieved 89.95% and the macerated ethanol extracts only showed 9.55% mortality. For the roots extracts, the highest concentration (1000 ppm) of roots acetone and chloroform caused 97.50% mortality of nauplii after 24 h of exposure. Whereas, macerated and reflux in ethanol achieved 69.85% and 74.87%, respectively. The overall lethality results show that the maximum mortalities occur at a concentration level of 1000 ppm, whereas least mortalities were at 50 ppm.

Table 4.4: Effect of *A. indica* crude extracts towards cytotoxic activity

Crude extract	Concentration (ppm)	Logarithm of concentration	Mean observed percentage mortality (%)	Corrected percentage mortality (%)
Bark				
Acetone	50	1.70	0.00	2.50
	100	2.00	10.00	9.55
	500	2.70	30.00	29.65
	1000	3.00	100.00	97.50
Chloroform	50	1.70	0.00	2.50
	100	2.00	15.00	14.57
	500	2.70	30.00	29.65
	1000	3.00	60.00	59.80
Maceration in ethanol	50	1.70	0.00	2.50
	100	2.00	10.00	9.55
	500	2.70	30.00	29.65
	1000	3.00	100.00	97.50
Reflux in ethanol	50	1.70	10.00	9.55
	100	2.00	10.00	9.55
	500	2.70	30.00	29.65
	1000	3.00	100.00	97.50
Leaves				
Acetone	50	1.70	0.00	2.50
	100	2.00	0.00	2.50
	500	2.70	50.00	49.75
	1000	3.00	100.00	97.50
Chloroform	50	1.70	10.00	9.55
	100	2.00	0.00	2.50
	500	2.70	55.00	54.77
	1000	3.00	90.00	89.95
Maceration in ethanol	50	1.70	0.00	2.50
	100	2.00	5.00	4.52
	500	2.70	5.00	4.52
	1000	3.00	10.00	9.55
Reflux in ethanol	50	1.70	0.00	2.50
	100	2.00	0.00	2.50
	500	2.70	20.00	19.60
	1000	3.00	100.00	97.50
Roots				
Acetone	50	1.70	0.00	2.50
	100	2.00	10.00	9.55
	500	2.70	60.00	59.80
	1000	3.00	100.00	97.50
Chloroform	50	1.70	0.00	2.50
	100	2.00	0.00	2.50
	500	2.70	50.00	49.75
	1000	3.00	100.00	97.50

Table 4.4: *Continues*

Crude extract	Concentration (ppm)	Logarithm of concentration	Mean observed percentage mortality (%)	Corrected percentage mortality (%)
Maceration in ethanol	50	1.70	0.00	2.50
	100	2.00	0.00	2.50
	500	2.70	20.00	19.60
	1000	3.00	70.00	69.85
Reflux in ethanol	50	1.70	0.00	2.50
	100	2.00	0.00	2.50
	500	2.70	25.00	24.62
	1000	3.00	75.00	74.87

The probit graph for the crude extracts were plotted to perform the empirical probit values to analyze the data of the bioassay. This method calculates the slope and intercepts in the form of linear regression to give out the LC values with the goodness-of-fit (Throne et al., 1995) and was represented by the Figure 4.1. Theoretically, the extract that exhibits the smaller LC_{50} value was noted to be more toxic. From the overall results of the crude extracts, bark of *A. indica* present to be the best part according to the LC_{50} value and the toxicity profile. Whereby the extracts of acetone (660.69 ± 0.87 ppm), maceration (660.69 ± 0.87 ppm) and reflux in ethanol (549.54 ± 0.79 ppm) present to provide LC_{50} values that lies between the toxicity level (≥ 500 ≤ 1000 ppm: weak toxicity). The second active part would be the roots as the acetone extracts present to be the best with the LC_{50} value of 457.09 ± 0.88 ppm that were listed in the toxic range (< 500 ppm: toxic) and the chloroform extract shows weak toxicity with LC_{50} values of 645.65 ± 0.94 ppm; whereas the maceration and reflux in ethanol present to be non-toxic (> 1000 ppm). Lastly, would be the leaves extract with a low toxicity level of acetone (645.65 ± 0.94 ppm) and chloroform (891.25 ± 0.71 ppm) while the toxic factors were not detected in the extracts of maceration and reflux in ethanol. But according to the previous studies, it had been reported that the leaves ethanol crude extract of *A. indica* showed LC_{50} value of 37.15 mg/mL (~37000 ppm) (Al-Emran et al., 2011) and 28 µg/mL (28 ppm) (Elhardallou, 2011) when tested against *A. salina*. Another cytotoxicity study on the ethanol extracts shows LC_{50} value of 36.813 mg/mL (~36000 ppm) when evaluated after 24 h of exposure (Chowdhury et al., 2008). The researcher had also suggested that the crude ethanol extracts of *A. indica* that were tested in the range of 10-1000 ppm had LC_{50} of 23 ppm after 24 h (Rahmani et al.,

1992). Therefore, with the comparison of the obtained results with the reviewed results of the past study, the current results were acceptable as the LC values achieved lies between the reported results.

The increasing chronological order of the solvent in extracting the crude with high toxic phytochemicals would be the maceration in ethanol < reflux in ethanol < chloroform < acetone. Acetone extract of the roots was the only extract that presents to fall between the toxic levels of LC_{50}. The maceration and reflux in ethanol was mostly non-toxic for both leaves and roots extracts. Chloroform extracts were listed to act better than the ethanol as only one extract of the bark chloroform was non-toxic.

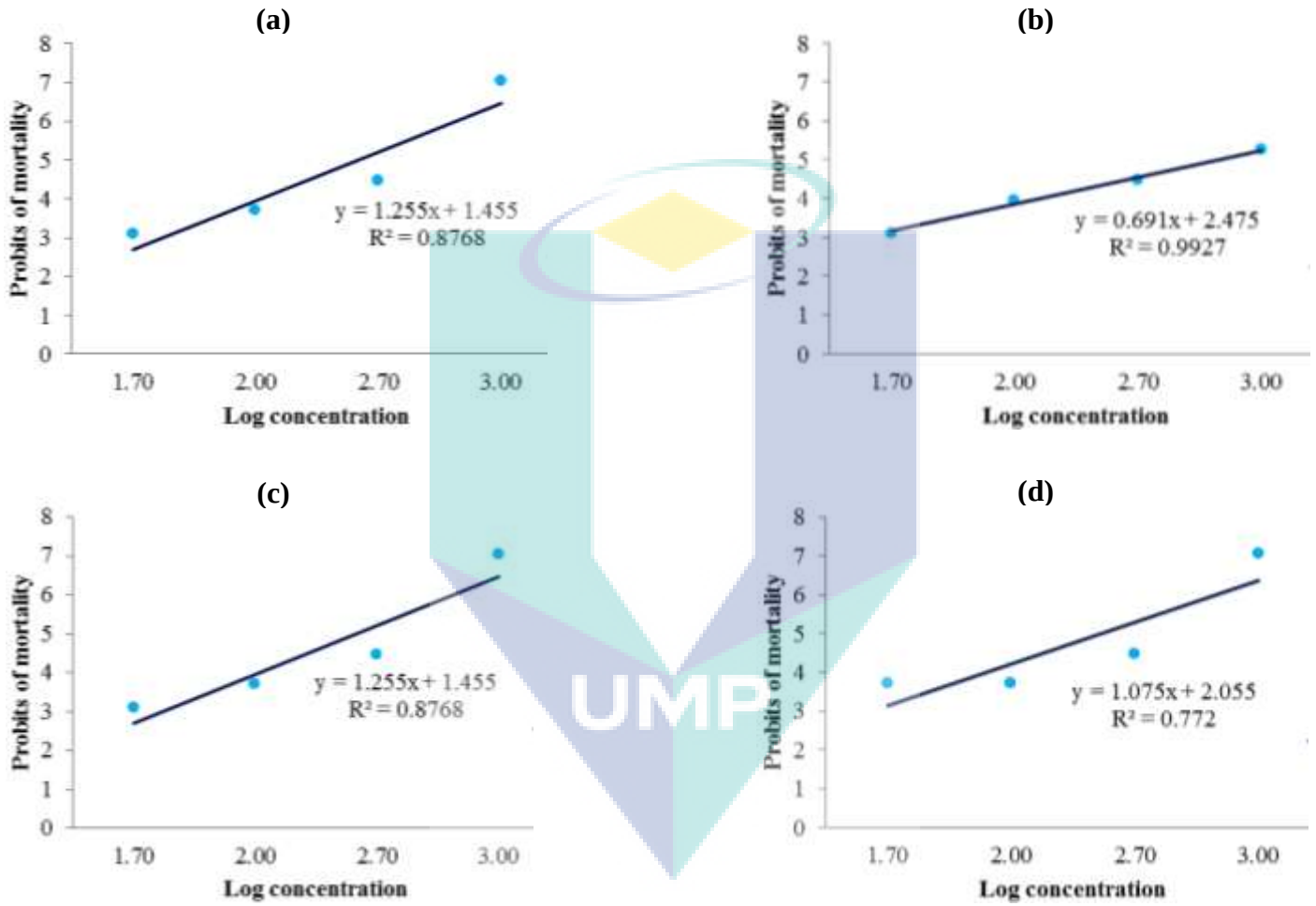


Figure 4.1: Probits plot for cytotoxic activity of *A. indica* crude extracts (a: bark acetone, b: bark chloroform, c: bark maceration in ethanol, d: bark reflux in ethanol, e: leaves acetone, f: leaves chloroform, g: leaves maceration in ethanol, h: leaves reflux in ethanol, i: roots acetone, j: roots chloroform, k: roots maceration in ethanol and l: roots reflux in ethanol)

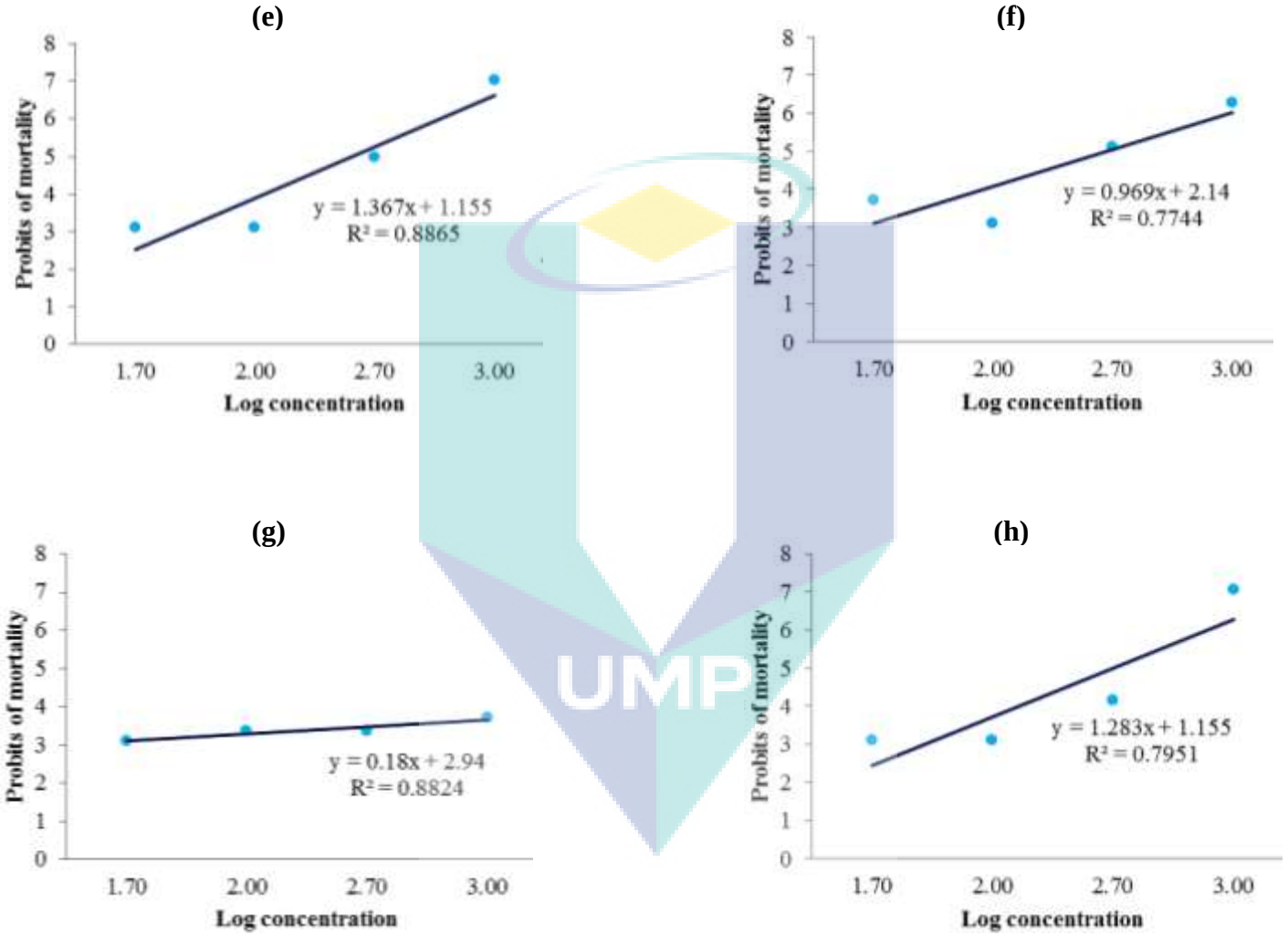


Figure 4.1: *Continues*

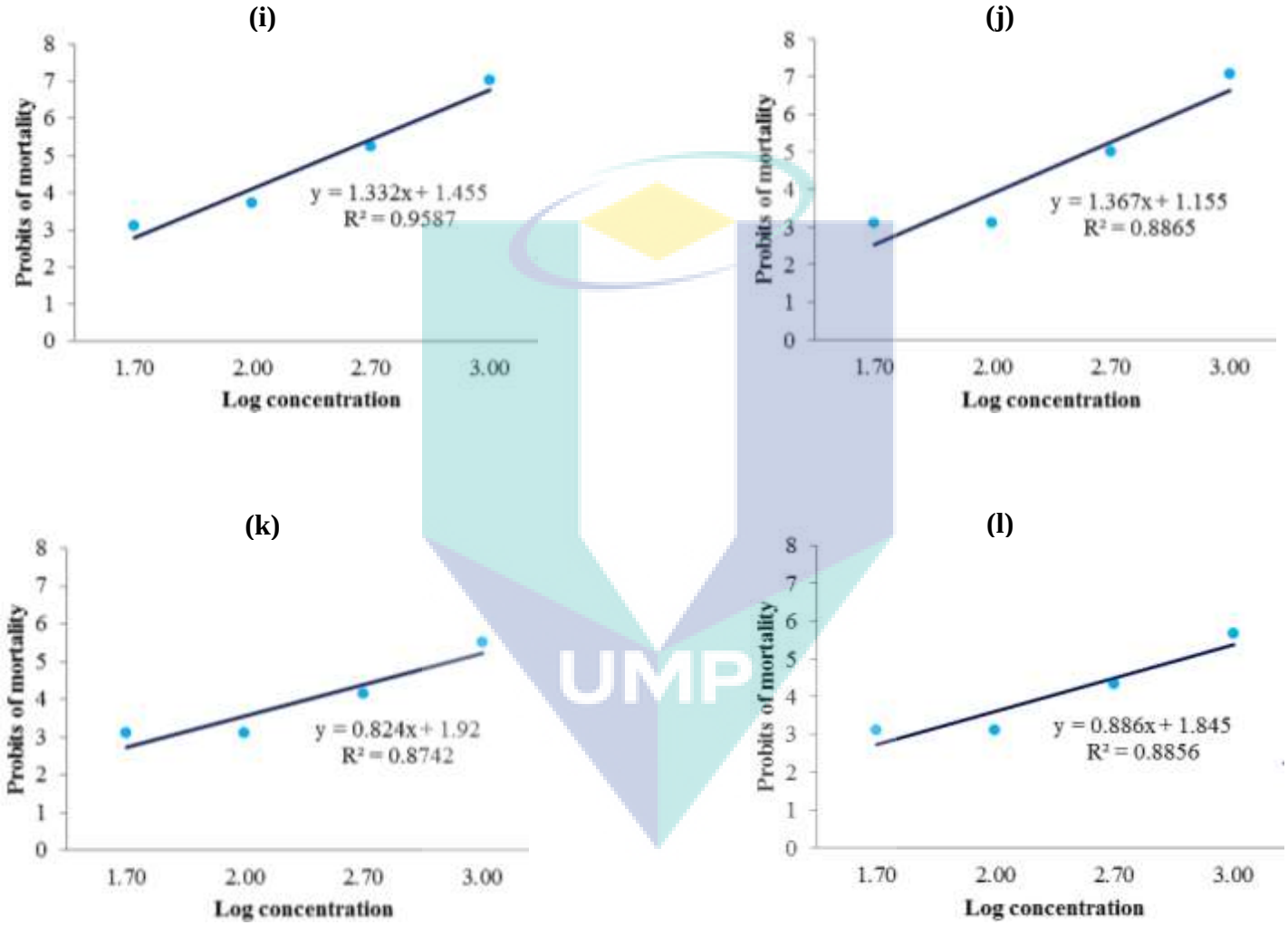


Figure 4.1: *Continues*

The two different extraction techniques of crude alone and partitioning of crude methanol extract to its subsequent solvent fractions resulted in toxicity activities that were variable in this study. The technique proves that the partitioning method present to exhibit more significant toxic effect whereby the overall mortality range lies between 25.71 to 97.50% as in Table 4.5. The fractions of two immiscible solvents obtain through partitioning were effective in separating the complex mixtures of the plant metabolites according to polarity that exhibits activities at different levels. The low concentration of the bioactive compounds in the crude might prejudice their activity detection. The highest mortality recorded for the bark extracts was the ethyl acetate with percentage mortality of 96.62% and followed by the chloroform (93.24%), aqueous (83.11%) and hexane (76.35%) at the concentration level of 1000 ppm at 24 h. For the leaves, ethyl acetate and chloroform fraction present to achieve the similar mortality rate of 97.50 % at 1000 ppm at 24 h, whereas the hexane and aqueous fraction achieves 89.86 and 86.49%, respectively. The roots fraction has different trend of arrangement compared with leaves and bark. Whereby the ethyl acetate and chloroform present to be the most active with similar mortality percentage of 89.86%, followed by the aqueous and hexane with the value of 76.35 and 66.22% at the concentration of 1000 ppm at 24 h.

Table 4.5: Effect of *A. indica* fractions towards cytotoxic activity

Part	Fraction	Concentration (ppm)	Logarithm of concentration	Observed percentage mortality (%)	Corrected percentage mortality (%)
Bark	Hexane	50	1.70	43.33	42.57
		100	2.00	53.30	52.67
		500	2.70	73.33	72.97
		1000	3.00	76.67	76.35
	Chloroform	50	1.70	60.00	59.46
		100	2.00	73.33	72.97
		500	2.70	90.00	89.86
		1000	3.00	93.33	93.24
	Ethyl acetate	50	1.70	63.33	62.84
		100	2.00	86.67	86.49
		500	2.70	93.33	93.24
		1000	3.00	96.67	96.62

Table 4.5: *Continues*

Part	Fraction	Concentration (ppm)	Logarithm of concentration	Observed percentage mortality (%)	Corrected percentage mortality (%)
Bark	Aqueous	50	1.70	53.33	52.70
		100	2.00	70.00	69.59
		500	2.70	76.67	76.35
		1000	3.00	83.33	83.11
Leaves	Hexane	50	1.70	43.33	42.57
		100	2.00	56.67	56.08
		500	2.70	86.67	86.49
		1000	3.00	90.00	89.86
	Chloroform	50	1.70	70.00	69.59
		100	2.00	76.67	76.35
		500	2.70	90.00	89.86
		1000	3.00	100.00	97.50
	Ethyl acetate	50	1.70	66.67	66.22
		100	2.00	86.67	86.49
		500	2.70	100.00	97.50
		1000	3.00	100.00	97.50
	Aqueous	50	1.70	33.33	32.43
		100	2.00	66.67	66.22
		500	2.70	73.33	72.97
		1000	3.00	86.67	86.49
Roots	Hexane	50	1.70	26.70	25.71
		100	2.00	50.00	49.32
		500	2.70	63.33	62.84
		1000	3.00	66.67	66.22
	Chloroform	50	1.70	43.33	42.57
		100	2.00	66.67	66.22
		500	2.70	70.00	69.59
		1000	3.00	90.00	89.86
	Ethyl acetate	50	1.70	56.67	56.08
		100	2.00	76.67	76.35
		500	2.70	86.67	86.49
		1000	3.00	90.00	89.86
	Aqueous	50	1.70	53.33	52.70
		100	2.00	60.00	59.46
		500	2.70	73.33	72.97
		1000	3.00	76.67	76.35

In this technique of fractionation, plant parts of the leaves present to be the best, followed by the bark and finally roots. The LC_{50} values of the fractions were determined via probits plot as in Figure 4.2. Leaves fractions have the lowest LC_{50} values of 1.35 ± 0.40, 2.14 ± 0.35, 25.12 ± 0.35 and 45.71 ± 0.32 ppm for all the solvents (ethyl acetate,

chloroform, hexane and aqueous, respectively) used. Islam et al. (2012) had also analyzed *A. indica* for cytotoxicity, and reported LC_{50} values of 1.3 µg/mL for hexane leaves extract, and the current study proves that the ethyl acetate extracts were almost at the similar toxic level (1.35 ± 0.40 ppm). According to Kirira et al. (2006), the *A. indica* aqueous and methanol extracts achieves LC_{50} of 101.26 ± 3.7 and 61.43 ± 2.9 µg/mL (101.26 ± 3.7 and 61.43 ± 2.9 ppm) which was higher compared to the value achieved in this study whereby the leaves aqueous fraction had LC_{50} of 45.71 ± 0.32 ppm.

The second active part of the fractions would be the bark that achieves LC_{50} values of 1.38 ± 0.33, 2.29 ± 0.29, 3.24 ± 0.18 and 35.48 ± 0.21 ppm for ethyl acetate, chloroform, hexane and aqueous, respectively This is also followed by the roots fractions with LC_{50} values of 2.29 ± 0.25, 4.68 ± 0.15, 23.44 ± 0.30 and 281.84 ± 0.24 ppm for ethyl acetate, aqueous, chloroform and hexane, respectively. A researcher, Nguta and Mbaria (2013) reported that the aqueous roots and bark extract of *A. indica* had achieved an LC_{50} of 285.8 ppm, and this value was far high compared to the current achieved data whereby the LC_{50} value of the bark and the roots aqueous extract were 3.24 ± 0.18 and 4.68 ± 0.15 ppm. Therefore, the LC values of the extracts were found to be lower than the previous studies and thus indicate that the prepared extracts were rich in bioactive compounds.

