Ng Wen Yih

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PHYTOCHEMICAL STUDY ON THE LEAVES OF *Muntingia calabura* AND ITS ANTIBACTERIAL ACTIVITY

NG WEN YIH

BACHELOR OF SCIENCE (HONS)
CHEMISTRY

FACULTY OF SCIENCE,
UNIVERSITI TUNKU ABDUL RAHMAN
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PHYTOCHEMICAL STUDY ON THE LEAVES OF *Muntingia calabura* AND ITS ANTIBACTERIAL ACTIVITY

By

NG WEN YIH

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ABSTRACT

PHYTOCHEMICAL STUDY ON THE LEAVES OF *Muntingia calabura* AND ITS ANTIBACTERIAL ACTIVITY

NG WEN YIH

Muntingia calabura is one of the commonly used traditional medicines in Southeast Asia to treat fever, cold and headache due to the presence of bioactive compounds found on various parts of plant with potent biological activities such as antioxidant, anticancer, antibacterial, anti-inflammatory, etc. The objective of this study is to isolate and elucidate the antibacterial compounds from the leaves of *Muntingia calabura*. The present study involved four main steps, including extraction, isolation, purification and structure characterization as well as to determine the antibacterial activity of pure isolated bioactive compounds from leaves of *Muntingia calabura* via broth microdilution method. There were total of three flavones and one chalcone successfully isolated from the active ethyl acetate crude extract. The structures of the compounds were characterized by modern spectroscopic techniques such as ^{1}H NMR, ^{13}C NMR, HMBC, HMQC, FTIR and UV/Vis and were compared with published data. The flavones and chalcone are 2', 4'-dihydroxy-3'-methoxychalcone **(I)**, 5-hydroxy-3,7-dimethoxyflavone **(II)**, 5-hydroxy-7-methoxyflavone **(III)**, 5-hydroxy-3, 7, 8-trimethoxyflavone **(IV)**. All

the compounds were not active against *B. cereus* and *S.* Typhimurium at highest concentration tested with both minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values greater than 400 mg/L.

ABSTRAK

PENGAJIAN FITOKIMIA PADA DAUN POKOK KERUKUP SIAM DAN AKTIVITI ANTIBAKTERIA

NG WEN YIH

Kerukup Siam adalah salah satu ubat-ubatan tradisional yang biasa digunakan di Asia Tenggara untuk merawat demam, sejuk dan sakit kepala kerana pelbagai bahagian daripada pokok ini mempunyai pelbagai kompaun bioaktif yang telah menunjukkan pelbagai aktiviti biologi seperti antioksidan, antikanser, anti-bakteria, anti-radang dan lain-lain. Objektif kajian ini adalah untuk mengasingkan dan mencirikan kompaun-kompaun tulen anti-bakteria dari daun Kerukup Siam. Kajian ini telah melibatkan empat langkah utama, termasuk pengekstrakan, pengasingan, penulenan dan pencirian serta untuk menguji potensi anti-bakteria bagi kompaun-kompaun tulen dari daun Kerukup Siam melalui kaedah "broth microdilution". Tiga flavon dan satu khalkon telah berjaya diperolehi daripada ekstrak mentah etil asetat. Struktur kompaun tersebut telah dikenalpastikan dengan teknik spektroskopi moden seperti ^{1}H NMR, ^{13}C NMR, HMBC, HMQC, FTIR and UV/Vis dan dibandingkan dengan data yang diterbitkan. Empat kompaun bioaktif adalah 2',4'-dihydroxy-3'-methoxychalcone **(I)**, 5-hydroxy-3,7-dimethoxyflavone **(II)**, 5-hydroxy-7-methoxyflavone **(III)**, 5-hydroxy- 3,7,8-trimethoxyflavone **(IV)** telah diasingkan dan didapati tidak aktif terhadap

B.cereus dan *S.*Typhimurium dengan nilai kepekatan perencatan minima (MIC) dan nilai kepekatan bakterisidal minima (MBC) lebih besar daripada 400 mg/L.