This data was supported by the similar extracting solvent itself, whereby the best solvent for all parts would be ethyl acetate with lowest LC_{50} values (1.35 ± 0.40, 1.38 ± 0.33, and 2.29 ± 0.25 ppm for leaves, bark and roots, respectively) followed by chloroform as the extracts present to achieve LC_{50} values closer to ethyl acetate in bark (2.29 ± 0.29 ppm) and leaves extract (2.14 ± 0.35 ppm). The aqueous and hexane fractions were in the third and fourth ranking. The LC_{50} value of hexane in roots fraction was very high (poor toxicity) 281.84 ± 0.24 ppm compared to the other fractions. The overall chronological order for the best solvent in fractionation of the plant material would be the ethyl acetate > chloroform > aqueous > hexane. Bulbul et al. (2011) had pointed out that the bioactive constituents differ in each extract depending on its solubility in the extraction solvent. Thus, extraction should be carried out in various solvent polarities. The overall LC_{50} values of *A. indica* crude extracts and fractions against brine shrimp nauplii, *A. salina* were as summarized in Table 4.6.

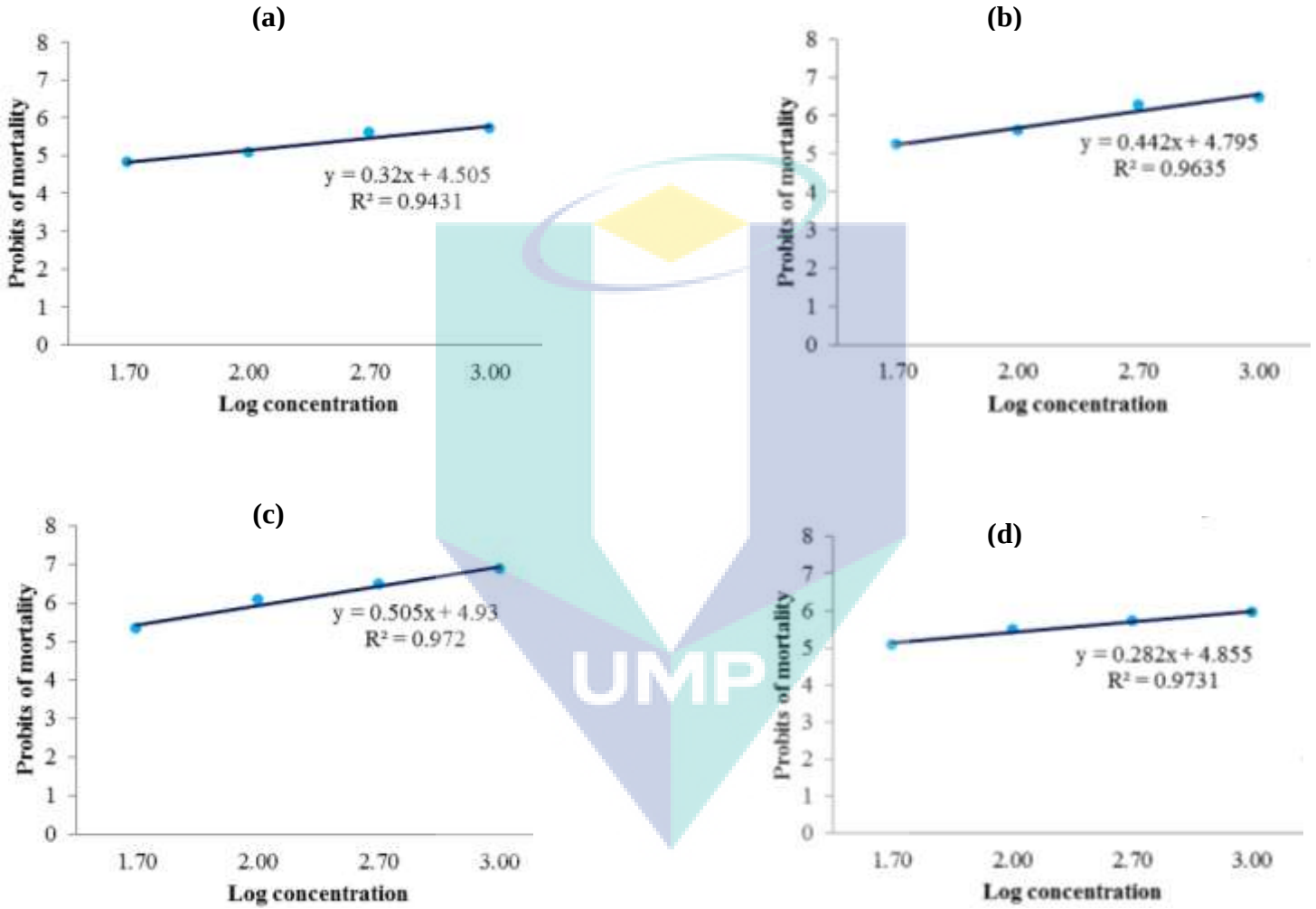


Figure 4.2: Probits plot for cytotoxic activity of *A. indica* fractions (a: bark hexane, b: bark chloroform, c: bark ethyl acetate, d: bark aqueous, e: leaves hexane, f: leaves chloroform, g: leaves ethyl acetate, h: leaves aqueous, i: roots hexane, j: roots chloroform, k: roots ethyl acetate and l: roots aqueous)

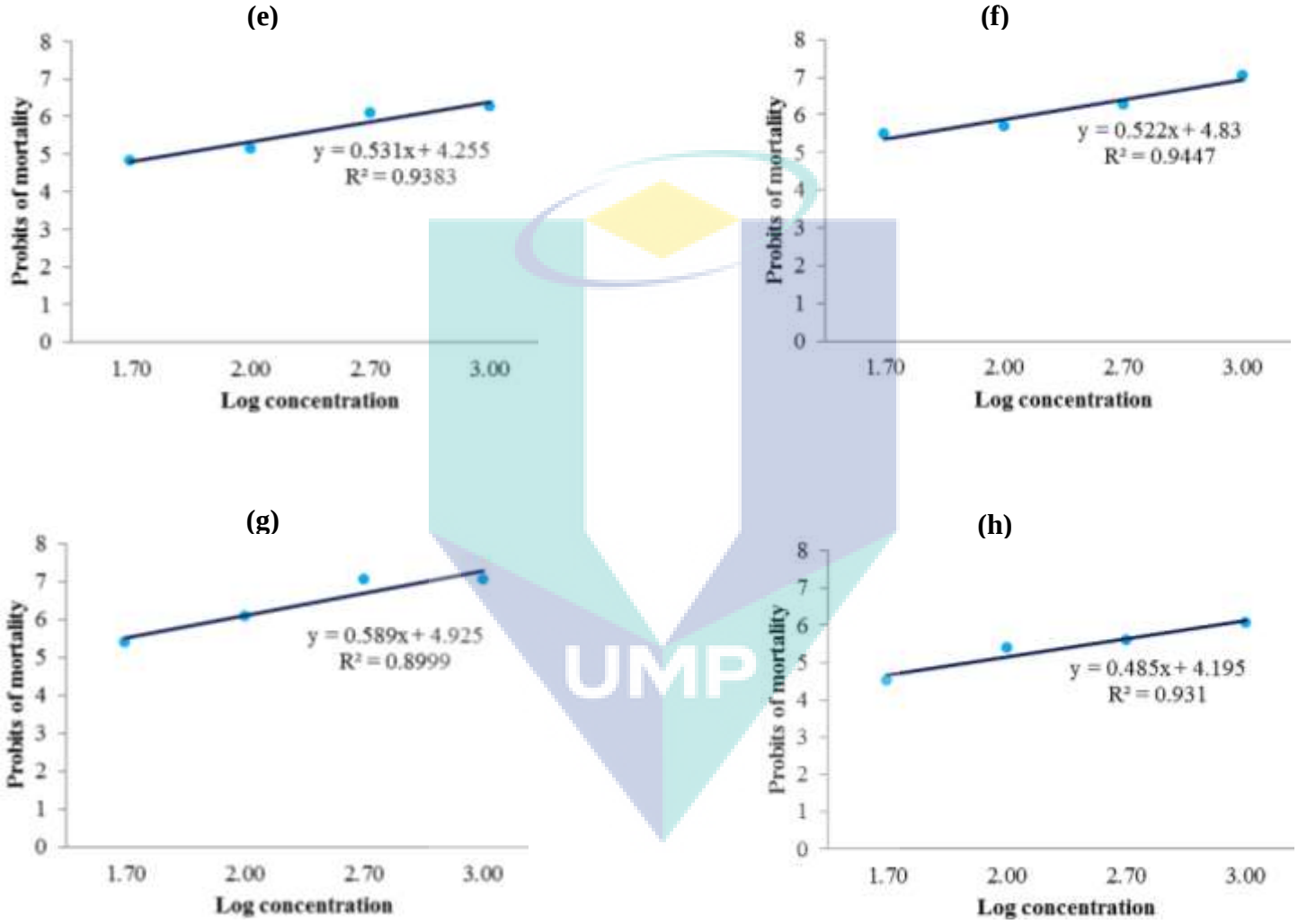


Figure 4.2: *Continues*

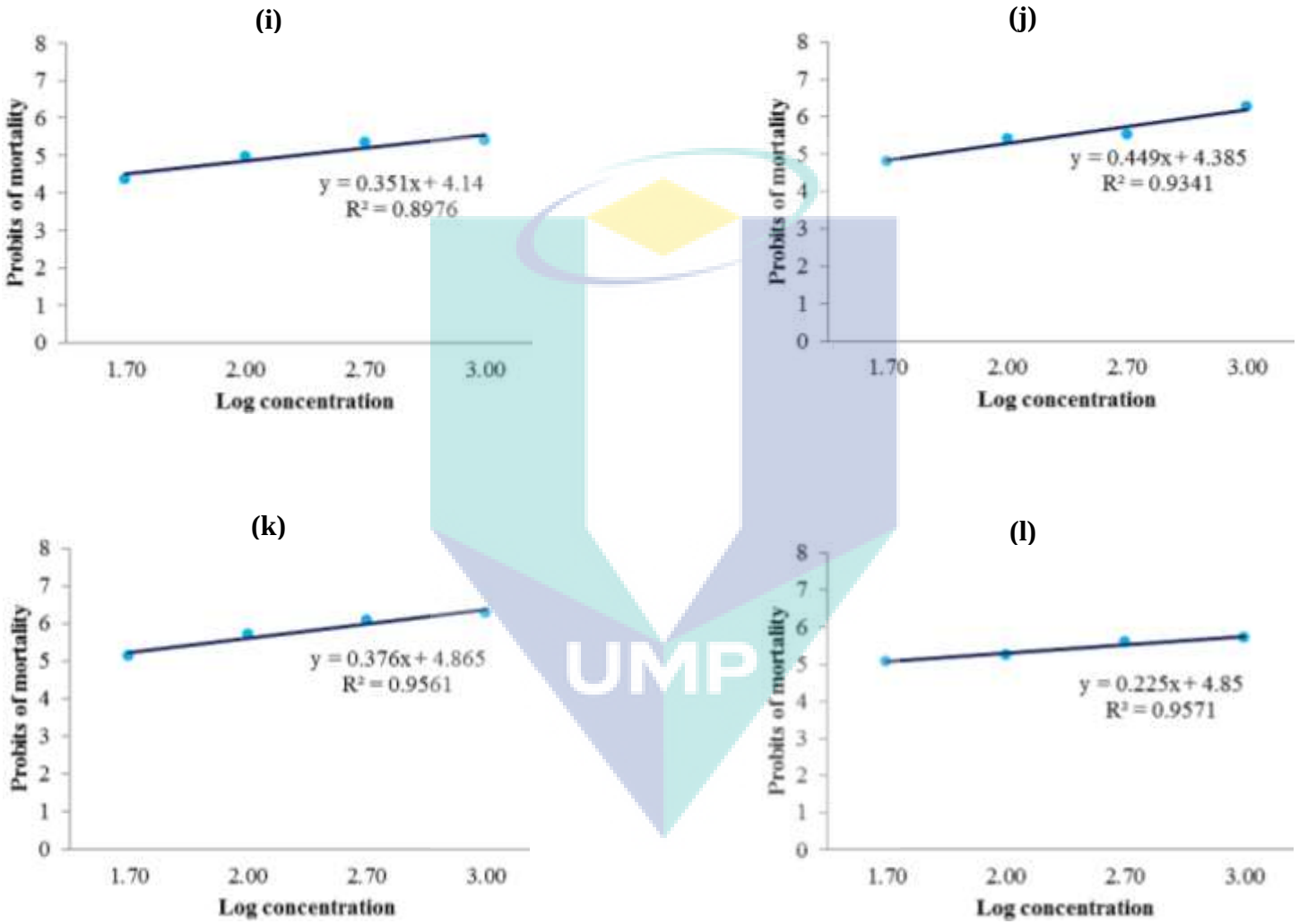


Figure 4.2: *Continues*

Table 4.6: LC_{50} values of *A. indica* crude extracts and fractions against *A. salina*

Lethal concentration values for crude extracts				Lethal concentration values for fractions			
Extract	**LC_{50} (ppm)**	**95% confidence interval**	**Toxicity level**	**Extract**	**LC_{50} (ppm)**	**95% confidence interval**	**Toxicity level**
Bark				***Bark***			
Acetone	660.69 ± 0.87 [a]	13.46 - 33140.81	Weak toxicity	Hexane	35.48 ± 0.21	13.49 - 91.20	Toxic
Chloroform	> 1000	NC	Non-toxic	Chloroform	2.29 ± 0.29 [e]	0.78 - 10.72	Toxic
Maceration in ethanol	660.69 ± 0.87 [a]	13.46 - 33140.81	Weak toxicity	Ethyl acetate	1.38 ± 0.33 [f]	0.31 - 6.17	Toxic
Reflux in ethanol	549.54 ± 0.79	15.55 - 19384.13	Weak toxicity	Aqueous	3.24 ± 0.18	1.41 - 7.59	Toxic
Leaves				***Leaves***			
Acetone	645.65 ± 0.94 [b]	9.46 - 44632.74	Weak toxicity	Hexane	25.12 ± 0.35	5.13 - 125.89	Toxic
Chloroform	891.25 ± 0.71	36.17 - 22111.72	Weak toxicity	Chloroform	2.14 ± 0.35 [e]	0.45 - 10.23	Toxic
Maceration in ethanol	> 1000	NC	Non-toxic	Ethyl acetate	1.35 ± 0.40 [f]	0.22 - 8.13	Toxic
Reflux in ethanol	> 1000	NC	Non-toxic	Aqueous	45.71 ± 0.32	10.47 - 199.53	Toxic
Roots				***Roots***			
Acetone	457.09 ± 0.88	8.72 - 24127.07	Toxic	Hexane	35.48 ± 0.21	95.50 - 831.76	Toxic
Chloroform	645.65 ± 0.94 [b]	9.46 - 44632.74	Weak toxicity	Chloroform	23.44 ± 0.30	6.03 - 91.20	Toxic
Maceration in ethanol	> 1000	NC	Non-toxic	Ethyl acetate	2.29 ± 0.25 [e]	0.74 - 7.08	Toxic
Reflux in ethanol	> 1000	NC	Non-toxic	Aqueous	4.68 ± 0.15	2.40 - 9.12	Toxic

The data represent the means ± standard deviation of three replicates. Means with the same letter were not significantly different at (Tukey's test, $p \leq 0.05$).
Toxicity level of extracts was established according to Bastos et al. (2009): LC_{50} values > 1000 ppm (non-toxic), ≥ 500 ≤ 1000 ppm (weak toxicity) and < 500 ppm (toxic).
Abbreviation: NC: Not calculated

According to Rana et al. (2012), the American National Cancer Institute had set up criteria that a sample should possess LC_{50} limit of at least 30 ppm to prove that the sample was promising and suitable for further purification. There for the crude extracts with LC_{50} values ranges from the 457.09 ± 0.88 to 891.25 ± 0.71 ppm were not suitable to be further purified. On the other aspect, all the fractions with LC_{50} values of 1.35 ± 0.40 to 25.12 ± 0.35 of the *A. indica* were suitable to be purified to isolate and further identify the bioactive phytochemical except for the hexane fraction of bark (35.48 ± 0.21 ppm) and roots (35.48 ± 0.21 ppm).

Jimenez et al. (2011) had listed out a research study that had achieved LC_{50} values of extracts less than 500 ppm together with its biological activity and some of them were the flowers of *Calendula officinalis* with LC_{50} of 245 µg/mL for anti-inflammatory and wound healing activity, whereas the leaves of *Vinca rosea* with LC_{50} of 170 µg/mL shows anti-diabetic and anti-cancer effect. Therefore, in comparison of the findings in this study, the acetone roots crude extract and all the fractions of the extract have potential bioactive compounds.

4.5 ISOLATION OF QUERCETIN-3-*O*-β-D-GLUCOPYRANOSIDE

A flavonol glucoside, quercetin-3-*O*-β-D-glucopyranoside was isolated via preparative-HPLC from leaves ethyl acetate fraction of 80% methanol extract of *A. indica*. The common name for this compound was isoquercetin or isoquercitrin (Valentova et al., 2014). This fraction was the most active fraction of the cytotoxicity study (Table 4.6). Analytical LC column separation was performed to screen for the presence of the compounds in the sample and thus exhibits five visible peaks from the wavelength of 210, 217 and 280 nm as indicated by Figure 4.3. The sample was then injected into the preparative column and the chromatogram results the same five different compounds but at different retention time and separation rate (Figure 4.3). The shift of the retention time and the separation was influenced by the bigger size of the preparative column. From the preparative analysis, the third peak was the most abundant at the retention time of 24.19 min and this peak was collected via the preparative collector. The collected compound was then verified to be a single compound via analytical technique as indicated by the zoomed peak in Figure 4.3.

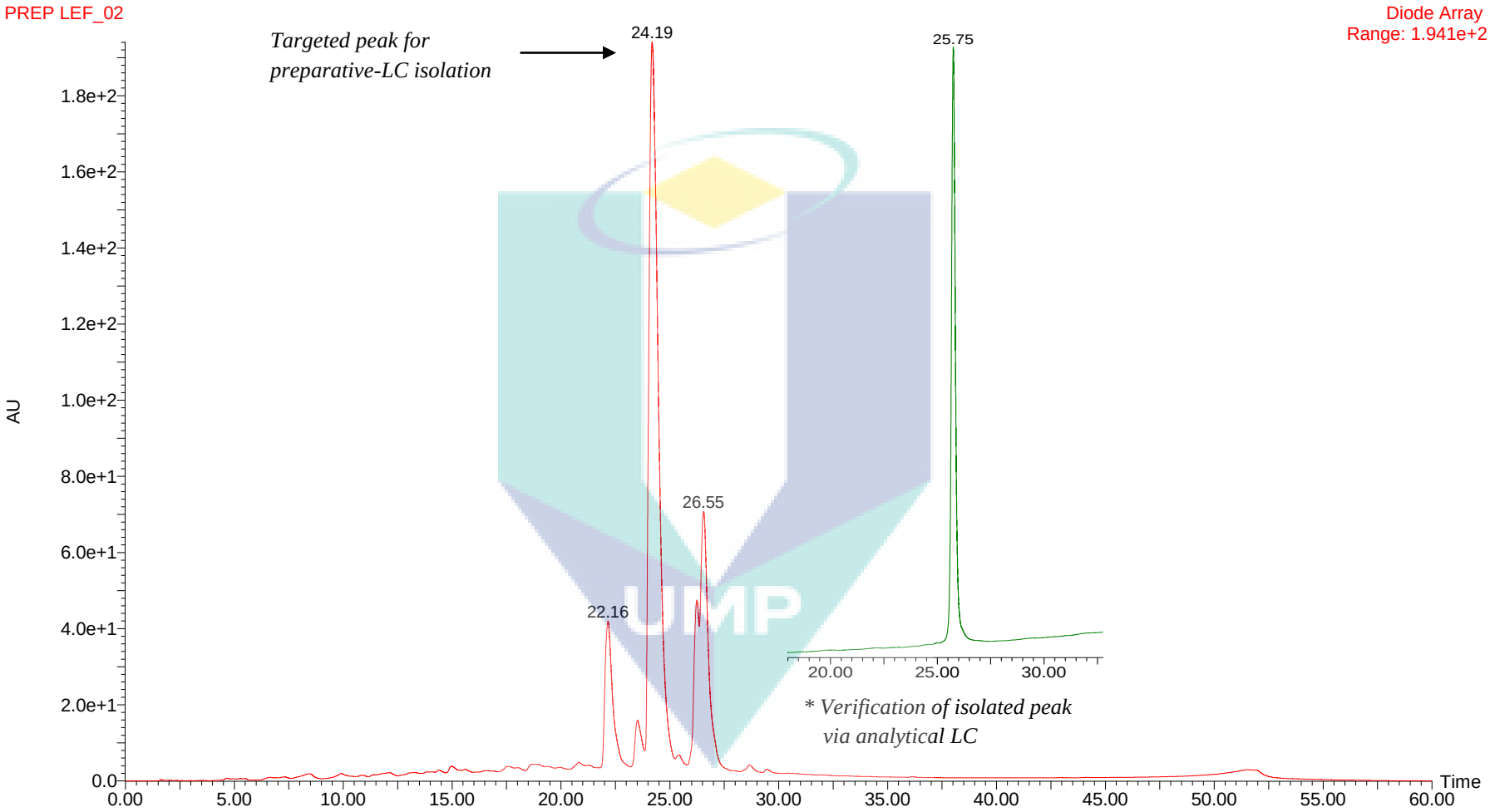


Figure 4.3: Compounds present in the leaves ethyl acetate fraction and peak of isolated quercetin-3-*O*-β-D-glucopyranoside

The isolated compound, quercetin-3-*O*-β-D-glucopyranoside belongs to the major group of flavonoids and was categorized under the minor group of flavonoid glycosides due to the presence of a sugar ring as represented in Figure 4.4. This compound was an amorphous yellow solid that was stable at room temperature (25 °C). The compound has high solubility in methanol, ethanol and DMSO and was slightly soluble in chloroform but insoluble in dichloromethane. The melting point of the compound was about 231-234 °C that was agreeable with reported values by Han et al. (2004) at around 230-232 °C.