ACKNOWLEDGEMENTS

A great successful on this final year project is not solely an individual's effort but with the kind support and assistance from many peoples and organizations. Firstly, I would like to take this opportunity to extend my sincere thanks to all of them. A special thanks to my final year project supervisor, Ms. Chang Chew Cheen, who has made great contribution in providing useful suggestions, guidance and advices continually throughout the entire course of this project. Again, thanks for giving me such attention and time in practicing before the viva presentation.

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Lastly, a million thanks to my grateful friends, lab mates, and lab assistants' wouldn't be enough to express how awesome they are in providing cooperation and guidance in dealing with various types of spectroscopic instruments. As learning is about sharing, exchanging information and ideas regarding the project with them has indeed stimulate my passion in learning which help the completion of this project smoothly.

DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

_____

NG WEN YIH

APPROVAL SHEET

The project report entitled **"PHYTOCHEMICAL STUDY ON THE LEAVES OF *Muntingia calabura* AND ITS ANTIBACTERIAL ACTIVITY"** was prepared by NG WEN YIH and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) Chemistry at Universiti Tunku Abdul Rahman.

Approved by:

_____

(Ms. Chang Chew Cheen) Date:……

Supervisor

Department of Chemical Science

Faculty of Science

Universiti Tunku Abdul Rahman

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: _____

PERMISSION SHEET

It is hereby certified that **NG WEN YIH** (ID No: **13ADB04873**) has completed this final year project entitled "**PHYTOCHEMICAL STUDY ON THE LEAVES OF *Muntingia calabura* AND ITS ANTIBACTERIAL ACTIVITY**" supervised by Ms. Chang Chew Cheen from the Department of Chemical Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

_____

(NG WEN YIH)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

α	Alpha
ATCC	American Type Culture Collection
A549	Human lung adenocarcinoma
β	Beta
C	Carbon
^{13}C	Carbon-13
CD	Circular dichroism
$CDCl_3$	Deuterated chloform
CD_3COCD_3	Deuterated acetone
COSY	Homonuclear Correlation Spectroscopy
^{o}C	Degree in Celsius
d	Doublet
^{1}D	One dimensional
^{2}D	Two dimensional
DEPT	Distortionless Enhancement by Polarisation
δ	Chemical shift in part per million
FTIR	Fourier Transform Infrared Spectroscopy
GC	Gas chromatography
HCT 116	Human colon cancer cells
Hepa lclc7	Mouse hepatoma cells

HL60	Human promyelocytic leukemia cells
HMBC	Heteronuclear Multiple Bond Coherence
HMQC	Heteronuclear Multiple Quantum Coherence
HPLC	High-performance liquid chromatography
HRMS	High Resolution Mass Spectrometry
HT-29	Human colorectal adenocarcinoma cell line
Hz	Hertz
IC_{50}	Half maximal inhibitory concentration of cell viability
INT	P-iodonitrotetrazolium violet
J	Coupling constant
MBC	Minimum bactericidal concentration
MCF-7	Human breast cancer cell line
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
NCI	National Cancer Institute
NMR	Nuclear magnetic resonance
NOE	Nuclear overhauser effect
P-388	Menogaril-resistant mouse leukemia cells lines
TLC	Thin layer chromatography
UV	Ultraviolet
WRL68	Human hepatic cell line

CHAPTER 1

INTRODUCTION

1.1 A Historical Overview of Natural Products as a Foundation in Drug Discovery

Definition of "natural product" varied according to the different field of science. The term natural product is defined as naturally occurring substance or organic chemical compound synthesize in all cells and living organism either through the primary or secondary pathways of metabolism in the point of view of organic chemistry. Secondary metabolites confer the organisms with evolutionary benefits in defense against predation as some exhibit toxicity (e.g. batrachotoxins in South American frogs) and anti-feedant properties (e.g. bitter taste of alkaloids found in plant being distasteful to grazer), in spite of the fact that they do not play a role in organisms' growth and development (Khalil, Diab and Moudgil, 2016). Natural products serve as important medical agents as it possess active pharmacological activities such as antioxidant, anticancer, antimicrobial, etc. that believe can treat ailments effectively with minimum side effects. This statement can be proven by the action of our earliest ancestors grinded or chewed on certain herbs to break into fine pieces or mashed into paste form to be applied on the area of injuries to relieve pain, or to improve healing (Ji, Li and Zhang, 2009). Thousand years of ancestors' precious medical knowledge was passed down from

parent to child over many generations has indeed contributed to the evolution of drug discovery and development from folk medicine to modern drug.