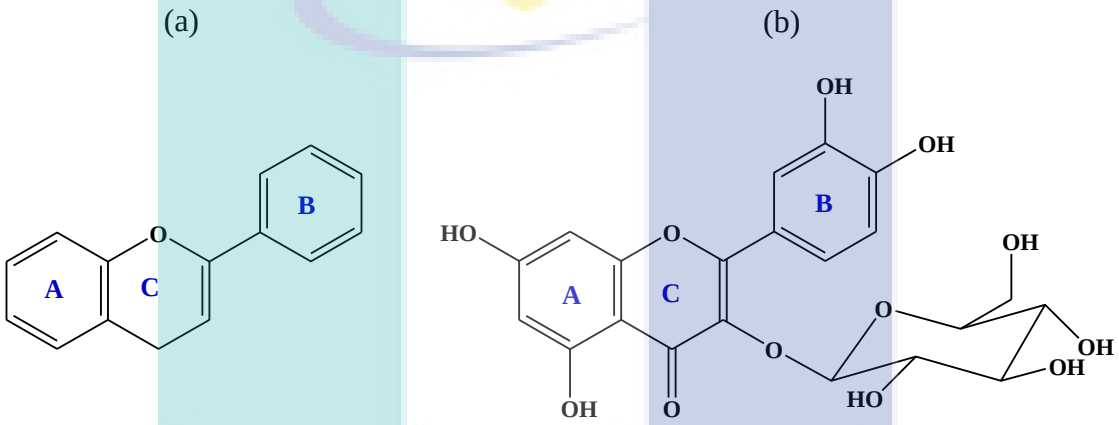


Figure 4.4: Basic structure of (a) flavonoid and (b) quercetin-3-*O*-β-D-glucopyranoside

The compound was studied for UV-Vis analysis to show the conjugation of the bonds in the compound. The flavonoids generally show high UV absorption due to the presence of the conjugated phenolic ring structure that behaves as the principle of the UV protection function. The UV-Vis spectrum of the 3-O-β-D-glucopyranoside of quercetin was as illustrated in Figure 4.5 and was obtained using methanol as the common solvents for flavonoids. The compound exhibits two maxima absorption band in this UV-Vis range (UV λ_{max} MeOH), at 256 nm and 281nm. This band was associated due the absorption of the benzoyl ring (~240-280 nm) system by the A and B ring (Figure 4.4) (Valentova et al., 2014). These transition was related to the $\pi \rightarrow \pi^*$ transition within the aromatic ring of the ligand molecule (Zsila et al., 2003).

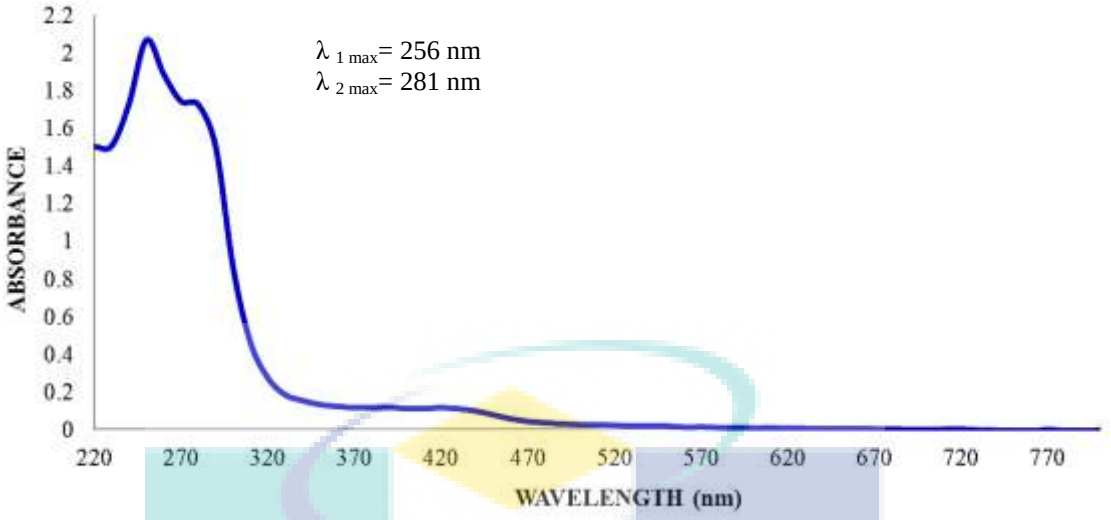


Figure 4.5: UV-Vis spectrum of quercetin-3-*O*-β-D-glucopyranoside

The compound quercetin-3-*O*-β-D-glucopyranoside was screened for functional groups via FTIR analysis with the IR transmittance ranges from 400 to 4000 cm^{-1}. The major absorption band observed for the O-H (3650-3160 cm^{-1}) stretching of the hydroxyl group. The hydroxyl group (-OH) was attached to ring (ring A and B) and exhibits a broad spectrum at 3393.02 cm^{-1}. The interatomic strong intensity bond of carbonyl (C=O) (1660-1600 cm^{-1}) correspond within the stretching point 1659.02 cm^{-1}. The carbonyl group (C=O) was bonded to two aromatic groups (ring C). The presence of three different rings of the quercetin moiety provides carbon double bonds, C=C (~1600-1400 cm^{-1}) (ring A and B), stretching in the aromatic compound with a transmittance value at point of 1607.27 cm^{-1}. The spectrum provides an evidence for the coordination between the flavonoid molecule and the sugar through the interatomic bond of C-O (1000-1300 cm^{-1}), stretching of aryl ether whereby an oxygen atom connects two aryl groups (between ring C and glucose) at 1201.58 and 1061.99 cm^{-1}. The covalent bonds between two carbons (C-C) (1500-1400 cm^{-1}) in aromatic rings were also observed at the transmittance value of 1493.19 cm^{-1}. The overall IR spectrum was as in Figure 4.6.

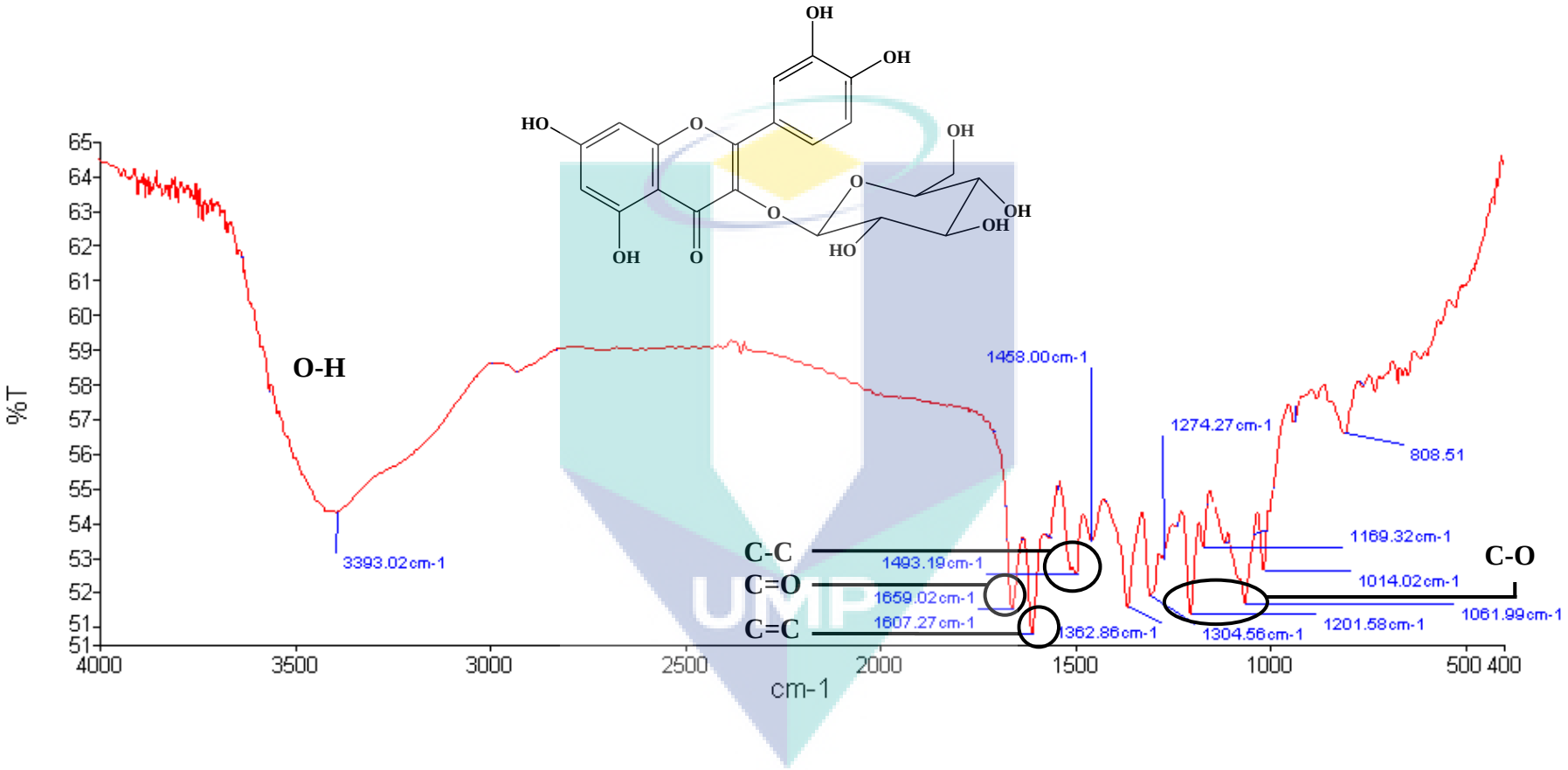
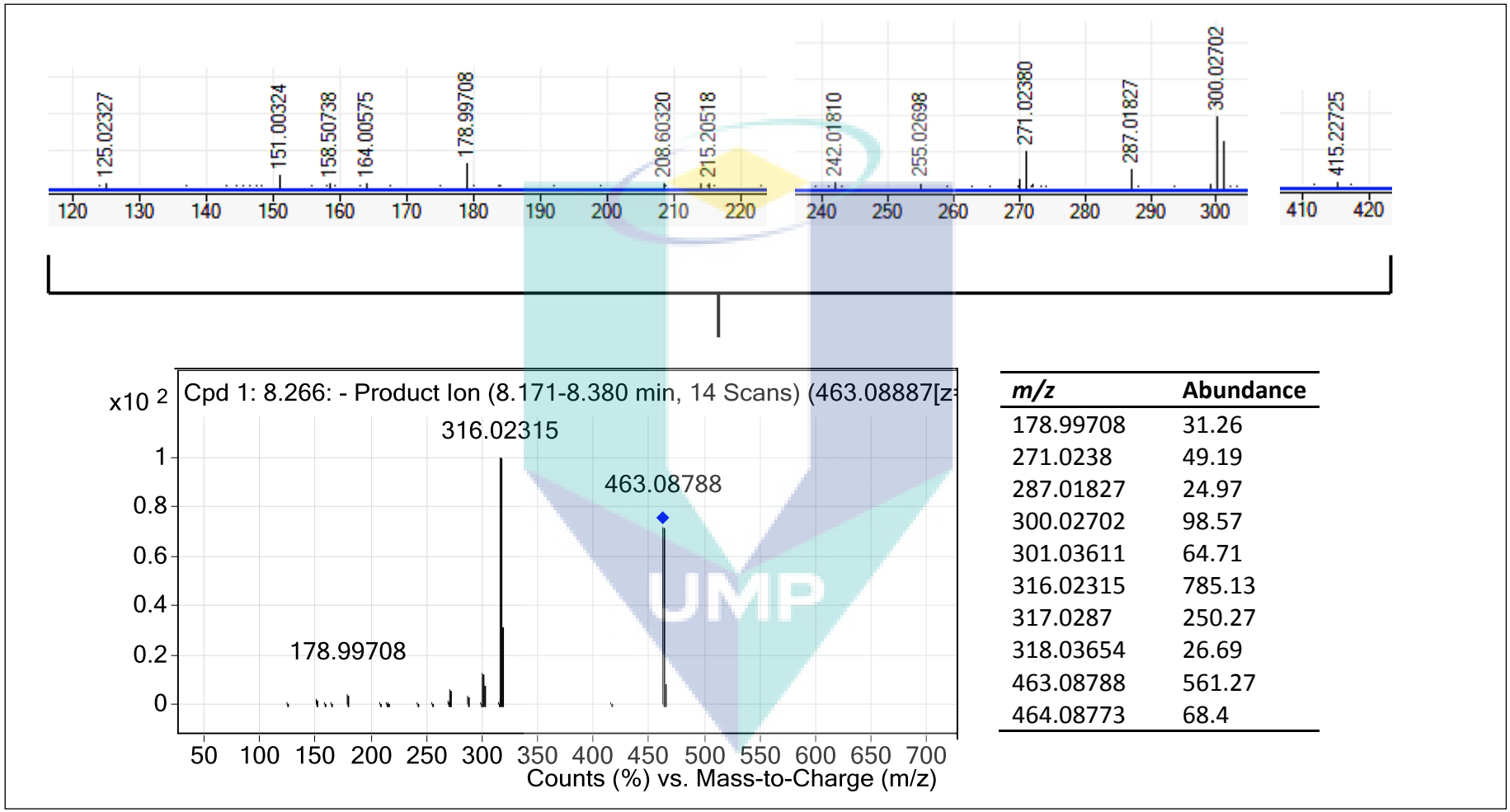


Figure 4.6: IR spectrum and bond assignments of quercetin-3-*O*-β-D-glucopyranoside

The mass spectrum obtained from the LC-MS analysis provides a data on the molecular formula, mass-to-charge ratio of negative ionization mode $[M-H]^-$ (*m/z*) and the average mass of $C_{21}H_{20}O_{12}$, 463.09 and 464.38 g/mol. Further analysis of the MS/MS was performed to confirm the structure of the compound via fragmentation value according to Tiberti et al. (2007). The $[M-H]^-$ ions was considered as presursors thus resulted in deprotonation of aglycone species by losing the sugar unit. The *m/z* value of the ions was 301.0361 which could represent the $[M-H]^-$ and $[M-H-163.149]^-$ ions of the one hexose moiety. The formation of *m/z* 179.107 $[M-H-121.115]^-$ was due to fragmentation of ring B and C. The fragmentation of ring C furthers yielding *m/z* 151.0032 with $[M-H-121.115-28.1018]^-$ ions. The $[M-H]^-$ ions could also undergo deprotonation of glycone by losing the quercetin (aglycone) unit resulting in *m/z* of 178.9971 of $[M-H-285.231]^-$ ions. Further fragmentation of the ions occurs and the spectrum showed formation of *m/z* 163.149 ion due to the $[M-H-15.8481]^-$ ions. The overall MS fragmentation spectrum of quercetin-3-*O*-β-D-glucopyranoside was as in Figure 4.7 and the fragments were shown in Figure 4.8.



m/z	Abundance
178.99708	31.26
271.0238	49.19
287.01827	24.97
300.02702	98.57
301.03611	64.71
316.02315	785.13
317.0287	250.27
318.03654	26.69
463.08788	561.27
464.08773	68.4

Figure 4.7: MS fragmentation spectrum of quercetin-3-*O*-β-D-glucopyranoside

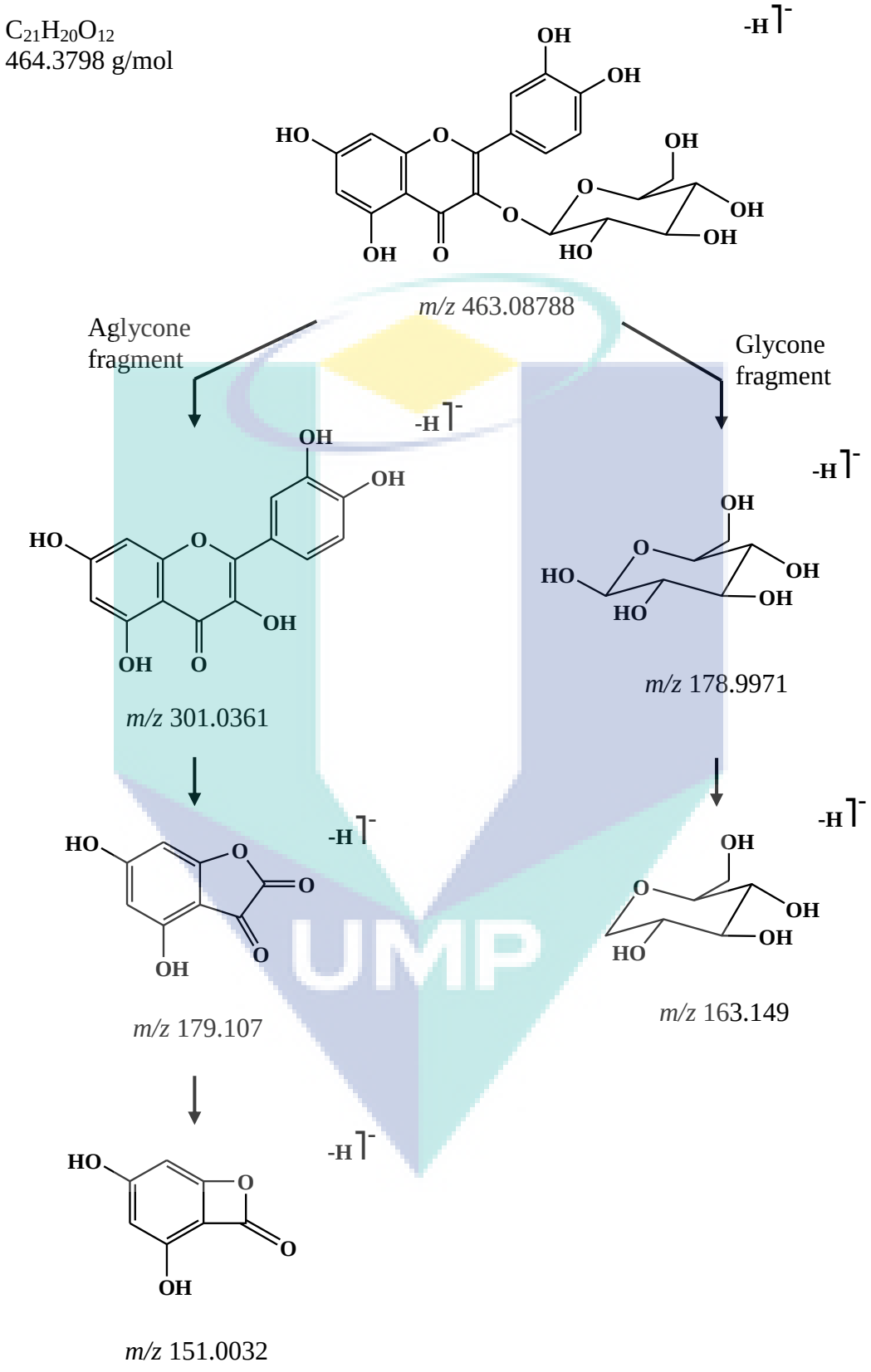


Figure 4.8: $[M-H]^-$ fragmentations of quercetin-3-*O*-β-D-glucopyranoside

The structure of the isolated compound, quercetin-3-*O*-β-D-glucopyranoside, was determined by ^{1}H NMR (Appendix A), ^{13}C NMR (Appendix B), COSY (Appendix C), HMQC (Appendix D) and HMBC (Appendix E) NMR data analysis together with the comparison of the suggested molecular formula and average mass of $C_{21}H_{20}O_{12}$ and 464.37981 g/mol from the LC-MS/MS analysis.

The ^{1}H NMR spectrum (Table 4.7) showed three aromatic proton signals at δ 7.61 (1H, d, *J* = 2, H-2'), 6.77 (1H, d, *J* = 8.45, H-5') and 7.49 (1H, dd, *J* = 8.45, 1.95, H-6') via A, B ring of flavonoid (Figure 4.4) and sugar ring spin-system suggesting a flavonol with 3', 4'-disubstituted B-ring and showed meta coupling proton signals at δ 6.10 (1H, d, *J* = 1.75, H-6) and 6.29 (1H, d, *J* = 1.5, H-8) for the A ring. The ^{1}H and ^{13}C NMR spectra provide an indication that the compound present to be a quercetin moiety attached with a sugar unit. The doublet at δ 5.16 (1H, d, *J* = 7.6, H-1") was a diaxial coupling and it was the anomeric proton of glucose whereby it provides a β-linked glucose with the quercetin moiety. Other proton signals at δ 3.37 (1H, dd, *J* = 9.75, 7.5, H-2"), 3.18 (1H, t, *J* = 9.45 ,3.9, H-3"), 3.45 (1H, dd, *J* = 9.65, 3.4, H-4"), 3.13 (1H, m), 3.62 (1H, dd, *J* = 11.85, 2.1, H-5"), 3.62 (1H, dd, *J* = 11.85, 2.1, H-6") and 3.48 (1H, dd, *J* = 11.85, 5.3, H-6") represents the proton of the glucose moiety.

The ^{13}C NMR spectrum showed 21 signals including the carbonyl signal at δ 178.07 (C-4). The spectrum do revealed chemical shifts at δ 134.20 (C-3), 161.64 (C-5), 164.67 (C-7), 144.50 (C-3') and 148.45 (C-4') that suggest that these carbons belongs to the oxygenated flavone nucleus. Other carbon signals, δ 157.59 (C-2), 104.26 (C-4a), 98.49 (C-6), 93.31 (C-8), 157.06 (C-8a), 121.79 (C-1'), 116.28 (C-2'), 116.14(C-5') and 121.70(C-6') belongs to the aglycone group of the compound. Significant glucose signals could be observed at the signals of δ 103.33 (C-1"), 74.32 (C-2"), 76.70 (C-3"), 70.82 (C-4"), 76.99 (C-5") and 61.13 (C-6").

The coupling of the protons between each other was confirmed by COSY. The proton in H-5', H-6', H-1", H-2", H-5" and H-6" were coupled with H-6', H-5', H-2", H-1", H-6" and H-5" respectively. The HMQC analysis suggest the one bond correlation of the proton and carbon whereby the observed correlation were for the H-6, H-8, H-2', H-5', H-6', H-1", H-2", H-3", H-4", H-5"; and H-6" with C-6, C-8, C-2', C-5', C-6', C-1",

C-2", C-3", C-4", C-5" and C-6", respectively. The HMBC analysis proves the exact bonding and location of the proton and carbon via two or three bond correlation whereby for this compound; H-6 provides correlation with C-4a, C-5, C-7 and C-8, H-8 with C-4a, C-6, C-7 and C-8a, H-2' with C-2, C-1', C-3' and C-4', H-5' with C-1', C-3' and C-4', H-6' with C-2, C-2' and C-4', H-2" with C-3", H-4" with C-2" and H-6" with C-5" (Appendix E).

On the basis of this NMR data and comparison with the spectral data of previously reported values (Islam et al., 2012 and Moco et al., 2006), this compound were identified as quercetin-3-*O*-β-D-glucopyranoside.

Table 4.7: NMR data of quercetin-3-*O*-β-D-glucopyranoside

Carbon	δ_C (ppm)	δ_H (ppm) (Int. Mult. *J* in Hz)	COSY (^{1}H-^{1}H)	HMQC (^{1}H-^{13}C)	HMBC (^{1}H-^{13}C)
2	157.59	-	-	-	-
3	134.20	-	-	-	-
4	178.07	-	-	-	-
4a	104.26	-	-	-	-
5	161.64	-	-	-	-
6	98.49	6.10 (1H, d, *J* = 1.75)	-	H-6	C-4a, C-5, C-7, C-8
7	164.67	-	-	-	-
8	93.31	6.29 (1H, d, *J* = 1.5)	-	H-8	C-4a, C-6, C-7, C-8a
8a	157.06	-	-	-	-
1'	121.79	-	-	-	-
2'	116.28	7.61 (1H, d, *J* = 2)	-	H-2'	C-2, C-1', C-3', C-4'
3'	144.50	-	-	-	-
4'	148.45	-	-	-	-
5'	116.14	6.77 (1H, d, *J* = 8.45)	H-6'	H-5'	C-1', C-3', C-4'
6'	121.70	7.49 (1H, dd, *J* = 8.45, 1.95)	H-5'	H-6'	C-2, C-2', C-4'
1"	103.33	5.16 (1H, d, *J* = 7.6)	H-2"	H-1"	-
2"	74.32	3.37 (1H, dd, *J* = 9.75, 7.5)	H-1"	H-2"	C-3"
3"	76.70	3.18 (1H, dd, *J* = 9.45, 3.9)	-	H-3"	-
4"	70.82	3.45 (1H, dd, *J* = 9.65, 3.4)	-	H-4"	C-2"
5"	76.99	3.13 (1H, m)	H-6"	H-5"	-
6"	61.13	3.62 (1H, dd, *J* = 11.85, 2.1)	H-5"	H-6"	C-5"
		3.48 (1H, dd, *J* = 11.85, 5.3)			

Appleton (2010) had reported that the compound quercetin generally present as glycoside in the plant; whereby glucoside and ethers were the main derivatives. Quercetin-3-*O*-β-D-glucopyranoside was the naturally occurring quercetin glucoside (Appleton, 2010). The occurance of quercetin in the form of glucosides was higher due to the capability of the sugar moiety that can additionally contain acyl and sulfate substituents.

The presence of hydroxyl groups in the structure determines the activity of the compound. The phenolic hydroxyl groups at the B-ring and position-3 (Figure 4.4) contribute towards free radical-scavenging activity. The free radical scavengers were able to destroy the free radicals that cause damage on the cell (Ebrahimzadeh et al., 2010). Besides, the aglycone group of quercetin, the nonsugar component of a glycoside molecule (Figure 4.4), reacts with the cancer receptors, commonly aryl hydrocarbon receptors to prevent the growth of cancer cells. This was also supported by Valentova et al. (2014) that the glucosides of quercetin were recently reported to exhibit toxic effect by reducing the size of tumor in mice. Besides, Wangchauy and Chanprasert (2012), proves the cytotoxicity of quercetin-3-*O*-β-D-glucopyranoside by inhibiting the growth of leukemic cells by 50% (IC_{50}) for 48 h at the concentration of 71.14 ± 4.49 µg/mL whereas Tharanath et al. (2013) reported that this compound contribute towards cytotoxicity and suppressed virus of the bluetongue with the IC_{50} value of 50 µM thus behaving as an antiviral agent.

Therefore, the isolated compound, quercetin-3-*O*-β-D-glucopyranoside from the most active fraction of the cytotoxicity study might have contributed as a toxic compound towards the *A. salina.*

4.6 PHYSICOCHEMICAL CHARACTERISTICS OF *A. indica* SEED OIL

The percentage yield of oil that was extracted from the plant seeds of *A. indica* was 37.03 %; and this value was represented in terms of lipid content as in Table 4.8. The obtained yield was agreeable with a literature stating that this plant seeds contains 25-45% oil on dry matter basis (Sathya and Manivannan, 2013). Various parameters of

the physicochemical properties had been studied and they were as listed in Table 4.8 and 4.9 and Figure 4.9.

Generally, the oil present to be liquid at room temperature of 25 °C and the color observed to be greenish brown. The literature reveals that the difference in the color intensity of oil from the same plant species, but from different location might be attributed due the presence of various pigments such as the chlorophyll content (Ayadi et al., 2009). The green color of the immature seeds disappears upon maturation resulting in chlorophyll retention. Besides, there was also a report stated that the presence of moisture contents at greater levels impacts the color of the oil whereby the moisture rises the chlorophyll content and thus contribute in increment of color intensity (Orhevba et al., 2013). The normal and thermal oxidation process of oil can also contribute towards the deterioration of lipids, and thus it might also influence the color changes of the oil (Aleksic and Knezevic, 2013; Ayadi et al., 2009).

Table 4.8: Physicochemical properties of *A. indica* seed oil

Parameters		**Units**	**Experimental Values***
Lipid Content		%	37.03
Physical State at 25 °C		-	Liquid
Color		-	Greenish brown
Odor		-	Combination of peanut and garlic
Density at 25 °C		g/cm^3	0.9480
RI at 25 °C		-	70.9
AV (% FFA as oleic)		mg KOH/g	4.8021
FFA	Oleic	%	4.74657
	Lauric	%	3.35925
	Palmitic	%	4.29986
IV		g I_2/100g	93.09
pH		-	4
PV		meq O_2/kg	8.4856
Moisture and volatile matter		wt %	0.8349
Unsaponifiable matter		wt %	1.8410
Total Saturated Fatty Acids		wt %	44.10
Total Unsaturated Fatty Acids		wt %	55.32

*Values were recorded as mean average

Table 4.9 Fatty acid composition of *A. indica* seed oil

Fatty Acid	Formula	Systematic name	Structure	Composition (%)
Saturated				
Stearic acid	$C_{16}H_{36}O_2$	Octadecanoic acid	C18:0	20.42
Palmitic acid	$C_{16}H_{32}O_2$	Hexadecanoic acid	C16:0	18.66
Arachidic acid	$C_{20}H_{40}O_2$	Eicosanoic acid	C20:0	3.59
Behenic acid	$C_{22}H_{44}O_2$	Docosanoic acid	C22:0	0.80
Lignoceric acid	$C_{24}H_{48}O_2$	Tetracosanoic acid	C24:0	0.55
Myristic acid	$C_{12}H_{28}O_2$	Tetradecanoic acid	C14:0	0.08
Unsaturated				
Linoleic acid	$C_{18}H_{32}O_2$	9,12-octadecadienoic acid	C18:2	34.69
Oleic acid	$C_{16}H_{34}O_2$	9-octadecenoic acid	C18:1	20.46
Palmiticoleic acid	$C_{16}H_{30}O_2$	9- hexadecenoic acid	C16:1	0.17

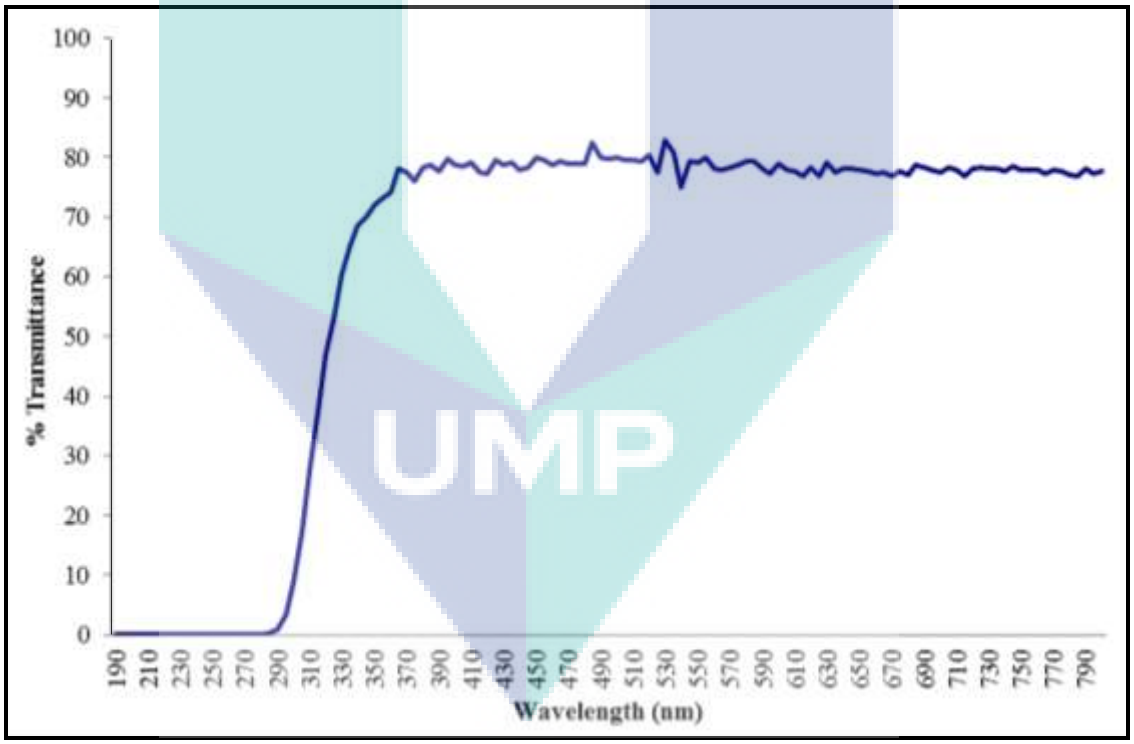


Figure 4.9: UV-Vis transmittance spectrum of *A. indica* seed oil

The oil smells like the combination of peanut and garlic. For the past several years, many compounds had been isolated and identified from *A. indica* seed oil and one of the compound, tignic acid (5-methyl-2-butanic acid) had been identified to be

responsible in the distinctive odor of the oil (Kumar et al., 2010; Rajwar and Khatri, 2013).

The density recorded for the oil was 0.9480 g/cm^3. A literature had reported a value, 1024 kg/m^3 (1.024 g/cm^3), that was almost near to the obtained results (Radha and Manikandan, 2011). The density differs as the concentration of the wall material varies at which more heavy material fits into spaces between the particles and causes an increase in mass and thus contribute towards high density (Fernandes et al., 2013).

The RI value was acceptable according to the amount of unsaturated fatty acids and long chain hydrocarbon. The RI of the oil was low, 70.9 and this was attributed by the low amount of unsaturated fatty acid, length of the hydrocarbon chain, molecular weight and degree of unsaturation as well as conjugation (Ibeto et al., 2012).

The AV was the relative measure of rancidity as FFA that were formed during decomposition or hydrolysis of oil glycerides due to the action of moisture, temperature and/or lypolytic enzyme lipase. The AV that was obtained in this study was 4.8021 mg KOH/g and was lesser compared to the past study with the value ranged from 7.7 to 410 mg KOH/g (0.77 to 41.0%). Therefore, oxidation and hydrolysis processes were also a factor that led towards increment in AV as the percentage of unsaturated fatty acids increase (Orhevba et al., 2013).

The pH value of the oil was 4.5. A study had been conducted for the optimization of *A. indica* seed oil extraction process using response surface methodology; and the research resulted in pH of oil that ranges from 3.98-4.7. The development and characterization of insecticidal soap from *A. indica* oil resulted in pH of 5.5. This result was in similar variation with this study in accordance of the pH value of 4.

Among various factors of oil classification, the drying quality of the oil was also being considered, whereby it could be drying, semi-drying or non-drying oil through the analysis of the IV (Talkit et al., 2012). The IV for current study was 93.09 gI_2/100 g; and it suggests that it was non-drying oil and it was comparable to the standard IV of

less than 100 gI_2/100g (Warra et al., 2011) in accordance with its physical state of being liquid at room temperature of 25 °C under expose air condition. The low IV represents the fewer amounts of unsaturated bonds and thus the oil has fewer tendencies to go through oxidative rancidity (Orhevba et al., 2013).

On the other hand, the oil had also undergone some chemical decomposition process whereby the obtained PV was 8.4856 meq O_2/kg, far higher than reported 6.8 meq O_2/kg. The PV indicates the rancidity process whereby the higher the PV, the higher was the oxidation level and the deterioration of lipids (Mohammed and Hamza, 2008). Theoretically, oil that shows a high amount of PV was more prone to undergo rancidity that affects the total quality of the oil (Ibeto et al., 2012).

Besides that, the moisture and volatile matter analysis prove that the oil contains a small amount of moisture and volatile matter whereby the value recorded was 0.8349 wt%. Thus, the presence of water or moisture contributes towards hydrolysis in breaking up of triglycerides into glycerol and FFA (Orhevba et al., 2013). This process might be accelerated due to the presence of the action of lipase enzyme. Therefore, these reactions, both oxidation and hydrolysis reduce the amount of unsaturated FFA and thus contributing towards the reducing of IV and average molecular weight and increasing in the AV (Orhevba et al., 2013).

Unsaponifiable matter consists of constituents such as sterols, higher molecular weight alcohols, pigments, waxes, and hydrocarbon which do not react with bases during formation of soap (AOCS Ca 6a-40, 1998). The value for unsaponifiable matter analysis was 1.84 wt% and was in the range of maximum value of 2 wt%. Due to the small value of unsaponifiable matter (< 2 wt%), *A. indica* had been studied in the application of biodiesel production (Tamboli et al., 2013).

From the current study, it could be said that the oil had undergone some oxidation and hydrolysis process as indicated by the low value of unsaturated fatty acids. This oxidation process might be influenced by storage of the oil whereby the presence of air in the bottle was in contact with the oil surface. Thus, the oxidation process converts the triglycerides into peroxides and hydro peroxides.

Table 4.9 shows the fatty acid composition of the seed oil. Generally fatty acid was a compound that contains carboxylic acids with long hydrocarbon chains was a main constituent of oil and was known to be a major parameter that differentiates the physicochemical properties of the oil. In this study, an amount of nine different fatty acids were detected and it includes both saturated and unsaturated. The sequence arrangement according to the increasing percentage of fatty acid was linoleic- (34.69%), oleic- (20.46%), stearic- (20.42%), palmitic- (18.66%), arachidic- (3.59%), behenic- (0.80%), lignoceric- (0.55%), palmiticoleic- (0.17%) and myristic acid (0.08%). The total percentage of fatty acid chains were 99.42 wt%. All the values were represented as the relative percentage area from the sum of all identified peaks. The overall results of this analysis show that the unsaturated fatty acid makes about half of the compositions, whereby the monounsaturated were 20.63 wt% and polyunsaturated were 34.69 wt%; and the saturated fatty acids were recorded to be the balance at the level of 44.10 wt%.

The preponderance chain detected in the oil was the polyunsaturated linoleic acid with the weight percentage of 34.69. Several studies had reported oleic acid as the dominance of *A. indica* seed oil, whereby the percentage lies between 25-61.9% (Atabani et al., 2013; Djenontin et al., 2012; Singh and Singh et al., 2010). But, the obtained results were supported by another reported study, in reporting that the major content was linoleic acid at 38.26% and followed by oleic acid at 34.09% (Muthu et al., 2010). The lowest content of fatty acid was represented by palmiticoleic acid, 0.17%, and myristic acid, 0.08%. These data were agreeing with some of the identified studies, whereby the percentage of palmiticoleic acid was between 0.1-0.2% (Atabani et al., 2013; Djenontin et al., 2012). Very less literature had reported the presence of Myristic acid in *A. indica* seed oil. Shivashankar et al. (2012), provides a result of the analysis of volatiles in the seed and cake of the same plant via Headspace Solid Phase Micro Extraction and GC-MS, and it reports the presence of small percentage of myristic- and lauric acid at the value of 0.127 and 0.191%. But, this current study does not reveal the presence of any lauric acid. The researcher had conducted a review study on the *A. indica* seed oil and had summarized that palmitic-, stearic-, oleic-, arachidic- and behenic acid lies between 17.3-34.3, 6.6-24, 25.4-57.9, 1.24-1.3, and 0.23-1.73%, respectively (Djenontin et al., 2012).

Overall, an author had stated that the various fatty acid composition of a same plant from different areas was varied due to its genetic make-up (Djenontin et al., 2012). The fatty acid profile could significantly change due to the storage and climatic conditions whether it could increase with period of storage (Dhar et al., 2012), air, heat, traces of metal, peroxides, light, or double bonds present in the oil (Sokoto et al., 2011) and thus leads towards the deterioration of the quality. *A. indica* seed oil had been reported to be one of the most suitable feedstock for biodiesel production, according to the fatty acid methyl ester profile that becomes one of the key factors (Ali et al., 2013). Therefore, some of the obtained results in this study were acceptable and similar to previous studies.

According to WHO (2013), the UV light was classified into three different categories namely, UV-A (400-315 nm), UV-B (315-280 nm), and UV-C (280-200 nm). According to its wavelength and the UV-Vis transmission, the profile of this oil was illustrated in Figure 4.9. The overall results on the UV-Vis analysis shows that the oil absorbs heavily as indicated by the lower transmittance value. The transmittance of the oil decreases as the wavelength decrease from UV-A, UV-B and UV-C. The UV-C ray was classified as the short wavelength radiation and was reported to be the most dangerous. But, this radiation could not reach the earth's surface as it could not penetrate through the atmosphere. Whereas UV-A and UV-B rays, a long and medium wavelength radiation could not be completely filtered and thus they do contribute towards consequent health effects up to cancer (WHO, 2013).

A past study had also pointed out that the *A. indica* seed oil from India does shows transmittance below 60% of the UV-B region and this oil were mostly used as an external applicant for skin in the southeastern part of India (Teressa et al., 2004) and this proves that this oil was non-harmful.

In terms of the overall quality of oil, it was said that the quality decreases as the storage lifetime was longer and the factors that influences were the decrease in IV and RI; and also increase in acid number. As comparison with the past studies, there might be a slight difference in the physicochemical properties as few factors might have influenced such as the geographical origin and environmental condition of the plant,

climate cultivation, soil composition, time of fruit harvesting and maturity and the drying process (Goja, 2013).

4.7 ANTIBACTERIAL ACTIVITY OF *A. indica* SEED OIL

The *A. indica* seed oil was found to be active against three different bacterial species listed as the *B. subtilis*, *E. coli* and *S. aureus* as shown in Figure 4.10 and Appendix F. The bioassay through well diffusion method provided an inhibition zones that lies between 1.23 ± 0.03 to 1.70 cm for *B. subtilis*, 1.33 ± 0.06 to 1.6 ± 1.57x10^{-16} cm for *S. aureus* and 1.4 ± 0.03 to 1.63 ± 0.03 cm for *E. coli* at the concentration range of 20 to 80% of *A. indica* seed oil. It had also been reported that, the seed oil extracted via hexane from this plant shows the inhibition zones of about similar diameters between 13-14 mm (1.3-1.4 cm) against bacteria strain of *E. coli* (El-Mahmood et al., 2010). Other than that, a study conducted on the comparison of *A. indica* oil and soap against *S. aureus*; and both tests show positive results (Chindo et al., 2011). The mixture of oil (1:1 v/v) with control drugs such as the Lamisil and Whitfield ointment resulted in a higher inhibition zone compared to pure *A. indica* seed oil or soap. This plant oil does show average inhibition of 17, 17.5, 19, and 19.5 mm (1.7, 1.75, 1.9 and 1.95 cm) on *Pseudomonas aeruginosa, Salmonella typhi, S. aureus,* and *E. coli* respectively (Jahan et al., 2007).

The tube dilution method that was adopted by past researcher to test the antibacterial activity of *A. indica* seed oil showed inhibition at different percentage; 21.24, 71.42, and 7.14% according to its concentration 500, 125, and 250 µL/ml respectively. These results were also influenced by the temperature and energy whereby many bacteria could be killed in the temperature range of 4-37 °C (Baswa et al., 2001).

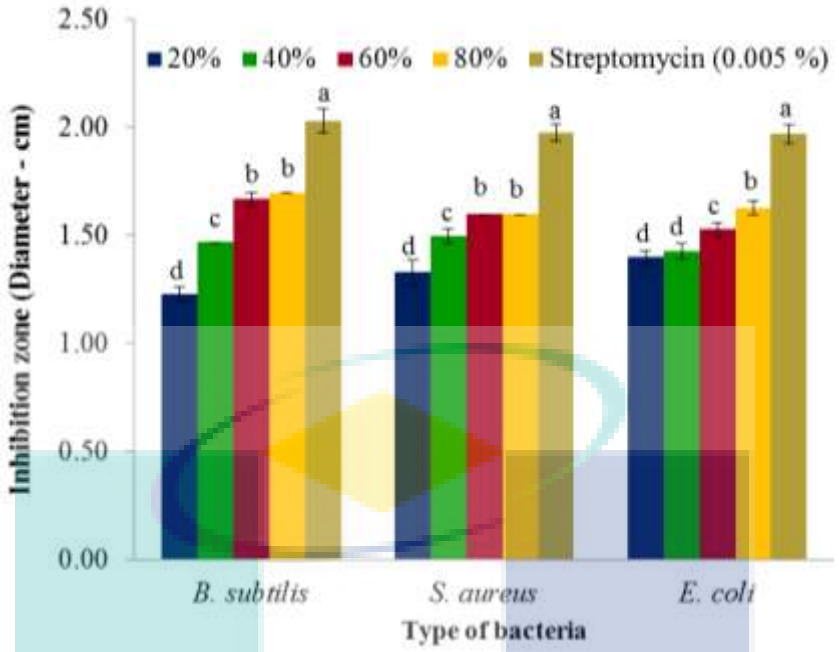
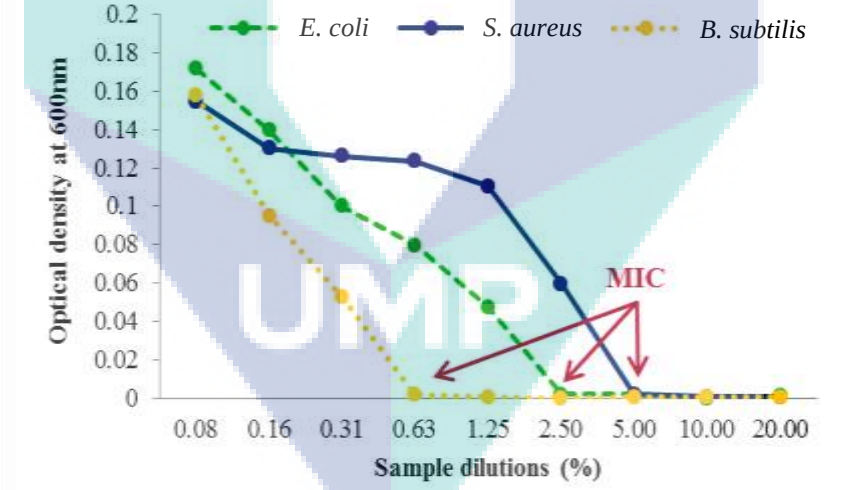


Figure 4.10: Growth inhibitory effect of *A. indica* seed oil against *B. subtilis*, *S.aureus* and *E. coli*

As in Figure 4.10 of this study, the standard antibiotic streptomycin at the concentration level of 0.005%, revealed higher inhibition activity with an inhibition zones of 2.03 ± 0.06, 1.97 ± 0.04 and 1.98 ± 0.04 cm against three strains of bacteria, *B. subtilis*, *E. coli* and *S. aureus* respectively. The obtained inhibition zones were higher compared to the seed oil of the plant. The activity of this antibiotic might be attributed by its condition whereby it present to be in pure condition and has gone through production process, thus been recognized as a standard antibiotic that could be commercially used. As a comparison with crude seed oil, the inhibitions exerted were lesser than the standard antibiotic used.