The oldest surviving written records of ancient Mesopotamia, dating from around 2600 B.C., illustrated on hundreds of clay tablets in cuneiform which indicated the beginning of medicine revolution. Besides that, it was also documented the discovery of about 1,000 medicinal plants and compounds derived from plant. For instance, the oils from *Cupressus sempervirens* (Cypress) and the resin from *Commiphora myrrha* (myrrh) which has found to treat coughs, colds and inflammation effectively are still widely used today. Moreover, the ancient Egyptian Ebers Papyrus pharmaceutical medicinal record, circa 1550 B.C. was depicted approximately 800 complex prescriptions and greater than 700 plant derived drugs including gargles, pills, infusions and ointments as well as natural products such as *Aloe vera* (aloe), *Ricinus communis* oil (castor) and the *Boswellia carteri* (frankincense). Likewise, the Compendium of Medicinal Materials also known as Ben Cao Gang Mu which is one of the prominent works of Li Shi-zhen during Ming dynasty in 1587 A.D. has provided a great insight into the significant medicinal development and revolution. It has recorded 1,892 natural agents and about 11,000 combinatorial recipes and is widely spread until today (Ji, Li and Zhang, 2009; Dias, Urban and Roessner, 2012).

Advancement in spectroscopic analytical technologies has played a central role in improving and extending the human life expectancy in the aspect of its

application in medicinal and pharmaceutical chemistry since natural products often serve as starting compounds in drug discovery and development. Once the chemical structure of the natural compound with high medicinal value was identified, semisynthetic and total synthetic analogs of natural products are readily attainable or replicable with improved effectiveness and safety by applying modification or possible rational design to reach the target compounds (Ji, Li and Zhang, 2009).

1.2 Classification of Phytochemicals

Phytochemicals are chemical compounds that found naturally in plants whereby “phyto” means "plant" in Greek. Plant kingdom provides a wide variety of bioactive species which are potential to serve as therapeutic pharmaceutical agents all around the world includes Asia, Africa, and South America. In recent years, a number of phytochemical studies have shown that plant kingdom is rich in secondary metabolites such as alkaloids, saponins, tannins, phenols, triterpenoids, flavonoids, steroids and coumarines which have found to be accumulated in various parts of plant such as leaves, flowers, bark, seeds, fruits, root, etc. Combination of these secondary plant metabolites has believed to exert synergism medicinal effect to alleviate or cure diseases efficiently. Phytochemicals protect plants against herbivores, UV light, pathogenic and environmental damage which also contribute to the plant’s aroma, flavor and coloration of blossom (Saxena, et al., 2013).

1.2.1 Flavonoids

Flavonoids are naturally occurring phenolic compounds that can be largely found in leaves, stem, flowers and bark of plants. These compounds occurred in the form of glycoside or aglycone. Flavonoids have been known as one of the major groups of plant secondary metabolites with 15 carbon skeleton which consist of one phenylpropane unit (C6-C3) derived from shikimate pathway which form the ring A and C6 unit derived from polyketide pathway. Flavonoids are usually soluble in water due to the present of oxygen atom which could form hydrogen bonds with water molecules. There are more than 4000 different structures reported and can be classified as flavones, flavonols, flavanones, dihydroflavonols, isoflavones, anthocyanins, catechins, aurones and chalcones based on their variety of chemical structures. These compounds play many significant roles such as pigmentation to attract insects for pollination, pathogen resistance, UV light protection, growth and development in plants. Isolation of the flavonoids can be done by using solvents with different polarity. Recently, attention has been given much to flavonoids for health benefits consideration due to their wide biological and pharmacological activities such as antimicrobial, anti-inflammatory, cytotoxicity, antioxidant, antitumor activities, etc (Saxena, et al., 2013). Figure 1.1 and Table 1.1 showed the basic structure of flavonoid skeleton and general description on sub-classes of flavonoids.

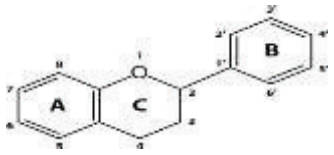
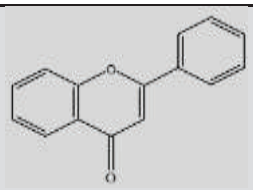
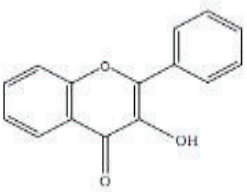
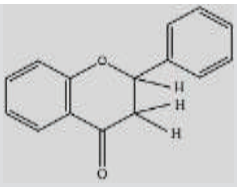
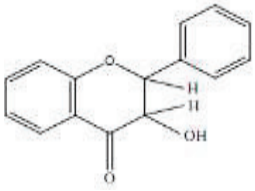
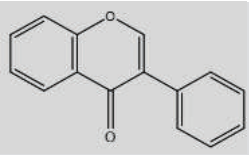
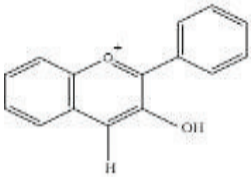


Figure 1.1: Basic structure of flavonoid skeleton with carbon numbering.

Table 1.1: Description of sub-classes of flavonoids (Tazzini, 2014).

Type (s)	Chemical structure	Description (s)
Flavone		- Double bond is located in between C-2 and C-3 and a carbonyl group at C-4 of the ring C. Most of flavones are hydroxylated at C-5/ C-7 of ring A or C-3'/C-4' of the ring B depends on the taxonomic classification of particular botany species.
Flavonol		- Hydroxyl group is located at C-3 of ring C as compared to flavones. Similar to flavones, flavonols are usually methylated and hydroxylated at various carbon positions.
Flavanone		- Consist of saturated ring C which can be differentiated from flavone with the absence of double bond between C-2 and C-3.
Dihydroflavonol		- Can be also known as flavanonols, which can be differentiated from flavanone with the presence of hydroxyl group located at C-3 of ring C.
Isoflavone		- Having rearranged flavonoid structure in which the ring B is located at C-3 of the ring C.
Anthocyanin		- They are flavylium cations which are water soluble due to their ionic characteristic. Anthocyanins are glycosides of anthocyanidins where the glusoce molecules usually attached to C-3 of ring C and often conjugated with phenolic acids.

1.2.2 Alkaloids

Alkaloids are plant secondary metabolites and consist of at least one nitrogen atom in its heterocyclic ring. The present of lone pair electrons on nitrogen atom have make the alkaloid serve as Lewis base and capable to donate electrons. Alkaloids can be derived from amino acid, terpene, steroid and also through shikimate pathway. They are organic compounds can be found from flowering plant, insect, marine organism, fungi, etc. For instance, nicotine from tobacco plant, cocaine from cocoa tree, morphine from opium poppy, which reported to be toxic but possess high medicinal value. Some alkaloids are employed as analgesic agent to relieve the pain such as morphine and cocaine while some serve as antitussive agent such as noscapine and codeine. Alkaloids are essential for the survival of plant as they provide protection against the attack from microorganism such as bacteria and fungi. The applications of alkaloids are dyes, spices, medical drugs, etc. due to their active pharmacological activities including antihypertensive effect (e.g. reserpine), antimalarial action (e.g. quinine), and anticancer activity (e.g. vincristine and vinblastine) (Saxena, et al., 2013).