Various compounds had been reported to be present in the seed oil and these compounds might have some other biological activities as verified by other studies (Biswas et al., 2002 and Kumar et al., 2010). El-Mahmood et al. (2010) reported that the seed oil inhibited the growth of both *E. coli* and *S. aureus* at different level of inhibition subjected on the concentration level. Therefore, many factors such as type of bacteria, type of solvent, extraction methods, temperature and pH of extracts were found out to be influencing the bacterial activity.

Minimum inhibitory concentration (MIC) values for the oil were also determined through the broth tube dilution method as represented by various absorbance values at different concentrations of samples with bacteria in Figure 4.11 and Appendix G. For the analysis nine different concentrations (0.08, 0.16, 0.31, 0.63, 1.25, 2.5, 5, 10 and 20%) of oil were tested against the strains of bacteria. MICs were determined as the lowest concentration of test samples that resulted in a complete inhibition of visible growth in the broth (Lee et al., 2013). The MIC was visually determined to be varied for each strain of bacteria whereby the MIC for *B. subtilis*, *E. coli* and *S. aureus* were 0.63± 0.0002, 2.50± 0.0010 and 5.00± 0.006% and respectively. From the analyzed absorbance value of the samples at 600 nm, the absorbance decreases as the dilution increases. The turbidity of the samples was compared with positive as well as negative controls after incubation for 48 h at 35 °C. Samples that could strongly inhibit the growth of bacteria have lower minimum inhibitory concentration values.



Positive control, Streptomycin 0.005% present to be a clear tube with no growth of microorganisms whereas negative control, DMSO 20% indicates the growth of microorganisms

Figure 4.11: MIC point according to the growth of bacteria at various concentrations of *A. indica* seed oil

Generally, the MIC values of these oil lie between 0.63 to 5.00% and *B. subtilis* recorded the lowest value. No further growth was detected beyond this point. The response of the bacteria's to different concentrations of the oil varies considerably. The

negative control treatment with 20% DMSO did not show any inhibitory effect on both well diffusion analysis and broth dilution method. Some studies have pointed that the sensitivity of those microorganisms to chemotherapeutic differs according to the type of bacteria strain (Jamal et al., 2012) and this study do also support the statement.

The *A. indica* seed oil used in this study to test the antibacterial activity, shows results that were acceptable when compared with previous studies; and at the same point, this plant seed oil on its own might not be able to behave as strong antibacterial agent. Thus, various plants have been identified in behaving as a therapeutic agent towards fighting the bacteria, such as the *Swietenia mahagoni* oil, Aniseed oil, Calamus oil, Camphor oil, Cedarwood oil, Clove oil, Lavender oil, Lemongrass oil, Lime oil, Nutmeg oil, Basil oil, Peppermint oil and Rosemary oil (Hammer et al., 1999; Inouyea et al., 2001; Majid et al., 2004; Prabuseenivasan et al., 2006; Sokovic et al., 2007; Ali et al., 2011; and Pandey et al., 2012). Therefore, suggestions on the combination of the oil of one plant to another and also a combination of plant oil with commercial drugs could be practiced as it proves that the synergistic combinations yielded in better outcomes in fighting against these bacteria that ruin human's life (Chindo et al., 2011). The overall results of the antibacterial activity of this study were as summarized in Table 4.10.

Table 4.10: Antibacterial activity of *A. indica* seed oil

Bacteria	Concentration of *A. indica* seed oil				Minimum inhibitory concentration (MIC)		Controls	
	20%	40%	60%	80%	Sample dilution ratio	Sample concentration (%)	Positive	Negative
B. subtilis	1.23 ± 0.03^{d}	1.47^{c}	1.67 ± 0.03^{b}	1.70^{b}	1:32	0.63 ± 0.0002	2.03 ± 0.06^{a}	NZ
E. coli	1.40 ± 0.03^{d}	1.43 ± 0.03^{c}	1.53 ± 0.03^{b}	1.63 ± 0.03^{b}	1:08	2.50 ± 0.0010	1.97 ± 0.04^{a}	NZ
S. aureus	1.33 ± 0.06^{d}	1.50 ± 0.03^{d}	$1.60 \pm 1.57 \times 10^{-16c}$	$1.60 \pm 1.57 \times 10^{-16b}$	1:04	5.00 ± 0.0060	1.98 ± 0.04^{a}	NZ

Positive control: Streptomycin 0.005%; Negative control: DMSO 20%
Abbreviation: NZ: No inhibition zone.
The diameter of the inhibition zone (cm) includes the diameter of the well (0.6 cm).
Values were means of three replicates (± Standard error mean).
The results were significant at 5% significance level when $p < 0.05$ and $F > f$ critical.
All inhibition values were intermediate (growth inhibition zone > 1.2 cm) according to Upadhyay et al. (2010).

4.8 SUMMARY

The extraction of the crude and fractions yielded in different amount with varying polarities of the solvent. The overall percentage of crude yield was the highest for the leaves reflux in ethanol extract at 5.46%. For the fraction, highest amount was obtained from the chloroform extract of leaves at 1.15% and overall; the solvent that produces the highest yield was chloroform followed by aqueous, hexane and ethyl acetate. The analyzed oil had undergone some chemical decomposition, rancidity, which was influenced by the oxidation and hydrolysis process. An amount of 73 volatile compounds was totally detected in the crude and fractions of *A. indica* that were reported to demonstrate various biological activities and the most occurring volatile compounds in all the extracts were n-hexadecanoic acid. The current study of the cytotoxic activity was also supported by the presence of the volatile toxic effect compounds, eicosane and octacosyl acetate together with the isolated compound of quercetin-3-*O*-β-D-glucopyranoside. The bark acetone and leaves ethyl acetate of fractions were identified to be the most toxic among the crude extracts and fractions respectively. The antibacterial activity of the seed oil proves that the oil could inhibit the growth of the *B. subtilis, E. coli* and *S. aureus* bacteria with almost the same level of inhibition. The study on the minimum inhibitory concentration proves that the *B. subtilis* has less resistancy towards the oil and followed by *E.coli* and *S. aureus.*

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

The two different extraction techniques of *A. indica* via crude extraction and fractionation demonstrate a quantitative analysis in the yield. The extraction of the crude yielded a total amount of extract of 13.94, 8.56 and 2.76% for leaves, bark and roots, respectively. Whereas the partitioning of the fractions produces yields in the amount of 3.31, 2.86 and 0.60 g of leaves, bark and roots, respectively. The solvent ethanol (reflux and maceration) present to be the best solvent in the extraction of the crude followed by acetone and chloroform with the total percentage of 9.04, 7.77, 4.79 and 3.66% respectively. Thus, the extraction of crude via acetone proves that the solvent could be used as good extractant for *A. indica.* The extraction of seed oil of *A. indica* via soxhlet method yielded in higher amount of 37.03% compared to the reported study by El-Mahmood et al. (2010) with 28.4% whereby the geographical area and the maturity of the seeds might be the factor.

The detection of most of the predominant volatile phytochemicals in the extracts proves the capability of the plant in synthesizing wide diversity of compounds with biologically active molecules that facilitate in many biological activities. Many of the compounds were previously identified by past researchers and n-hexadecanoic acid was the most frequent compound present in all the extracts. The study also suggests that the different solvent extracts produced different ratios of bioactive compounds whereby the hexane fraction was found to yield the highest amount (42 constituents) of volatiles. Some of the volatiles of the roots extracts that were not reported were found to similar with other extracts but 2-tetradecene, (*E*)-; alpha-cadinol; dichloroacetic acid,

heptadecyl ester; hentriacontane; hexadecanoic acid, butyl ester and nimbiol were present only in crudes of roots whereas 2-methoxy-4-vinylphenol; 2-propenal, 3-(4-hydroxy-3-methoxy phenyl)-; 9,12-octadecadienoic acid (*Z*,*Z*); 9-octadecenoic acid (*Z*)-, methyl ester; gamma-sitosterol were only detected in fractions of roots. These compounds were also been proven to exhibit variety of biological activities. Therefore, *A. indica* plant was listed to be a potential source of biologically active constituents added with pharmaceutical value.

The present cytotoxicity study on the bark, leaves, and roots of *A. indica* could be used for the treatment of the pharmacological effects and diseases as it was supported by some of the past studies. The part of the plant that shows the highest cytotoxic effect in the crude was the bark and followed by the roots and the leaves. Maceration in the ethanol present to be the best solvent in the extraction of the crude cytotoxic phytochemicals followed by reflux in ethanol, chloroform and acetone; whereas for the fractions ethyl acetate present to be the best solvent followed by chloroform, aqueous and hexane. Cytotoxic activity were not been reported for the roots and thus this study proves that the acetone crude extracts (LC_{50} 457.09 ± 0.88 ppm), fractions of hexane (LC_{50} 35.48 ± 0.21 ppm), chloroform (LC_{50} 23.44 ± 0.30 ppm), ethyl acetate (LC_{50} 2.29 ± 0.25 ppm) and aqueous (LC_{50} 4.68 ± 0.15 ppm) does falls under the toxic category. Isolation of quercetin-3-*O*-β-D-glucopyranoside from the most cytotoxic fraction of leaves ethyl acetate was correlated with previous reports to support the toxicity study. The results obtained from this work proves that the *A. indica* plant possesses chemical constituents that behave as effective and natural antibacterial and cytotoxicity agent supported by vary volatile chemical compounds.

The study of the physicochemical properties provides insight characteristics of the *A. indica* seed oil. Overall, the *A. indica* seed oil demonstrates promising properties that could be a potential source for unlimited applications. UV-Visible transmittance of the oil suggests these oils for applications to solve current skin problem that was attributed to the penetration of UV light. Moreover, the geographical variation of the plant source and storage duration of the oil has an impact on many properties that would influence in the quality of seed oil as per compared with the literature.

The seed oil of *A. indica* had demonstrated the antibacterial activity against *B. subtilis*, *E. coli* and *S. aureus* that were promising with a variety of resistance levels. These antibacterial activities against the pathogenic bacteria depend on the correlation of its concentration. The problem associated with pneumonia (Labandeira-Rey et al., 2007), foodborne diseases (Elaine et al., 2011) and urinary tract infections (Manges et al., 2001) contributed by pathogenic bacteria had increased resistance to commercial drugs and therefore could be solved by developing *A. indica* seed oil as one of the active drug to fight against these bacteria. Many researches had supported that this oil had been used for countless purposes in the traditional medicines and had been proven to be effective as medicinal agents.

5.2 RECOMMENDATIONS FOR FUTURE WORK

This study should not be only listed as a part of research alone, but instead it should be further investigated and applied in order to solve the occurring problems. The activities obtained from this work could be considered with further investigation for the production of pharmaceutical drugs from *A. indica*.

The cytotoxicity analysis via *A. salina* proves that the plant extracts have a toxic effect, but the same level of effect might not be the same towards the insects or human cell. Therefore, the direct biological studied with insect and human cell line should be performed to confirm the effect of the drug. The study would be more supportive with the isolation and identification of the active compounds in each of the crude extracts of different parts of *A. indica* through the separation and purification method. The active compound alone could be tested to prove the biological activity to enhance the activity of the compound.

Further investigations and study on the many other local trees from the same family of *Meliaceae* could be done to study the synergistic effect of the plant and thus contribute towards the production of drugs with higher efficiencies.

REFERENCES

Abbott, W.S. 1987. A method of computing the effectiveness of an insecticide. *Journal of the American Mosquito Control Association*. **3**(2): 302-303.

Abou-Zeid, A.M. 2012. Review on citrinin: Synthetic methods, molecular biosynthesis and effect of plant extracts. *British Microbiology Research Journal.* **2**(2): 108-122.

Adeosun, C.B., Olaseinde, S., Opeifa, A.O. and Atolani, O. 2013. Essential oil from the stem bark of *Cordia sebestena* scavenges free radicals. *Journal of Acute Medicine*. **3**: 138-141.

Akpuaka, A., Ekwenchi, M.M., Dashak, D.A. and Dildar, A. 2013. Biological activities of characterized isolates of n-Hexane extract of *Azadirachta indica* A. Juss (Neem) leaves. *New York Science Journal*. **6**(6): 119-124.

Aleksic, V. and Knezevic, P. 2013. Antimicrobial and antioxidative activity of extracts and essential oils of *Myrtus communis* L. *Microbiological Research*. Doi: 10.1016/j.micres.2013.10.003

Al-Emran, A., Shahed, S.M., Farzana Ahmed, Saha, S.K., Das, S.C. and Bachar, S.C. 2011. Evaluation of brine shrimp lethality and antimicrobial activity of *Azadirachta indica* leaf extract on some drug resistance bacteria in Bangladesh. *Pharmacognosy Journal*. **3**(20): 66-71.

Ali, M.A., Sayeed, M.A., Islam, M.S., Yeasmin, M.S., Khan, G.R.M. and Ida I.M. 2011. Physicochemical and antimicrobial properties of *Trichosanthes anguina* and *Swietenia mahagoni* seeds. *Bulletin of Chemical Society of Ethiopia.* **25**: 427-436.

Ali, M.H., Mashud, M., Rubel, M.R. and Ahmad, R.H. 2013. Biodiesel from Neem oil as an alternative fuel for diesel engine. *Procedia Engineering*. **56**: 625-630.

Al-Rofaai, A., Rahman, W.A., Sulaiman, S.F. and Yahaya, Z.S. 2012. In vitro activity of Neem (*Azadirachta indica*) and Cassava (*Manihot esculenta*) on three pre-parasitic stages of susceptible and resistant strains of *Teladorsagia* (*Ostertagia*) *circumcincta*. *Veterinary Parasitology*. **188**: 85–92.

Al-Samarrai, G., Singh, H. and Syarhabil, M. 2013. Evaluating eco-friendly botanicals (natural plant extracts) as alternatives to synthetic fungicides. *Annals of Agricultural and Environmental Medicine.* **19**(4): 673-676.

AOAC.1990. International Method 921.08: Helrich K ed. Official methods of analysis of the Refractive index. Arlington, USA, Edition 15 Revision 2.

AOAC. 1999. International Method 993.20: P Cuniff ed. Official Methods of analysis of Iodine value. Gaithersburg, MD, Edition 16 Revision 5.

AOAC. 2002. International Method 965.33: Horwitz W ed. Official methods of analysis of the peroxide value. Gaithersburg, MD, Edition 17.

AOCS. 1998. Method Ca 6a-40: Official methods and recommended practices of the AOCS unsaponifiable matter. Press Champaign IL, Edition 5.

AOCS. 2009. Method Ca 2c-25: Official methods and recommended practices of the AOCS moisture and volatile matter: Hot air-oven method. Press Champaign IL, Edition 16.

AOCS. 2009. Method Cd 3a-63: Official methods and recommended practices of the AOCS acid value. Press Champaign IL, Edition 6.

AOCS. 2009. Method Ca 5a-40: Official methods and recommended practices of the AOCS free fatty acids. Press Champaign IL, Edition 6.

Appleton, J. 2010. Evaluating the bioavailability of isoquercetin. *Natural Medicine Journal*. **2**(1): 1-6.

Ara, I., Bukhari, N.A., Aref, N.M., Shinwari, M.M.A. and Bakir, M.A. 2012. Antiviral activities of *streptomycetes* against tobacco mosaic virus (TMV) in *Datura* plant: Evaluation of different organic compounds in their metabolites. *African Journal of Biotechnology*. **11**(8): 2130-2138.

Arunkumark, V. and Paridhavi, M. 2013. Evaluation of the components and antimicrobial activity of volatile oil from *Zanthoxylumlimonella* fruit. *International Journal of Pharma and Bio Sciences.* **4**(2): 777-787.

Arya, S.S., Ramanujam, S. and Vijayaraghavan, P.K. 1969. Refractive index as an objective method for evaluation of rancidity in edible oils and fats. *The Journal of American Oil Chemists' Society.* **46**: 28-30.

Atabani, A.E., Silitonga, A.S., Ong, H.C., Mahlia, T.M., Masjuki, H.H., Badruddin, I.A. and Fayaz, H. 2013. Non-edible vegetable oils: A critical evaluation of oil extraction, fatty acid compositions, biodiesel production, characteristics, engine performance and emissions production. *Renewable and Sustainable Energy Reviews*. **18**: 211-245.

Atawodi, S. E. and Atawodi, J. C. 2009. *Azadirachta indica* (neem): a plant of multiple biological and pharmacological activities. *Phytochemistry Reviews*. **8**(3): 601–620.

Ayadi, M.A., Grati-Kamoun, N. and Attia, H. 2009. Physico-chemical change and heat stability of extra virgin olive oils flavoured by selected Tunisian aromatic plants. *Food and Chemical Toxicology*. **47**: 2613–2619.

Azmir, J., Zaidul, I.S.M., Rahman, M.M., Sharif, K.M., Mohamed, A., Sahena, F., Jahurul, M.H.A., Ghafoor, K., Norulaini, N.A.N. and Omar, A.K.M. 2013.

Techniques for extraction of bioactive compounds from plant materials: A review. *Journal of Food Engineering*. **117**: 426–436

Bachheti, R.K., Rai, I., Joshi, A. and Rana, V. 2012. Physico-chemical study of seed oil of *Prunus armeniaca* L. grown in Garhwal region (India) and its comparison with some conventional food oils. *International Food Research Journal.* **19**(2): 577-581.

Bastos, M.L.A., Lima, M.R.F., Conserva, L.M., Andrade, V.S., Rocha, E.M.M. and Lemos, R.P.L. 2009. Studies on the antimicrobial activity and brine shrimp toxicity of *Zeyheria tuberculosa* (Vell.) Bur. (Bignoniaceae) extracts and their main constituents. *Annals of Clinical Microbiology and Antimicrobials*. **8**(16): 1-6.

Baswa, M., Rath, C.C., Dash, S.K. and Mishra, R.K. 2001. Antibacterial activity of Karanj (*Pongamia pinnata*) and Neem (*Azadirachta indica*) seed oil: a preliminary report. *Microbiology.* **105**: 183-189.

Benavides, S., Villalobos-Carvajal, R. and Reyes, J.E. 2012. Physical, mechanical and antibacterial properties of alginate film: Effect of the crosslinking degree and oregano essential oil concentration. *Journal of Food Engineering.* **110**: 232–239.

Biswas, K., Chattopadhyay, I., Banerjee, R. K. and Bandyopadhyay, U. 2002. Biological activities and medicinal properties of Neem (*Azadirachta indica*). *Current Science*. **82**(11): 1336-1345.

Boursier, C.M., Bosco, D., Coulibaly, A. and Negre, M. 2011. Are traditional Neem extract preparations as efficient as a commercial formulation of azadirachtin A? *Crop Protection*. **30**: 318-322.

Bulbul, I.J., Zulfiker, A.H.M., Hamid, K., Khatun, M.H. and Begum, Y. 2011. Comparative study of *in vitro* antioxidant, antibacterial and cytotoxic activity of two Bangladeshi medicinal plants- *Luffa cylindrica* L. and *Luffa acutangula. Pharmacognosy Journal*. **3**(23): 59-66.

Carballo, J.L., Hernandez-Inda, Z.L., Perez, P. and Garcia-Gravalos, M.D. 2002. A comparison between two brine shrimp assays to detect *in vitro* cytotoxicity in marine natural products. *BioMedical Central Biotechnology*. **2**: 1-5.

Carneiro, S. M.P., Carvalho, F.A.A., Santana, L.C.L.R., Sousa, A.P.L., Neto, J.M.M. and Chaves, M.H. 2012. The cytotoxic and antileishmanial activity of extracts and fractions of leaves and fruits of *Azadirachta indica* (A. Juss). *Biological Research*. **45**(2): 111-116.

Chandrawathani, P., Sariman, Z. Y., Premalatha, B., Rahimah, H., Norhafiza, N.H., Nurulaini, R., Andilla, N.I. and Wahab, A.R. 2013. Evaluation of Neem leaf (*Azadirachta indica*) product for worm control on goats. *Malaysian Journal of Veterinary Research*. **4**(1): 5-12.

Chemat, F., Vian, M.A. and Cravotto, G. 2012. Green extraction of natural products: Concept and principles. *International Journal of Molecular Sciences*. **13**: 8615-8627.

Chaisawangwong, W. and Gritsanapan, W. 2009. Extraction method for high free radical scavenging activity of Siamese Neem tree flowers. *Songklanakarin Journal of Sciences and Technology*. **31**(4): 419-423.

Chen, Y., Zhou, C., Ge, Z., Liu, Y., Liu, Y., Feng, W., Li, S., Chen, G. and Wei, T. 2013. Composition and potential anticancer activities of essential oils obtained from myrrh and frankincense. *Oncology Letters*. **6**: 1140-1146.

Chester, T.L. and Winefordner, J.D. 1977. Throughput advantage and disadvantage in analytical ultraviolet-visible spectrometry by considerations of signal and noise spectral bandpasses. *Analytical Chemistry*. **48**(1): 119-123.

Chindo, I.Y., Osiude J.O. and Yongabi, K.A. 2011. *Azadirachta indica* (Neem) seed oil as adjuvant for antimicrobial activity. *International Research Journal of Applied and Basic Sciences*. **2**: 299-302.

Chourasiya, A., Upadhayay, A. and Shukla, R.N. 2012. To Assess Isolation of quercetin- from the leaves of *Azadirachta indica* and antidiabetic study of the crude extracts. *Journal of Pharmaceutical and Biomedical Sciences*. **25**(25):179-181.

Chowdhury, N., Ghosh, A. and Chandra, G. 2008. Mosquito larvicidal activities of *Solanum villosum* berry extract against the dengue vector *Stegomyia aegypti*. *BoiMed Central Complementary and Alternative Medicine*. **8**:10-17.

Conforti, F., Statti, G.A. and Menichini, F. 2007. Chemical and biological variability of hot pepper fruits (*Capsicum annuum var. acuminatum* L.) in relation to maturity stage. *Food Chemistry*. **102**: 1096–1104.

Dai, J., Yaylayan, V.A., Raghavan, G.S.V. and Pare, J.R. 1999. Extraction and colorimetric determination of azadirachtin-related limonoids in Neem seed kernel. *Journal of Agricultural, Food and Chemistry*. **47**: 3738-3742.

Dai, J. 1999. *Microwave-assisted extraction (MAE) of Neem and the development of a colorimetric method for the determination of azadirachtin related limonoids* (*AZRL*). M.Sc. Thesis. Department of Agricultural & Biosystems Engineering Macdonald Campus of McGill University Montreal, QC, Canada.

Deng, Y., Shi, D., Yin, Z., Guo, J., Jia, R., Xu, J., Song, X., Lv, C., Fan, Q., Liang, X., Shi, F., Ye, G. and Zhang, W. 2012. Acaricidal activity of petroleum ether extract of Neem (*Azadirachta indica*) oil and its four fractions separated by column chromatography against *Sarcoptes scabiei var. cuniculi* larvae in vitro. *Experimental Parasitology*. **130**: 475–477.

Deng, Y.X., Cao, M., Shi, D.X., Yin, Z.Q., Jia, R.Y., Xu, J., Wang, C., Lv, C., Liang, X.X., He, C.L., Yang, Z.R. 2013. Toxicological evaluation of neem (*Azadirachta indica*) oil: Acute and subacute toxicity. *Environmental Toxicology and Pharmacology*. **35**: 240-246.

Dhar, A., Kevin, R. and Agarwal, A.K. 2012. Production of biodiesel from high-FFA Neem oil and its performance, emission and combustion characterization in a single cylinder DICI engine. *Fuel Processing Technology.* **97**:118–129.

Diao, W.R., Hu, Q.P., Zhang, H. and Xu, J.G. 2014. Chemical composition, antibacterial activity and mechanism of action of essential oil from seeds of Fennel (*Foeniculum vulgare Mill.*). *Food Control*. **35**: 109-116.

Djenontin, T.S., Wotto, V.D., Avlessi, F., Lozano, P., Sohounhloue, D.K.C. and Pioch, D. 2012. Composition of *Azadirachta indica* and *Carapa procera* (Meliaceae) seed oils and cakes obtained after oil extraction. *Industrial Crops and Products*. **38**: 39-45.

Du, Y.H., Li, J.L., Jia, R.Y., Yin, Z.Q., Li, X.T., Lv, C., Ye, G., Zhang, L. and Zhang, Y.Q. 2009. Acaricidal activity of four fractions and octadecanoic acid-tetrahydrofuran-3,4-diyl ester isolated from chloroform extracts of Neem (*Azadirachta indica*) oil against *Sarcoptes scabiei var. cuniculi* larvae in vitro. *Veterinary Parasitology*. **163**: 175–178.

Dua, V. K., Pandey, A. C., Raghavendra, K., Gupta, A., Sharma, T. and Dash, A. P. 2009. Larvicidal activity of Neem oil (*Azadirachta indica*) formulation against mosquitoes. *Malaria Journal*. **8**(1): 124-129.

Dutta, R., Sarkar, U. and Mukherjee, A. 2014. Extraction of oil from *Crotalaria juncea* seeds in a modified soxhlet apparatus: Physical and chemical characterization of a prospective bio-fuel. *Fuel* **116**: 794–802.

Ebrahimzadeh, M.A., Nabavi, S.M., Nabavi, S.F., Bahramian, F. and Bekhradnia, A.R. 2010. Antioxidant and free radical scavenging activity of *H. Officinalis* L. *Var. Angustifolius, V. Odorata, B. Hyrcana* and *C. Speciosum*. *Pakistan Journal of Pharmaceutical Sciences*. **23**(1): 29-34.

Ekiert, R.J., Krzek, J. and Talik, P. 2010. Chromatographic and electrophoretic techniques used in the analysis of triazole antifungal agents - a review. *Talanta*. **82**: 1090–1100.

Elaine, S., Robert, H.M., Frederick, A.J., Robert, T.V., Marc-Alain, W., Sharon, R.L., Jeffrey, J.L. and Patricia, G.M. 2011. Foodborne illness acquired in the United States - Major pathogens. *Emerging Infectious Diseases.* **17**(1): 7-15.

Elavarasu, S., Abinava, P., Elanchezhivan, S., Thangakumaran, Vennila, K. and Naziya, K.B. 2012. Evaluation of anti-plaque microbial activity of *Azadirachta indica* (Neem oil) *in-vitro*: A pilot study. *Journal of Pharmacy and Bioallied Sciences*. **4**(2): 394-396.

Elhardallou, S.B. 2011. Cytotoxicity and biological activity of selected Sudanese medicinal plants. *Research Journal of Medicinal Plant.* **5**: 201-229.

El-Hawary, S.S., El-Tantawy, M.E. Rabeh, M.A. and Badr, W.K. 2013. Chemical composition and biological activities of essential oils of *Azadirachta indica* A. Juss. *International Journal of Applied Research in Natural Products.* **6**(4). 33-42.

El-Mahmood, A. M., Ogbonna, O. B. and Raji, M. 2010. The antibacterial activity of *Azadirachta indica* (Neem) seeds extracts against bacterial pathogens associated with eye and ear infections. *Journal of Medicinal Plants Research*. **4**(14): 1414-1421.

Erakhrumen, A.A. 2011. Selected physical and chemical properties of mechanically extracted Neem seed oil sourced as a preservative for ligno-cellulose in southwestern Nigeria. *Forestry Studies in China*. **13**(4): 263-269.

Fernandes, R.V.B., Borges, S.V. and Botrel, D.A. 2013. Influence of spray drying operating conditions on microencapsulated Rosemary essential oil properties. *Food Science and Technology*. **33**:171-178.

Ferreira-Dias, S., Valente, D.G. and Abreu, J.M.F. 2003. Comparison between ethanol and hexane for oil extraction from *Quercus suber* L. fruits. *Grasas y Aceites*. **54**(4): 378-383.

Finney D.J. 1971. Probit Analysis: A Statistical Treatment of the Sigmoid Response Curve. 3rd edn. Cambridge University Press.

Gandhi, M., Lal, R., Sankaranarayanan, A., Banerjee, C.K. and Sharma, P.L. 1988. Acute toxicity study of the oil from *Azadirachta indica* seed (Neem oil). *Journal of Ethnopharmacology*. **23**(1): 39-51.

Ghimeray, A.K., Jin, C.W., Ghimire, B.K. and Cho, D.H. 2009. Antioxidant activity and quantitative estimation of azadirachtin and nimbin in *Azadirachta Indica* A. Juss grown in foothills of Nepal. *African Journal of Biotechnology*. **8**(13): 3084-3091.

Girish, K. and Shankara Bhat, S. 2008. Neem - A Green Treasure. *Electronic Journal of Biology*. **4**(3): 102-111.

Goja, A.M. 2013. Physico-chemical properties of oil produced from *Moringa oleifera, Jatropha curcas* and *Carthamus tinctorius* L seeds. *International Journal of Advanced Research.* **1**:181-187.

Growther, L., Savitha, N. and Niren Andrew, S. 2012. Antibacterial activity of *Punica granatum* peel extracts against shiga toxin producing *E. coli*. *International Journal of Life Sciences Biotechnology and Pharma Research*. **1**(4): 164-172.

Gupta, A., Naraniwal, M. and Kothari, V. 2012. Modern extraction methods for preparation of bioactive plant extracts. *International Journal of Applied and Natural Sciences*. **1**(1): 8-26.

Hammer, K. A., Carson, C.F. and Riley, T.F. 1999. Antimicrobial activity of essential oils and other plant extracts. *Journal of Applied Microbiology*. **86**: 985–990.

Han, J.T., Bang, M.H., Chun, O.K., Kim, D.O., Lee, C.Y. and Baek, N.I. 2004. Flavonol glycosides from the aerial parts of *Aceriphyllum rossii* and their antioxidant activities. *Archibes of Pharmatal Research*. **27**(4): 390-395.

Hanaa, R.M.F., Abdou, Z.A., Salama, D.A, Ibrahim, M.A.R. and Sror, H.A.M. 2011. Effect of Neem and willow aqueous extracts on *fusarium wilt* disease in tomato seedlings: Induction of antioxidant defensive enzymes. *Annals of Agricultural Science*. **56**: 1–7.

Handa, S.S., Khanuja, S.P.S., Longo, G. and Rakesh, D.D. 2008. *Extraction technologies for medicinal and aromatic plants*. ICS-UNIDO, Italy.

Harborne, J.B. 1998. Phytochemical methods. 3rd ed. London, UK: Chapman & Hall.

Helmy, W.A., Abd-Alla, H.I., Amer, H. and El-Safty, M.M. 2007. Chemical composition and in vitro antiviral activity of *Azadirachta indica* A. Juss (Neem) leaves and fruits against Newcastle disease virus and infectious Bursal disease virus. *Australian Journal of Basic and Applied Sciences*. **1**(4): 801-812.

Hind, D., Amar, Z. and Noureddine, G. 2013. Germacrene-D, a characteristic component of the essential oils from the leaves of *Juniperus oxycedrus* ssp. *macrocarpa* (S. et Sm.) BalI Growing in El-Kala, Algeria. *Journal of Natural. Products. Plant Resources.* **3**(1):40-44.

Holopainen, J.K. and Blande, J.D. 2012. *Sensing in Nature*. Carlos Lopez-Larrea. *Sensing in Nature*. Advances in Experimental Medicine and Biology. **739**. United States; Landes Bioscience and Springer.

Hossain, M.A., Al-Toubi, W.A.S., Weli, A.M., Al-Riyami, Q.A. and Sabahi, J.N. 2013. Identification and characterization of chemical compounds in different crude extracts from leaves of Omani Neem. *Journal of Taibah University for Science*. **7**: 181–188.

Ibeto, C.N., Okoye, C.O.B. and Ofoefule, A.U. 2012. Comparative study of the physicochemical characterization of some oils as potential feedstock for biodiesel production. *International Scholarly Research Network*. **2012**: 1-5.

Inouyea, S., Takizawab, T. and Yamaguchia, H. 2001. Antibacterial activity of essential oils and their major constituents against respiratory tract pathogens by gaseous contact. *Journal of Antimicrobial Chemotherapy*. **47**: 565-573.

Islam, M., Al-Amin, M., Ali Siddiqi, M.M., Akter, S., Haque, M.M., Sultana, N. and Chowdhury, A.M.S. 2012. Isolation of quercetin-3-*O*-β-D-glucopyranoside from the leaves of *Azadirachta indica* and antimicrobial and cytotoxic screening of the crude extracts. *Dhaka University Journal of Science*. **60**(1): 11-14.

Ivanova, A., Kostova, I., Navas, H.R. and Villegas, J. 2004. Volatile components of some *Rutaceae* species. *Zeitschrift fur Naturforschung C*. **59**(3-4): 169-173.

Jadeja, G.C., Maheshwari, R.C. and Naik, S.N. 2011. Extraction of natural insecticide azadirachtin from Neem (*Azadirachta indica* A. Juss) seed kernels using pressurized hot solvent. *The Journal of Supercritical Fluids*. **56**: 253–258.

Jahan, T., Begum, Z.A. and Sultana, S. 2007. Effect of Neem oil on some pathogenic bacteria. *Bangladesh Journal of Pharmacology*. **2**: 71-72.

Jain, D., Jayaram, L., Prabhu, V.M. and Bhat, G.K. 2013. Antibacterial effect of Neem (*Azadirachta indica*) oil on multidrug resistant bacteria isolated from human infections. *International Journal of Biological and Medical Research*. **4**(4): 3544-3546.

Jamal, M.A.H.M., Rahman, S., Islam, M.A., Karim, M.R., Alam, M.S. and Rahman, M.Z. 2012. Minimum inhibitory concentration analysis of *Nerium oleander* against bacterial pathogens. *Asian Pacific Journal of Tropical Biomedicine*. **2**(3): 1664-1666.

Jimenez, C.D.C., Flores, C.S., He, J., Tian, Q., Schwartz, S.J. and Giusti, M.M. 2011. Characterisation and preliminary bioactivity determination of *Berberis boliviana Lechler* fruit anthocyanins. *Food Chemistry*. **128**: 717-724.

Kalaiarasan, A. and John, S.A. 2011. GC-MS Analysis of *Bulbophyllum Kaitense Rechib. Pseudobulbs* Estern Ghats of India. *International Journal of Chemistry and Applications*. **3**(3): 215-220.

Kalaivani, C.S., Sathish, S.S., Jankiraman, N. and Johnson, M. 2012. GC-MS studies on *Andrographis paniculata* (Burm.f.) Wall. ex Nees - A medicinally important plant. *International Journal of Medicinal and Aromatic Plants*. **2**(1): 69-74.

Kaufmann, B. and Christen, P. 2002. Recent extraction techniques for natural products: Microwave-assisted extraction and pressurized solvent extraction. *Phytochemical Analysis*. **13**: 105-113.

Kebede, Y., Gebre-Michael, T. and Balkew, M. 2010. Laboratory and field evaluation of Neem (*Azadirachta indica* A. Juss) and Chinaberry (*Melia azedarach* L.) oils as repellents against *Phlebotomus orientalis* and *P. bergeroti* (Diptera: *Psychodidae*) in Ethiopia. *Acta Tropica*. **113**: 145–150.

Khoddami, A., Wilkes, M.A. and Roberts, T.H. 2013. Techniques for analysis of plant phenolic compounds. *Molecules*. **18**: 2328-2375.

Kirira, P.G., Rukunga, G.M., Wanyonyi, A.W., Muregi, F.M., Gathirwa, J.W., Muthaura, C.N., Omar, S.A., Tolo, F., Mungai, G.M. and Ndiege, I.O. 2006. Anti-plasmodial activity and toxicity of extracts of plants used in traditional malaria therapy in Meru and Kilifi Districts of Kenya. *Journal of Ethnopharmacology*. **106**: 403–407.

Kittiphoom, S and Sutasinee, S. 2013. Mango seed kernel oil and its physicochemical properties. *International Food Research Journal*. **20**: 1145-1149.

Kosma, P., Ambang, Z., Begoude, B.A.D., Ten Hoopen, G.M., Kuate, J. and Akoa, A. 2011. Assessment of nematicidal properties and phytochemical screening of neem seed formulations using *Radopholus similis*, parasitic nematode of plant in Cameroon. *Crop Protection*. **30**: 733-738.

Krishnaraju, A.V., Raoa,T.V.N., Sundararajua, D., Vanisreeb, M., Tsayb, H.S. and Subbarajua, G.V. 2005. Assessment of bioactivity of Indian medicinal plants using brine shrimp (*Artemia salina*) lethality assay. *International Journal of Applied Science and Engineering*. **3**(2): 125-134.

Kumar, P.S., Mishra, D., Ghosh, G. and Panda, C.S. 2010. Biological action and medicinal properties of various constituent of *Azadirachta indica* (Meliaceae) an Overview. *Annals of Biological Research*. **2013**: 24-34.

Kumar, K.A. and Viswanathan, K. 2013. Study of UV Transmission through a Few Edible Oils and Chicken Oil. *Journal of Spectroscopy*. **2013**: 1-5.

Kumar, V.S. and Navaratnam, V. 2013. Neem (*Azadirachta indica*): Prehistory to contemporary medicinal uses to humankind. *Asian Pacific Journal of Tropical Biomedicine*. **3**(7): 505-514.

Kumar, V.S., Navaratnam, V., Rajasekaran, A. and Nair, N.K. 2011. Neem leaf glucosamine stimulates Interleukin-2 (IL-2) in swiss albino mice. *Natural Proceedings*. 1-25.

Kurekci, C., Padmanabha, J., Bishop-Hurley, S.L., Hassan, E., Al Jassim, R.A.M. and McSweeney, C.S. 2013. Antimicrobial activity of essential oils and five terpenoid compounds against *Campylobacter jejuni* in pure and mixed culture experiments. *International Journal of Food Microbiology*. **166**: 450–457.

Kutama, I.U. 2008. Correlation between brine shrimp test (BST) and some bioassays using Neem (*Azadirachta indica* A. Juss) and Wild custard-apple (*Annona senegalensis Pers*). *Nigerian Journal of Basic and Applied Sciences*. **16**(2): 123-127.

Labandeira-Rey, M., Couzon, F., Boisset, S., Brown, E.L., Bes, M., Benito, Y., Barbu, E.M., Vazquez, V., Hook, M., Etienne, J., Vandenesch, F. and Bowden, M.G. 2007. *Staphylococcus aureus* panton-valentine leukocidin causes necrotizing pneumonia. *Science Magazine*. **315**(5815): 1130-1133.

Lale, N.E.S. and Abdulrahman, H.T. 1999. Evaluation of Neem (*Azadirachta indica* A. Juss) seed oil obtained by different methods and Neem powder for the management of *Callosobruchus maculatus* (F.) (Coleoptera: *Bruchidae*) in stored cowpea. *Journal of Stored Products Research*. **35**: 135-143.

Lampman, G.M., Pavia, D.L., Kriz, G.S. and Vyvyan, J.R. 2010. *Spectroscopy*. 4th ed. USA: Mary Finch.

Lee, K.Y., Jeong, M.R., Choi, S.M., Na, S.S. and Cha, J.D. 2013. Synergistic effect of fucoidan with antibiotics against oral pathogenic bacteria. *Archives of Oral Biology*. **58**: 482-492.

Liu, H., Wang, K., Zhao, J., Wang, M. and Zhou, L. 2012. Secondary metabolites from *Halostachys caspica* and their antimicrobial and antioxidant activities. *Records of Natural Products.* **6**(1): 57-61.

Majid, M.A., Rahman, I.M.M., Shipar, M.A.H., Helal Uddin, M. and Chowdhury, R. 2004. Physico-chemical characterization, antimicrobial activity and toxicity analysis of *Swietenia mahagoni* seed oil. *International Journal of Agriculture and Biology*. **6**(2): 350-354.

Manges, A.R., Johnson, J.R., Foxman, B., O'Bryan, T.T., Fullerton, K.E., and Riley, L.W. 2001. Widespread distribution of urinary tract infections caused by a multidrug-resistant *Escherichia coli* clonal group. *New England Journal of Medicine.* **345**: 1007-1013.

Meghna, S.V. and Minal, R.C. 2013. Novel Techniques for isolation and extraction of phyto-constituents from herbal plants. *American Journal of Phytomedicine and Clinical Therapeutics*. **1**(3): 338-350.

Meyer, B.N., Ferrighi, N.R., Putnam, J.E., Jacobsen, L.B., Nichols, D.E. and McLaughlin, J.L. 1982. Brine shrimp: A convenient general bioassay for active plant constituents. *Journal of Medicinal Plant*. **45**: 31-34.

Michael, A.S., Thompson, C.G., Abramovitz, M. 1956. *Artemia salina* as a test organism for a bioassay. *Science*. **123**(3194): 464.

Moco, S., Tseng, L.H., Spraul, M., Chen, Z. and Vervoort, J. 2006. Building-up a comprehensive database of flavonoids based on nuclear magnetic resonance data. *Chromatographia*. **64**(9-10): 503-508.

Mohammed, M.I. and Hamza, Z.U. 2008. Physicochemical properties of oil extracts from *Sesamum indicum* L. seeds grown in Jigawa state - Nigeria. *Journal of Applied Science and Environmental Management.* **12**: 99-101.

Mohammed, A.M.A., Coombes, P.H., Crouch, N.R. and Mulholland, D.A. 2013. Chemical constituents from *Fadogia homblei* De Wild (*Rubiaceae*). *International Letters of Chemistry, Physics and Astronomy.* **9**(2): 116-124.

Molina-Salinas, G.M. and Said-Fernandez, S. 2006. A modified microplate cytotoxicity assay with brine shrimp larvae (*Artemia salina*). *Pharmacologyonline*. **3**: 633-638.

Mondali, N. K., Mojumdar, A., Chatterje, S. K., Banerjee, A., Datta, J. K. and Gupta, S. 2009. Antifungal activities and chemical characterization of Neem leaf extracts on the growth of some selected fungal species *in vitro* culture medium. *Journal of Applied Sciences and Environmental Management*. **13**(1): 49-53.

Moorthy, V. and Boominathan, M. 2011. The antimicrobial activities of crude extracts and fraction of *Psidium guajava* and *Azadirachta indica* against *Staphylococcus aureus* in chronic disease affected patients. *International Journal of Universal Pharmacy and Life Sciences.* **1**(2): 160-173.

Murugesan, S., Vijayakumar, R. and Panneerselvam, A. 2011. Evaluation of phytochemical constituents from the leaves of *Memecylon umbellatum Burm*.f. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*. **2**(4): 1145-1152.

Muthu, H., SathyaSelvabala, V., Varathachary, T.K., Selvaraj, S.D., Nandagopal, J. and Subramaniam, S. 2010. Synthesis of biodiesel from Neem oil using sulfated Zirconia via transesterification. *Brazilian Journal of Chemical Engineering.* **27**(4): 601-608.

Nand, P., Drabu, S. and Gupta, R.K. 2012. Insignificant anti-acne activity of *Azadirachta indica* leaves and bark. *Journal of Pharmaceutical Negative Results.* **3**(1): 29-33.

Nathan, S. S., Kalaivani, K. and Murugan, K. 2005. Effects of Neem limonoids on the malaria vector *Anopheles stephens* Liston (Diptera: *Culicidae*). *Acta Tropica*. **96**(1): 47-55.

Nathan, S.S., Choi, M.Y., Paik, C.H., Seo, H.Y., Kim, J.D. and Kang, S.M. 2007. The toxic effects of Neem extract and azadirachtin on the brown planthopper, *Nilaparvata lugens* (Stal) (BPH) (Homoptera: *Delphacidae*). *Chemosphere.* **67**: 80-88.

Nayan, N.K., Kumar, S. and Singh, R.K. 2013. Production of the liquid fuel by thermal pyrolysis of Neem seed. *Fuel* **103**: 437-443.

Ngan, D.H., Hoai, H.T., Huong, L.M., Hansen, P.E. and Vang, O. 2008. Bioactivities and chemical constituents of a Vietnamese medicinal plant Che Vang, *Jasminum subtriplinerve Blume* (*Oleaceae*). *Natural Products Research*. **22**(11): 942-949.

Nguta, J.M. and Mbaria, J.M. 2013. Brine shrimp toxicity and antimalarial activity of some plants traditionally used in treatment of malaria in Msambweni district of Kenya. *Journal of Ethnopharmacology*. **148**: 988–992.

Orhevba, B.A., Chukwu, O., Oguagwu, V. and Osunde, Z.D. 2013. Effect of moisture content on some quality parameters of mechanically expressed Neem seed kernel oil. *The International Journal of Engineering and Science*. **2**: 1-7.