1.2.3 Terpenoids

Terpenoids are organic compounds can be found from plants, marine organisms and fungi through head to tail joining of smallest five-carbon isoprene units. They are also one of the largest groups of plant secondary metabolites and may appear

in open chain or cyclic structures with one or more double bond which vary from each other based on their functional groups and basic carbon skeletons. Terpenoids serve as important natural products in cosmetic and perfumery industries as fragrant liquids (e.g. menthol and sclareol). Beside that, in agricultural field, terpenoids contribute to fruits flavor and the fragrance of flowers (e.g. linalool) which are the major components in essential oil. Terpenes play a significant role in promoting plant reproduction by attracting particular insects for pollination and also providing protection against herbivores. They have found to exhibit pharmacological activities such as anticarcinogenic (e.g. perilla alcohol), anti-ulcer, antimalarial (e.g. artemisinin), anticancer (e.g. diterpenoid drug, taxol), antimicrobial, etc (Saxena, et al., 2013).

1.3 Botany of Plants Species Studied

1.3.1 Taxonomy

The taxonomy of plant studied in this project is *Muntingia calabura* as shown in Table 1.2 below.

Table 1.2: Taxonomy of *Muntingia calabura* (Team, no date).

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta

Table 1.2: Continued.

Division	Magnoliophyta
Class	Monocotyledonae
Subclass	Rosidae
Order	Malvales
Family	Elaeocarpaceae
Genus	*Muntingia*
Species	*calabura*

1.3.2 Features of *Muntingia calabura*

Muntingia calabura also known as “kerukup siam” or “buah ceri kampong” to Malay and can also addressed as Singapore or Jamaica cherry. This fruit tree can be ripening very fast and the height could reach 7.5 to 12 m with horizontal branches distributed widely. It is drought resistant but not salt tolerant. The leaves are evergreen, oblong-lanceolate, tiny sticky hair underneath and alternate along the stem which long-pointed at the ends 5.0 to 12.5 cm. The flowers comprised of five white petals and five green sepals with numerous prominent yellow stamens located in the center of petals with lightly foul-smelling as shown in Figure 1.3. The flowers are around 1.25 to 2.00 cm wide and grow in 2's or 3's from where leaves attached to the branch. The fruits are round, 1.00 to 1.25 cm in diameter and usually in red or yellow color as shown in Figure 1.4. The fruits taste sweet and consist of a lot tiny yellowish seeds and the skin are thin and smooth (Masudur, Solaiman and Mustafizur, 2010).

Figure 1.2: Tree (Left) and Flower (Right) of *Muntingia calabura.*

Figure 1.3: Fruits of *Muntingia calabura.*

1.3.3 Geographical Distribution

Muntingia calabura is native to southern Mexico, tropical South America, Central America, and the Caribbean. It is also broadly cultivated in warm region of the New World and in India, Southeast Asia, Indonesia, Philippines, including Malaysia. It is being considered as potential candidate for reforestation project for environmental benefits as the seeds of fruits are normally rapid spread by bats and birds and high potential to grow quickly in poor soil environment as shown in Figure 1.5 (Masudur, Solaiman and Mustafizur, 2010; Yusof, et al., 2013).

Figure 1.4: Seeds of fruits are rapidly spread by birds.

1.3.4 Ethnomedicinal and Pharmacological Review

Various parts on *Muntingia calabura* including leaves, barks and flowers have been reported to exhibits useful medicinal values as claimed in Peru. The leaves are believed to possess antiseptic properties to relieve headache, treat pain, cold and gastric ulcer. Others parts of this plant such as roots and flowers have also found to display antidyspeptic, antispasmodic activities and used as an emmenagogue, abortifacient and diaphoretic agents in Vietnam and Philippines (Yusof, et al., 2013). A number of studies have further been made to illustrate the medicinal and pharmacological potential of *Muntingia calabura.* The leaves of this plant have revealed to possess antinociceptive (Yusof, et al., 2013), antioxidant and anti-inflammatory activities (Zakaria, et al., 2014), antimicrobial activity (Zakaria, et al., 2010), cytotoxic and antibacterial activities (Sufian, et al., 2013). Studies have shown that the high potency of this plant with active pharmacological activities is due to the plant has found to be rich in flavonoids secondary metabolites such as flavones, flavanones, etc. Apart from this, extract

of *Muntingia calabura* has also play an important role in cosmetic industry as it has found to have an inhibitory effect on melanogenesis and minimum side effects. Hence, the plant would be a potential candidate as skin whitening agent (Balakrishnan, 2011).

1.3.5 Commercial use of *Muntingia calabura*

The fruits are sweet and widely eaten by children. It can be further processed to tarts and made into jam as shown in Figure 1.6. The leaves can also treat as tea leaves for making tea beverage. The timber is useful for carpentry application since it is compact, hard, durable and low density. Besides, the timber can also use as firewood as it can be lighted easily and provide intense heat for warmth with the release of very little smoke. The bark also can be made into ropes (Lim, 2012).

Figure 1.5: Fruits can be made into jam.

1.4 Methods of Antimicrobial Susceptibility Testing

In vitro study of microbial growth inhibition was performed outside the living organism based on the growth response of microbes when come in contact with compound suspected to possess potential antimicrobial activity. Methods of antimicrobial susceptibility testing can be divided into several types which including disk diffusion, agar dilution, broth dilution and Epsilometer test. Diffusion methods are widely used to determine the antimicrobial susceptibility of substances isolated from clinical samples because it is simple and inexpensive but have their limitations. For instance, obtain equivocal results, unable to obtain precise quantitation of antibiotic action with regard to the pathogen as potential antibacterial may have low rate of diffusion, whether a drug is bactericidal or bacteriostatic and etc. Hence, more precise assessment has been introduced to determine the minimum inhibitory concentration (MIC) of antibiotic to the organisms concerned which involving broth dilution and agar dilution methods. In this project, a simple procedure, broth dilution method has been chosen as antimicrobial susceptibility testing for small number of isolates in triplicates by using 96-well Plates to determine the lowest concentration of antimicrobial to inhibit or kill the microorganism. It has an added advantage as sample from the same wells can further subject to minimum bactericidal concentration (MBC) tests. It is important to determine both MIC and MBC to prevent excessive use of expensive antibiotics and reduces the side effect of larger dose than necessary due to toxicity (Gerard, Berdell and Christine, 2010).

1.5 Hypothesis

The plant *Muntingia calabura,* selected in this study serves as an important source of medicinal agent as it is rich in flavonoids compounds, thus making it active in biological and pharmacological activities such as antioxidant, antibacterial, anticancer and etc. Hence, this project provides an opportunity to isolate the flavonoids compounds (e.g. chalcones and flavones) from leaves of *Muntingia calabura* with significant potential antibacterial activity.

1.6 Objectives of Study

Three main purposes of this study were:

- To isolate bioactive compounds from the leaves of *Muntingia calabura.*
- To identify and elucidate the chemical structures of isolated compounds by using modern spectroscopic techniques.
- To investigate the antibacterial activity of the pure isolates via broth micro-dilution method.

CHAPTER 2

LITERATURE REVIEW

2.1 Phytochemical Investigation on *Muntingia calabura* Leaves Extracts

According to the study of Ridhowi, et al. (2014), qualitative and quantitative analysis on the phytochemical constituents of leaves extracts of *Muntingia calabura* was conducted. The extraction methodology involved extraction of bioactive compounds from dried leaves powder with various solvents including water, methanol, ethanol, chloroform, ether and citric acid. It was reported that polar solvent (water, ethanol and methanol) showing higher potency to extract large amount of bioactive constituents as compared to non-polar solvent (chloroform, ether and citric acid). The results showed that methanol consists of highest percentage of extract concentration, followed by water, ethanol, chloroform, ether and citric acid with 13.15 %, 11.93 %, 9.57 %, 9.04 %, 4.52 % and 3.69 %, respectively. The major phytochemical constituents extracted from plant material in all solvent were flavonoids, tannin and saponins. On quantitative analysis, aqueous and organic solvent revealed that flavonoids are the major components which appeared in great amount compared to tannin and saponin.