Othman, F., Motalleb, G., Peng, S.L.T., Rahmat, A., Basri, R. and Pei, C.P. 2011. Effect of Neem leaf extract (*Azadirachta indica*) on c-Myc oncogene expression in 4T1 breast cancer cells of BALB/c Mice. *Cell Journal*. **14**(1): 53-60.

Pandey, I.P., Ahmed, S.F., Shhimwal, S. and Pandey, S. 2012. Chemical composition and wound healing activity of volatile oil of leaves of *Azadirachta indica* A. Juss. *Advances in Pure and Applied Chemistry*. **1**(3): 62-66.

Pawar, B., Kanyalkar, M. and Srivasta, S. 2010. Search for novel antifungal agents by monitoring fungal metabolites in presence of synthetically designed fluconazole derivatives using NMR spectroscopy. *Biochimica et Biophysica Acta*. **1798**: 2067–2075.

Prabuseenivasan, S., Jayakumar, M. and Ignacimuthu, S. 2006. In vitro antibacterial activity of some plant essential oils. *BMC Complementary and Alternative Medicine*. **6**: 39-46.

Prakash, O., Gondwal, M. and Pant, A.K. 2011. Essential oils composition and antioxidant activity of water extract from seeds and fruit pulp of *Skimmia anquetilia* N.P. Taylor & Airy Shaw. *Indian Journal of Natural Products and Resources*. **2**(4): 435-441.

Radha, K.V. and Manikandan, G. 2011. Novel production of biofuels from Neem oil. Working paper. World Renewable Energy Congress Sweden. Linkoping: 8-13 May.

Rahmani, M., Mohd Ismail, H., Ahmad, F., Manas, A.R. and Sukari, M.A. 1992. Screening of tropical plants for the presence of bioactive compounds. *Pertarnka* **15**(2): 131-135.

Rajwar, S. and Khatri, P. 2013. Diabetic effects of polyherbal formulation of *Lawsonia inermis* and *Azadirachta indica. Journal of Drug Delivery and Therapeutic.* **3**(2): 45-51.

Rana, S., Bhatt, C., Kanaki, N. and Zaveri, M. 2012. Evaluation of cytotoxicity of some selected medicinal plants. *Advanced Research in Pharmaceuticals and Biologicals.* **2**(3): 290-295.

Rathinamala, J., Anjana, J.C., Sruthy, P.B. and Jayashree, S. 2013. Phytochemical screening and GC-MS analysis of bioactive compounds from *Mimusops elengi. Annals of Pharma Research.* **1**(2): 32-34.

Robertson, S., Narayanan, N. and Nargis, N.R.R. 2012. Toxicity evaluation on hydroalcoholic extract of leaf and stem bark of *Prosopis cineraria.*

International Journal of Pharmacy and Pharmaceutical Sciences. **4**(3): 113-118.

Rodriguez, J.A., Theoduloz, C., Yanez, T., Becerra, J. and Schmeda-Hirschmann, G. 2006. Gastroprotective and ulcer healing effect of ferruginol in mice and rats: assessment of its mechanism of action using in vitro models. *Life Sciences.* **78**(21): 2503-2509.

Samaram, S., Mirhosseini, H., Tan, C.P. and Ghazali, H.M. 2014. Ultrasound-assisted extraction and solvent extraction of papaya seed oil: Crystallization and thermal behavior, saturation degree, color and oxidative stability. *Industrial Crops and Products*. **52**: 702-708.

Sarker, S.D., Latif, Z. and Gray, A.I. 2006. Natural products isolation. 2nd ed. Totowa, New Jersey: Humana Press, Inc.

Sathya, T. and Manivannan, A. 2013. Biodiesel production from Neem oil using two step transesterification. *International Journal of Engineering Research and Application.* **3**: 488-492.

Saravanamuttu, S. and Sudarsanam, D. 2012. Antidiabetic plants and their active ingredients: A review. *International Journal of Pharmaceutical Sciences and Research*. **3**(10): 3639-3650.

Scottish Sensory Centre, 2013. Laboratory video (Quicktime) - distillation, boiling, condensation. Url: http://www.ssc.education.ed.ac.uk/bsl/chemistry/boilingd. html (accessed: 18 March 2014)

Senthilkumar, N., Murugesan, S. and Vijayalakshmi, K.B. 2012. GC-MS-MS analysis of *Trichilia connaroides* (Wight & Arn.) Bentv (Meliaceae): A tree of ethnobotanical records. *Asian Journal of Plant Science and Research*. **2**(2): 207-211.

Sewram, V. 1997. Supercritical fluid extraction and analysis of indigenous medicinal plants for uterotonic activity. Ph.D. Thesis. Department of Chemistry and Applied Chemistry, University of Natal, Durban, South Africa.

Shahid, A.A. 2012. Biological activities of extracts and isolated compounds from *Bauhinia galpinii* (*Fabaceae*) and *Combretum vendae* (*Combretaceae*) as potential antidiarrheal agents. Ph.D Thesis, University of Pretoria.

Siddiquee, S., Cheong, B.E., Taslima, K., Kausar, H. and Hasan, M.M. 2012. Separation and Identification of Volatile Compounds from Liquid Cultures of *Trichoderma harzianum* by GC-MS using Three Different Capillary Columns. *Journal of Chromatographic Science*. **50**: 358-367.

Shivashankar, S., Roy, T.K. and Moorthy, P.N.K. 2012. Headspace Solid Phase Micro Extraction and GC/MS Analysis of the Volatile Components in Seed and Cake

of *Azadirachta indica* A. Juss. *Chemical Bulletin of "Politehnica" University of Timisoara*. **57**(71): 1-6.

Singh, S.P. and Singh, D. 2010. Biodiesel production through the use of different sources and characterization of oils and their esters as the substitute of diesel: A review. *Renewable and Sustainable Energy Reviews*. **14**: 200-216.

Sithisarn, P., Supabphol, R. and Gritsanapan, W. 2006. Comparison of free radical scavenging activity of Siamese Neem tree (*Azadirachta indica* A. Juss var. *siamensis* Valeton) leaf extracts prepared by different methods of extraction. *Medical Principles and Practice*. **15**: 219-222.

Sokoto, M.A., Hassan, L.G., Dangoggo, S.M., Ahmad, H.G. and Uba, A. 2011. Influence of fatty acid methyl esters on fuel properties of biodiesel produced from the seeds oil of *Curcubita pepo*. *Nigerian Journal of Basic and Applied Science*. **19**: 81-86.

Sokovic, M., Marin, P.D., Brkic, D. and Griensven, L.J.L.D.V. 2007. Chemical composition and antibacterial activity of essential oils of ten aromatic plants against human pathogenic bacteria. *Global Science Books*. **1**(2): 220-226.

Solanki, R. and Nagori, B.P. 2012. New method for extracting phytoconstituents from plants. *International Journal of Biomedical and Advance Research*. **3**(10): 770-774.

Sticher, O. 2008. Natural product isolation. *Natural Products Reports*. **25**: 517-554.

Takagi, M., Tachi, Y., Zhang, J., Shinozaki, T., Ishii, K., Kikuchi, T., Ukiya, M., Banno, N., Tokuda, H. and Akihisa, T. 2014. Cytotoxic and melanogenesis-inhibitory activities of limonoids from the leaves of *Azadirachta indica* (Neem). *Chemical Biodiversity*. **11**(3): 451-468.

Talkit, K.M., Mahajan, D.T. and Masand, V.H. 2012. Study on physicochemical properties of vegetable oils and their blends use as possible ecological lubricant. *Journal of Chemical and Pharmaceutical Research*. **4**: 5139-5144.

Tamboli, Y., Selokar, G.R., Paul, A. and Zala, J: 2013. Feasibility testing of VCR engine using various blend of Neem oil. *International Journal of Innovations in Engineering and Technology* **2**: 170-182.

Teressa, S., Kumar, G.M. and Sampath, S. 2004. Ultraviolet transmission through a few edible oils in the context of changing solar insolation. *Journal of Indian Geophysical Union*. **8**: 267-271.

Tharanath, V., Peddanna, K., Kotaiah and Gopal, D.V.S. 2013. Flavonoids isolated from *Foeniculum vulgare* (Fennel) have virostatic efficiency against bluetongue virus. *International Journal of Pharmaceutical Sciences Review and Research*. **23**(1): 237-242.

Throne, J.E., Weaver, D.K., Chew, V. and Baker, J.E. 1995. Probit analysis of correlated data: Multiple observationsa over time at one concentration. *Journal of Economic Entomology*. **88**(5): 1510-1512.

Tiberti, L.A., Yariwake, J.H., Ndjoko, K. and Hostettmann, K. 2007. On-line LC/UV/MS analysis of flavonols in the three apple varieties most widely cultivated in Brazil. *Journal of the Brazilian Chemical Society*. **18**(1): 100-105.

Tistaert, C., Dejaegher, B., Heyden, Y.V. 2011. Chromatographic separation techniques and data handling methods for herbal fingerprints: A review. *Analytica Chimica Acta*. **690**: 148–161.

Tonthubthimthong, P., Chuaprasert, S., Douglas, P. and Luewisutthichat, W. 2001. Supercritical CO_2 extraction of nimbin from Neem seeds - An experimental study. *Journal of Food Engineering*. **47**: 289-293.

Upadhyay, R.K., Dwivedi, P. and Ahmad, S. 2010. Screening of antibacterial activity of six plant essential oils against pathogenic bacterial strains. *Asian Journal of Medical Sciences*. **2**(3): 152-158.

USDA. 2011. Classification for kingdom plantae down to species *Azadirachta indica* A. Juss. *Plant database, United States Department of Agriculture* (online). http://plants.usda.gov/java/ClassificationServlet?kingdom=Plantae&name=Neem&options=1&options=2&options=4&options=8&options=16&options=32&source=start&sort=sciname&Submit.x=34&Submit.y=10 (accessed: 10 January 2011)

Valentova, K., Vrba, J., Bancirova, M., ULrichova, J. and Kren, V. 2014. Isoquercitrin: Pharmacology, toxicology, and metabolism. *Food and Chemical Toxicology*. **68**: 267-282.

Volli, V. and Singh, R.K. 2012. Production of bio-oil from de-oiled cakes by thermal pyrolysis. *Fuel*. **96**: 579-585.

Wangchauy, C. and Chanprasert, S. 2012. Effects of Houttuynia cordata Thunb extract, Isoquercetin and Rutin on cell growth inhibition and apoptotic induction in K562 human leukemic cells. *Journal of Chemical and Pharmaceutical Research*. **4**(5): 2590-2598.

Warra, A.A., Wawata, I.G., Gunu, S.Y. and Aujara, K.M. 2011. Extraction and physicochemical analysis of some selected Northern Nigerian industrial oils. *Archives of Applied Science Research*. **3**: 536-541.

Waters. 2014. How does high performance liquid chromatography work?.Url: http://www.waters.com/waters/en_US/How-Does-High-Performance-Liquid-Chromatography-Work%3F/nav.htm? cid= 10049055&locale= en_US (accessed February 20, 2014).

WHO: 2013. Ultraviolet radiation and the INTERSUN Programme. Geneva: World Health Organization 2013. Url: http://www.who.int/uv/uv_and_health/en/ (accessed April 26, 2013).

Wu, Q., Kohli, M., Bergen, H.R., Cheville, J.C., Karnes, R.J., Cao, H., Young, C.Y., Tindall, D.J., McNiven, M.A. and Donkena, K.V. 2014. Preclinical evaluation of the supercritical extract of *Azadirachta indica* (Neem) leaves in vitro and in vivo on inhibition of prostate cancer tumor growth. *Molecular Cancer Therapy*. **13**(5): 1067-1077.

Yamini, Y., Khajeh, M., Ghasemi, E., Mirza, M. and Javidnia, K. 2008. Comparison of essential oil compositions of *Salvia mirzayanii* obtained by supercritical carbon dioxide extraction and hydrodistillation methods. *Food Chemistry.* **108**: 34-346.

Yogeswari, S., Ramalakshmi, S., Neelavathy, R. and Muthumary, J. 2012. Identification and comparative studies of different volatile fractions from *Monochaetia kansensis* by GC-MS. *Global Journal of Pharmacology*. **6**(2): 65-71.

Ziyada, A.K. and Elhussein, S.A. 2008. Physical and chemical characteristics of *Citrullus lanatus* Var. *Colocynthoide* seed oil. *Journal of Physical Science.* **19**(2): 69-75.

Zhang, Y.Q., Xu, J., Yin, Z.Q., Jia, R.Y., Lu, Y., Yang, F., Du, Y.H., Zou, P., Lv, C., Hu, T.X., Liu, S.L., Shu, G. and Yi, G. 2010. Isolation and identification of the antibacterial active compound from petroleum ether extract of Neem oil. *Fitoterapia*. **81**: 747–750.

Zhu, Y. and Fang, Q. 2013. Analytical detection techniques for droplet microfluidics - A review. *Analytica Chimica Acta*. **787**: 24-35.

Zsila, F., Bikadi, Z. and Simonyi, M. 2003. Probing the binding of the flavonoid, quercetin to human serum albumin by circular dichroism, elcetronic absorption spectroscopy and molecular modelling methods. *Biochemical Pharmacology*. **65**: 447-456.

APPENDICES
Appendix A

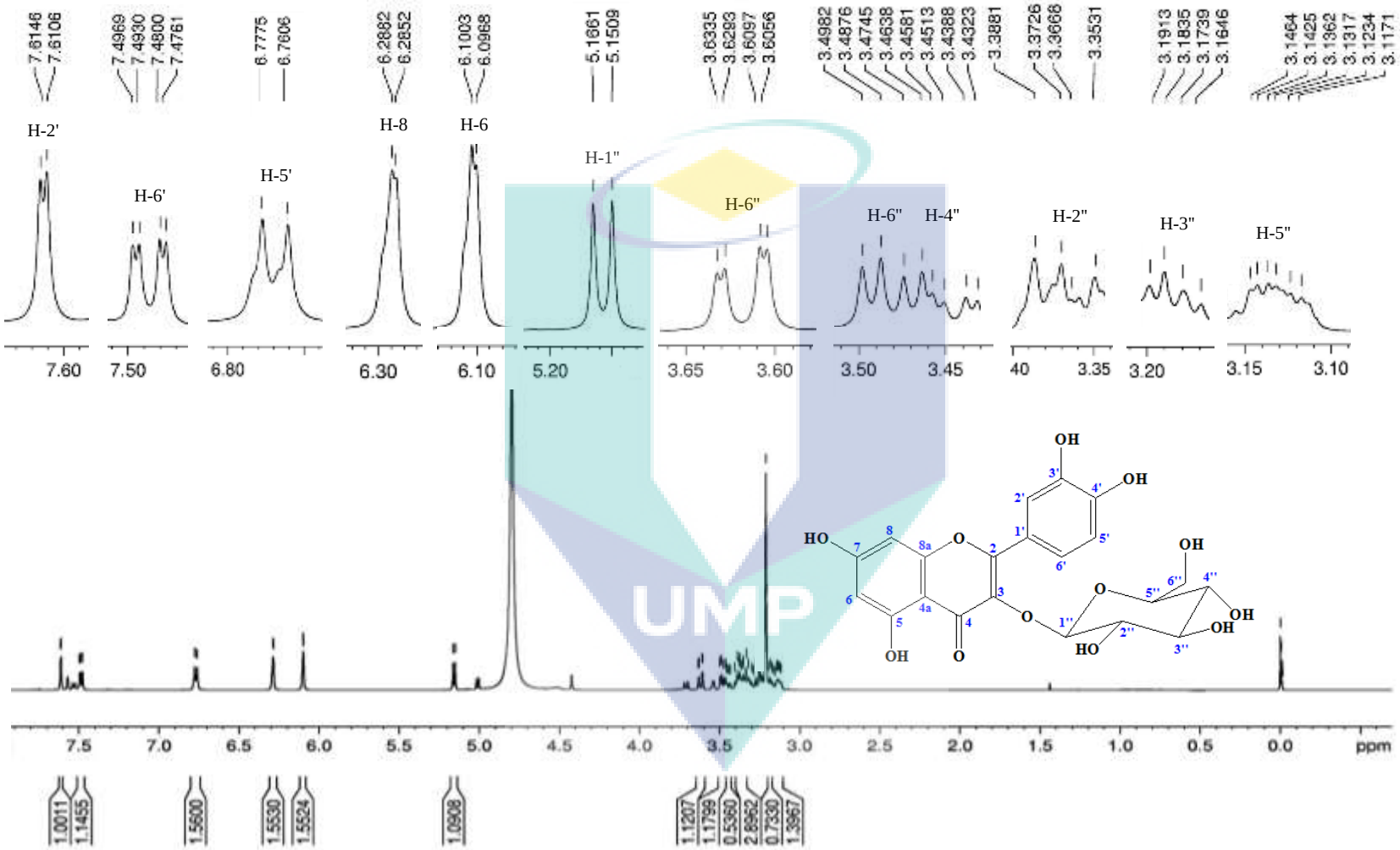


Figure A: ^{1}H NMR spectrum of quercetin-3-*O*-β-D-glucopyranoside

Appendix B

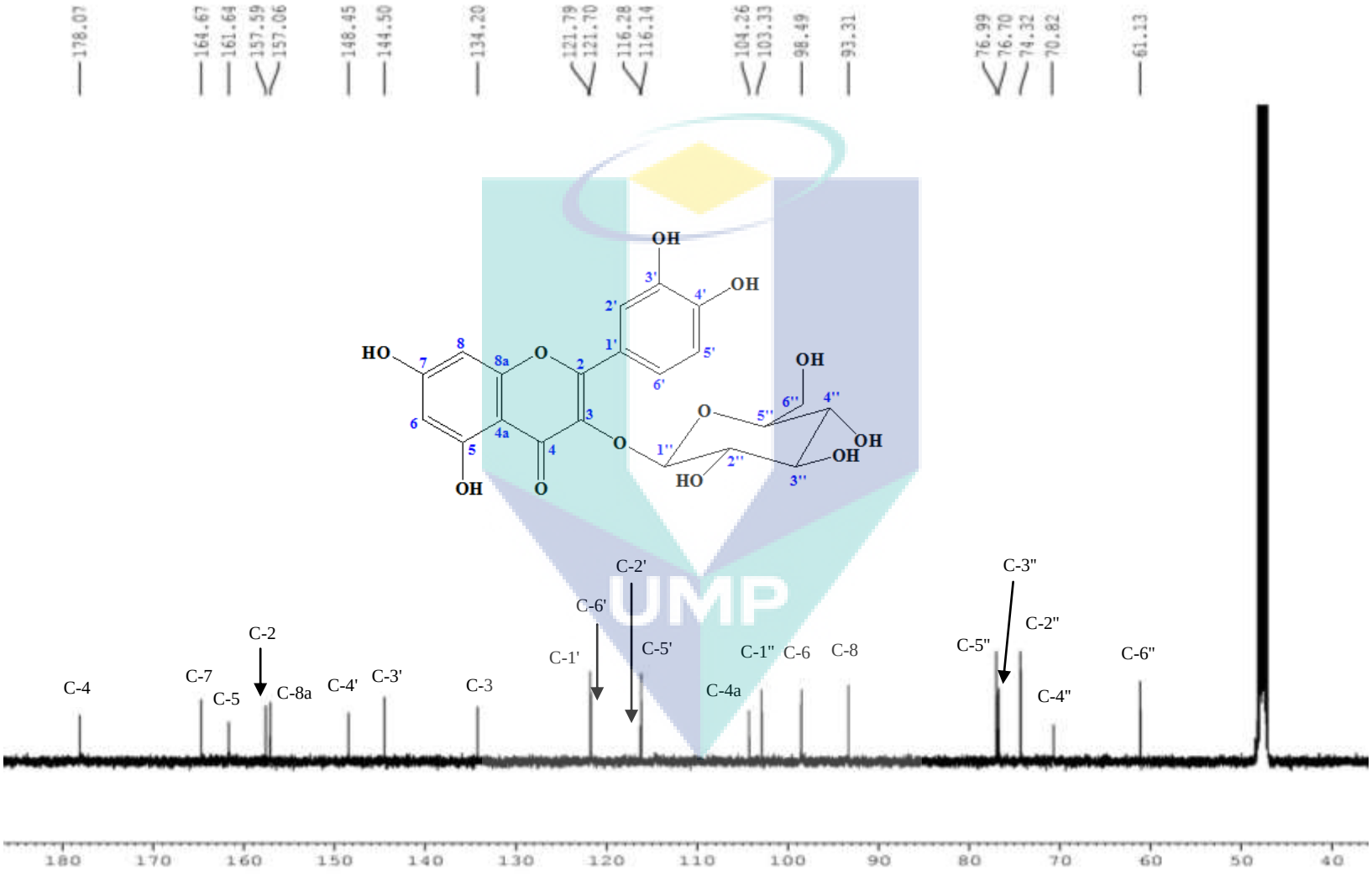


Figure B: ^{13}C NMR spectrum of quercetin-3-*O*-β-D-glucopyranoside

Appendix C

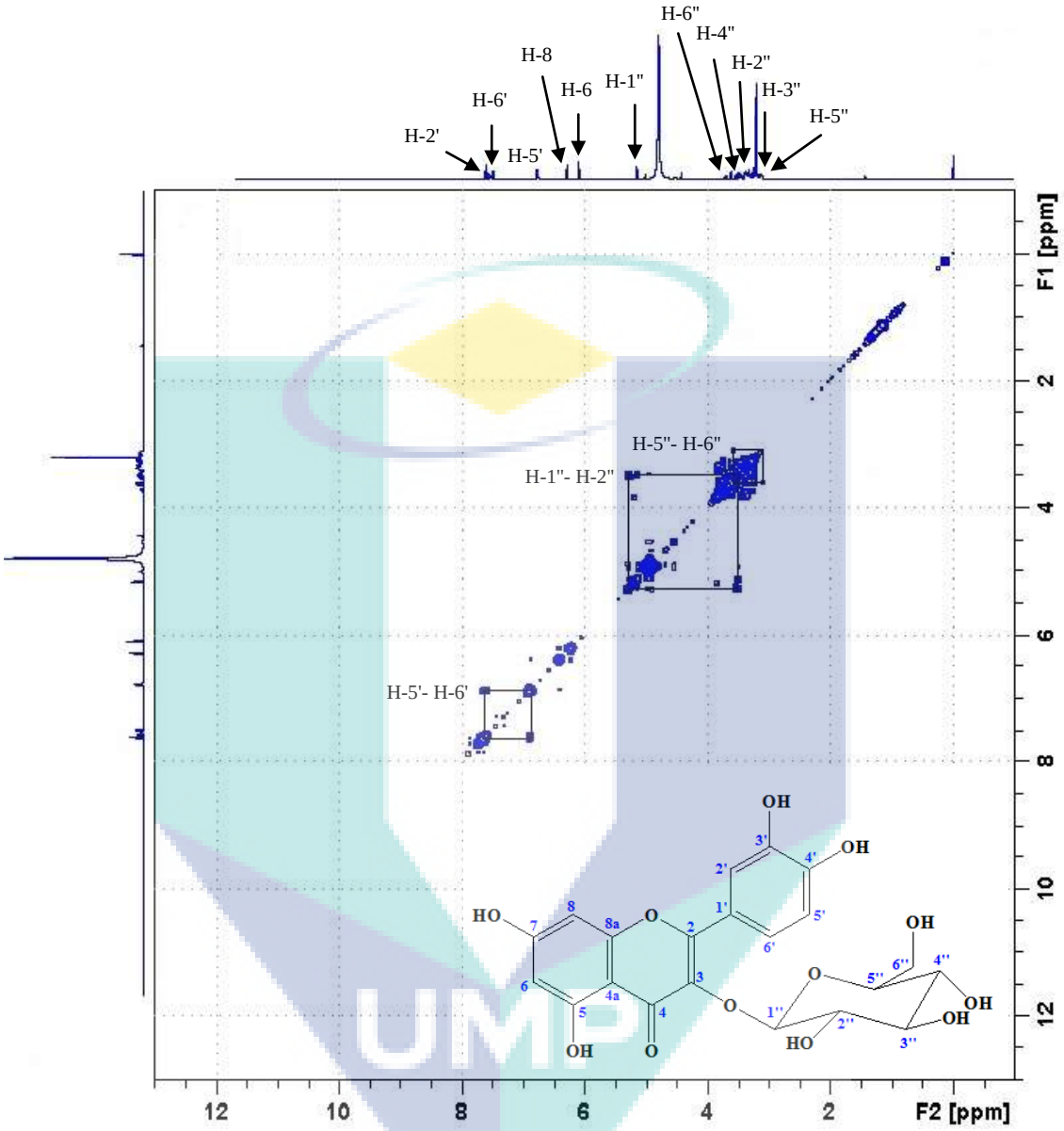


Figure C: COSY spectrum of quercetin-3-*O*-β-D-glucopyranoside

Appendix D

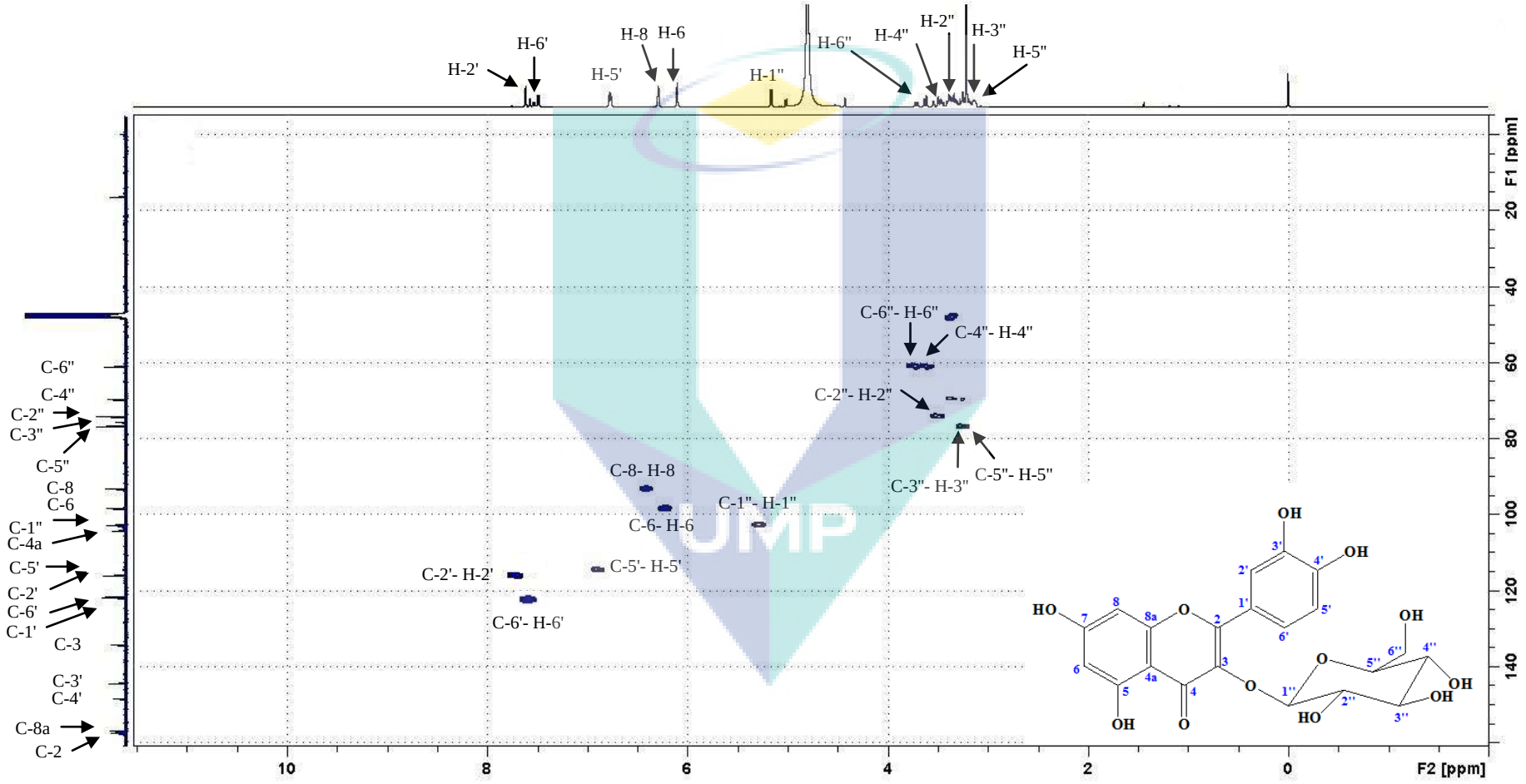


Figure D: HMQC spectrum of quercetin-3-*O*-β-D-glucopyranoside

Appendix E

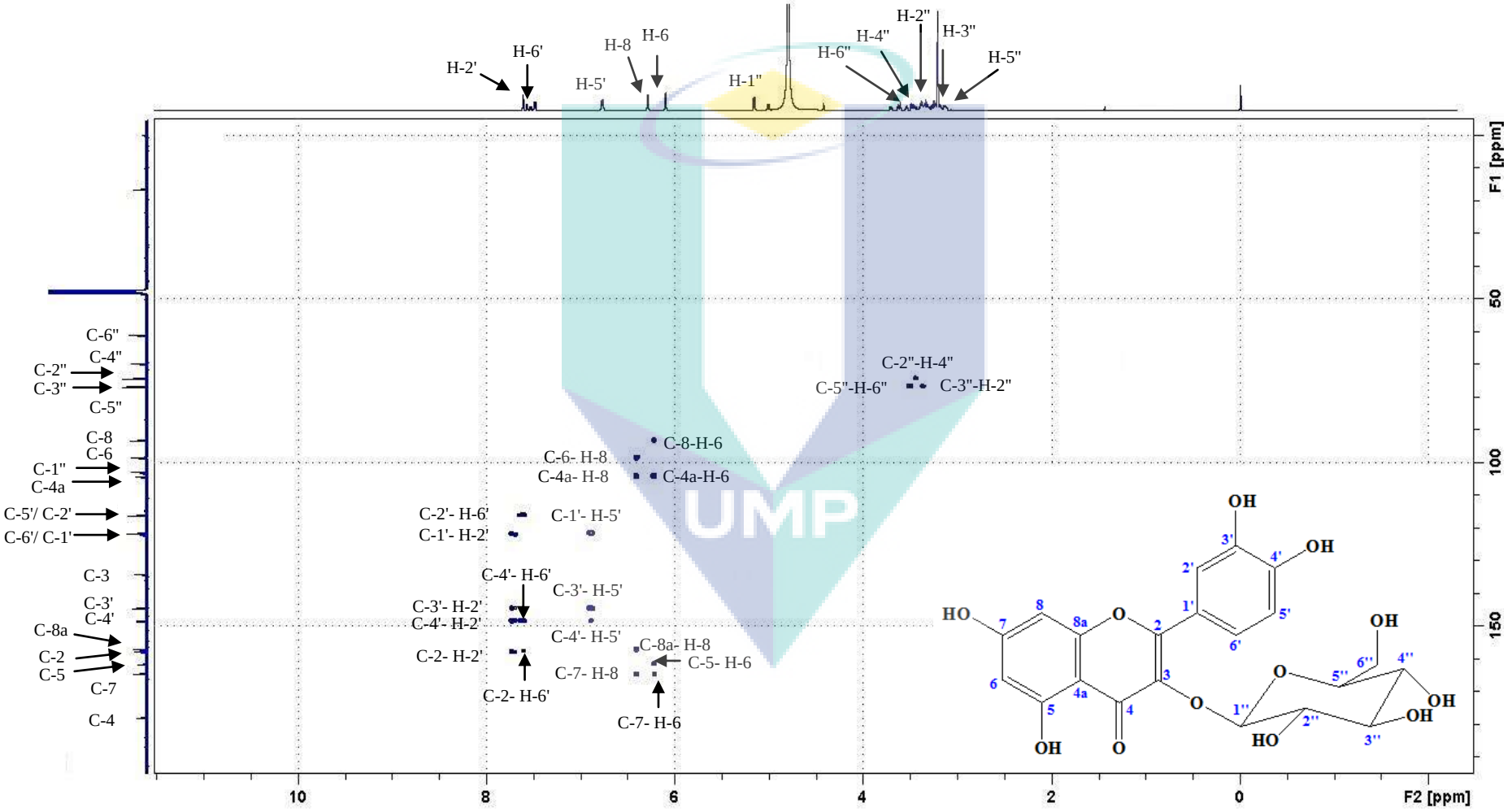


Figure E: HMBC spectrum of quercetin-3-*O*-β-D-glucopyranoside

Appendix F

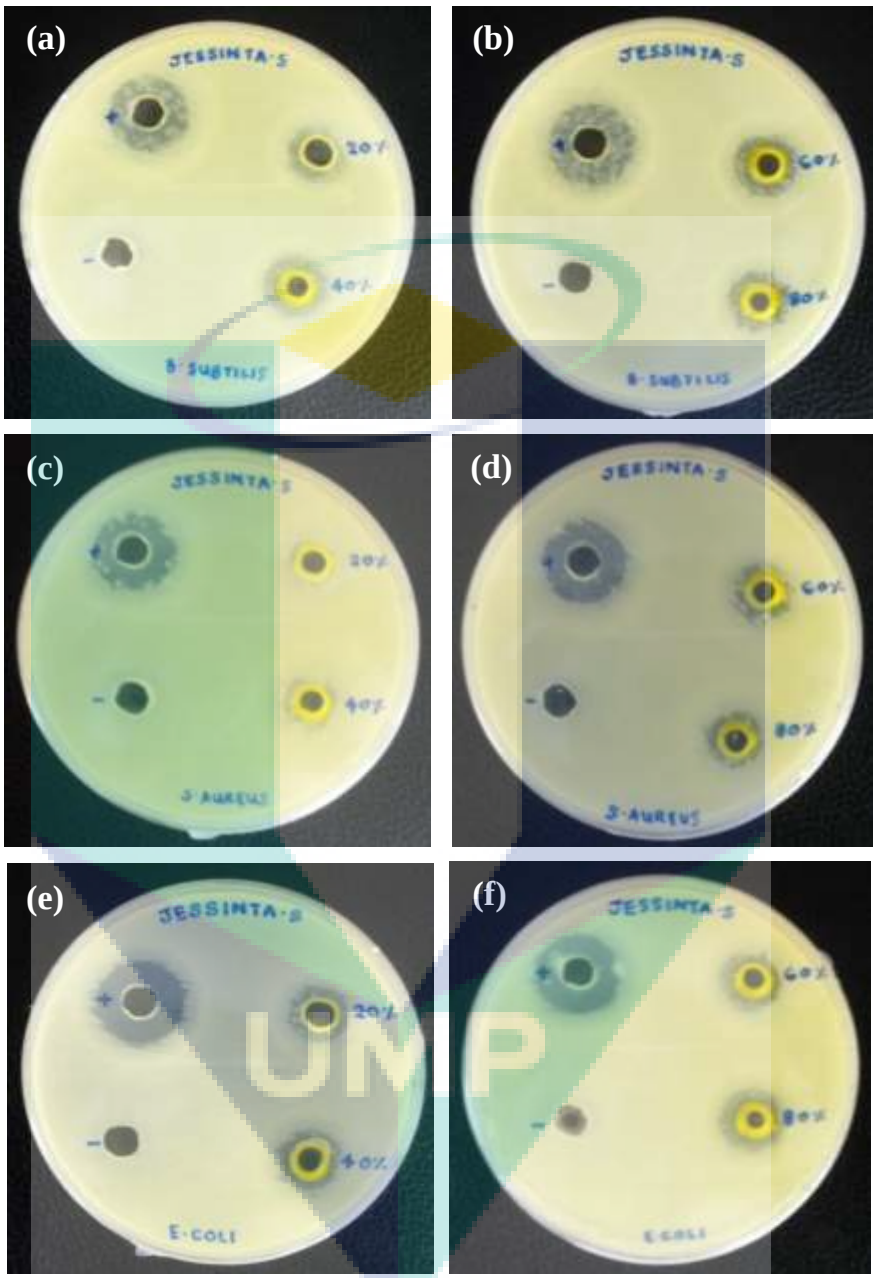


Figure F: Inhibition zone of *A. indica* seed oil against different bacteria
(a and b: *B. subtilis*, c and d: *S. aureus* and; e and f: *E. coli*)

Appendix G

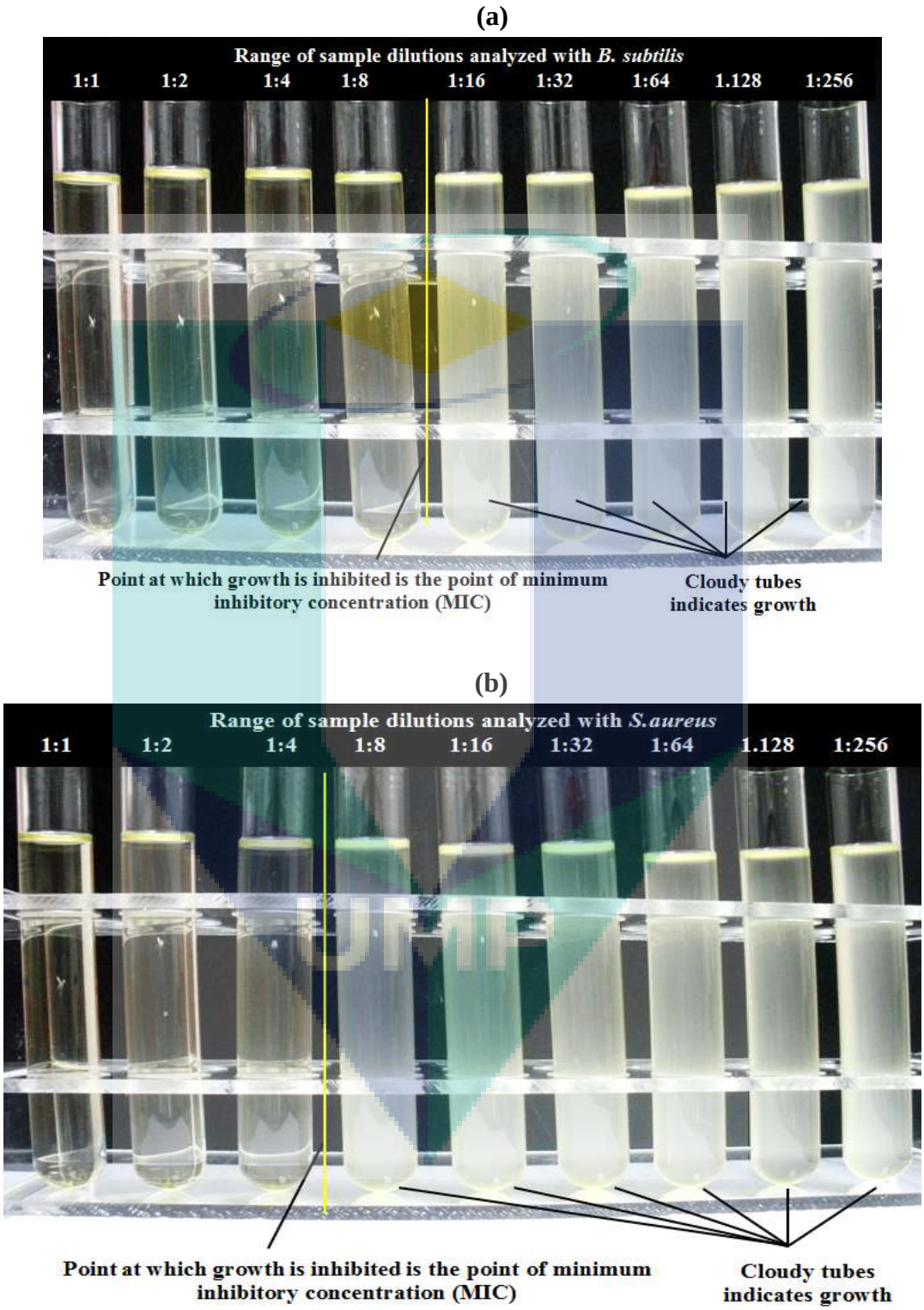


Figure G: MIC of *A. indica* seed oil against bacteria through broth tube dilution technique (a: *B. subtilis*, b: *S. aureus* and c: *E. coli*).

(c)

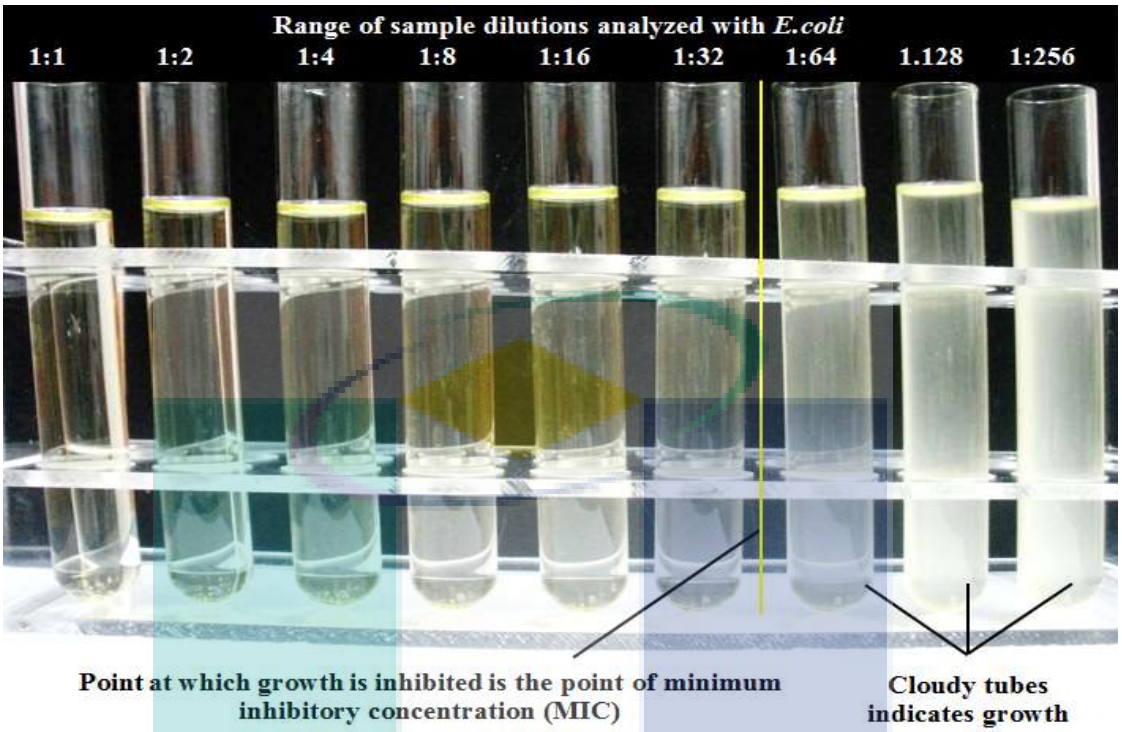


Figure G: *Continues*

Appendix H

List of publications

Awards

Jessinta D/O Sandanasamy, Nour, A.H, Tajuddin, S.N. and Nour, A.H. 2013. *A.salina* Toxicity Assay of Extracts from Different Parts of *A. indica* Seed Oil. Third Prize Best Paper Award ICCEIB2013. Zenith Hotel, Kuantan, Malaysia: 28-29 August.

Jessinta D/O Sandanasamy, Nour, A.H, Tajuddin, S.N. and Nour, A.H. 2014. Harmless and Effective Antibacteria and Larvicide from Neem for Solving Global Problems. Silver medal CITREX. UMP, Kuantan, Malaysia: 4-6 March.

Conferences

Jessinta D/O Sandanasamy, Nour, A.H, Tajuddin, S.N. and Nour, A.H. 2013. *A. salina* Toxicity Assay of Extracts from Different Parts of *A. indica* Seed Oil. ICCEIB2013. The Zenith Hotel, Kuantan, Malaysia: 28-29 Aug. pp. 52

Jessinta D/O Sandanasamy, Nour, A.H, Tajuddin, S.N. and Nour, A.H. 2013. Fatty Acid Composition and Antibacterial Activity of *A. indica* Seed Oil. ICNP 2013. Universiti Teknologi Mara, Selangor. 4-6 March.

Journals

Jessinta, S., Azhari, H.N., Saiful, N.T. and Abdurahman, H.N. 2014 Impact of Geographical Variation on Physicochemical Properties of Neem (*Azadirachta indica*) Seed Oil. *International Journal of Pharmaceutical Sciences and Research.* **5**(10): 4406-4413.

Jessinta, S., Azhari, H.N., Saiful, N.T. and Abdurahman, H.N. 2014. Chemical Characterization and Biological Study of *Azadirachta indica* Extracts. *European Journal of Academic Essays*. **1**(10): 9-16.

Jessinta, S., Azhari, H.N., Saiful, N.T., Abdurahman, H.N., Hazrulrizawati, A.H. and Ahmad Ziad S. Bioassay Guided Isolation and Identification of Cytotoxic Compound from *Azadirachta indica* Leaves. *Journal of the Chinese Chemical Society*. **(Wiley Publication)** ***(In Progress)***

Proceeding

Jessinta D/O Sandanasamy, Nour, A.H, Tajuddin, S.N. and Nour, A.H. 2013. Fatty Acid Composition and Antibacterial Activity of *A. indica* Seed Oil. *The Open Conference Proceedings Journal*. **4** (Suppl-2, M11): 43-48.