2.2 Isolation of Secondary Metabolites from Leaves of *Muntingia calabura* and Study on their Biological Activities

According to Sufian, et al. (2013), there were three flavones and one chalcone being isolated which were 5,7-dihydroxy-3,8-dimethoxyflavone **[1]** (yellow crystals), 2',4'-dihydroxychalcone **[2]** (yellow needle crystals), 5-hydroxy-3,7-dimethoxyflavone **[3]** (yellow solid), and 3,5,7-trihydroxy-8-methoxyflavone **[4]** (yellow crystal) as shown in Appendix A Figure A1. On preliminary screening process, ethyl acetate extract was found to possess notable antibacterial activity against MSSA and MRSA with lowest MIC and MBC values among all the extracts which are 156 μg/mL; 313 μg/mL and 313 μg/mL; 625 μg/mL, respectively and also displayed strongest cytotoxicity against cancer cell lines of HL 60 with lowest IC_{50} value of 17.26 μg/mL but less effective toward MCF 7, HCT 116 and WRL 68 cell lines. It was then subjected to vacuum liquid chromatography to obtain total of seven fractions with fractions 5 revealed as the most effective fraction with the lowest MIC value of 100 μg/mL and 200 μg/mL against MSSA and MRSA, respectively and this fraction was also noted to be the most cytotoxic active toward HL 60 cell line with lowest IC_{50} value of 3.98 ppm within the minimum requirement set by NCI (≤ 20 μg/mL). Further purification of fraction chosen and sub-fractions obtained with radial chromatography had eventually led to the isolation of four known flavonoids compounds **1**, **2**, **3** and **4**. The results for antibacterial activity investigation on these isolated compounds revealed that compounds **1** and **3** were ineffective against MSSA and MRSA. Isolated compounds with MIC value ≤ 100 μg/mL are only considered to be

effective and thus compound **2** considered as an active antibacterial compound against MSSA and MRSA with the MIC values recorded as 50 μg/mL and 100 μg/mL respectively. Chloramphenicol was used as positive control in this study. Study on cytotoxic activity of isolated compounds shown that compounds **2** and **3** exhibit very strong cytotoxic activity against HL60 cell line with lowest IC_{50} values of 3.43 μg/mL and 3.34 μg/mL, respectively whereas compounds **1** and **4** are weak in inhibit the growth of cancer cell lines of HL60 and MCF7 with IC_{50} values exceed the cutoff point set by NCI (less than 4 μg/mL) which ranging from 34.47 to 40.53 μg/mL. Doxorubicin was used as positive control in this study. Note that chemical structure of compounds **1**, **3** and **4** are closely similar with slight different in which compound **3** consists of one hydroxyl group and compounds **1** and **4** bearing two and three hydroxyl groups, respectively but with compound **3** displayed higher cytotoxic properties. Hence, this has concluded that the number of hydroxyl groups present in a compound is not positive correlated with its cytotoxic ability. Besides, compound **4** with the hydroxyl group at carbon position 3 has believed to decrease its cytotoxic potential.

In 2013, Yusof et al had conducted the first study on antinociceptive property of chemical compounds isolated from leaves of *Muntingia calabura* and successfully isolated total of four compounds namely as 5-hydroxy-3,7,8-trimethoxyflavone **[5]** (yellow solid), 3,7-dimethoxy-5-hydroxyflavone **[6]** (yellow needle crystals), 2',4'-dihydroxy-3'-methoxychalcone **[7]** (yellow needle crystals) and calaburone **[8]** (orange crystals) as shown in Appendix A Figure A1. This is the first report on

the isolation of one new compound which is calaburone **[8]** from *Muntingia calabura*. The antinociceptive activities of the isolated compounds were determined by using formalin test in which rats were used as the test subject. The period of time that the rats spent on licking the formalin injected paw reflected the intensity of pain and was recorded in both early and late phases. Initially, petroleum ether extract gave notable antinociceptive activities in both early and late phase amongst the rest of extracts. Seven fractions including from A to G, three flavones and one chalcone were obtained from active petroleum ether extract using activity-guided isolation method. Aspirin and morphine were used as positive control in this antinociceptive study which serves as analgesic potentiater in clinical practice. Results shown that fraction D has displayed useful antinociceptive activity in both phases with no significant difference in the intensity of antinociceptive activity as compared to morphine and aspirin in first phase and second phase, respectively. Further isolation had eventually led to identification of compounds **5**, **6**, **7** and **8**. At the dose of 50 mg/kg, compound **7** has shown the most effective antinociceptive properties with the highest percentage of pain inhibition at 34.5 % and 43.8 % in both early and late phases, respectively in formalin test, followed by compounds **8**, **6** and **5**. Besides, compound **8** displayed higher percentage of antinociceptive inhibition at the dosage of 100 mg/kg which gave 43.5 % and 57.2 % in both first and second phases respectively. According to the study, it has found that there is no positive correlation between the antinociceptive activity with chemical structure of flavonoid derivative compounds (chalcone and flavones). Yet, the findings

obtained has shown that more hydroxylated and less methylated compound would be more active in antinociceptive property as compared to their less active analogue.

In 2005, Chen, et al. (2005) had conducted a research on phytochemical constituents of the leaves of *Muntingia calabura* and their cytotoxic activity against the cancer cell lines of P-388 and /or HT-29 *in vitro*. There were total of twenty flavonoids compounds being isolated from the chloroform and butanol soluble fractions of the leaves which including two new dihydrochalcones, 2',4'-dihydroxy-3'-methoxydihydrochalcone **[9]** (colorless needles), (-)-3'-methoxy-2',4',β-trihydroxydihydrochalcone **[10]** (colorless amorphous solid), one new flavanone, (2*S*)-(-)-5'-hydroxy-7,3',4'-trimethoxyflavanone **[11]** (yellow amorphous solid) and one new flavonol derivative, known as muntingone **[12]** (yellowish powder) as shown in Appendix A Figure A1. The method of isolation involved the use of column chromatography and preparative TLC for further purification. There were also sixteen others known compounds being isolated aside from the four new compounds isolated which including ten known flavones, 5-hydroxy-7-methoxyflavone **[13]**, compound **6**, 5-hydroxy-3,6,7-trimethoxyflavone **[14]**, 6,7-dimethoxy-5-hydroxyflavone **[15]**, 3,5-dihydroxy-7-methoxyflavone **[16]**, 3,5-dihydroxy- 6,7-dimethoxyflavone **[17]**, 8-methoxy-3,5,7-trihydroxyflavone **[18]**, compound **1**, galangin **[19]**, and chrysin **[20]**, three known flavanones, 7-hydroxyflavanone **[21]**, 7-hydroxy-8-methoxyflavanone **[22]**, and 4'-hydroxy-7-methoxyflavanone **[23]**, two chalcones, 2',4'-

dihydroxychalcone **[24]** and compound **7**, and one dihydrochalcone, 2',4'-dihydroxydihydrochalcone **[25]** as shown in Appendix A Figure A1. Study on cytotoxic activity indicated that compounds **11**, **23**, **24** and **7** exhibited notable activity with IC_{50} values of 3.50 μg/mL, 3.88 μg/mL, 0.21 μg/mL and 0.30 μg/mL respectively against P-388 cell line. According to the study, isolates with IC_{50} values below than 4 μg/mL in against P-388 and/or HT-29 cell lines are considered noteworthy. Compounds **11** and **23** have found to exert less cytotoxic effect against HT-29 cell line whereas compounds **24** and **7** were very active against this cancer cell line at lowest concentration tested. Mithramycin has been served as positive control in this study. The findings had shown positive correlation between structure-activity relationships in estimating the cytotoxic potential of isolates. Note that the chemical structures of compounds **9**, **24**, **7** and **25** are likely similar and only differ at substitution and presence of double bond between C-α and C-β which might reveal the significant of this double bond characteristic that causes them possess different range of cytotoxic potency with compounds **24** and **7** showing the stronger effect to against P-388 and HT-29 cell lines as compared to compounds **9** and **25**. Furthermore, compounds **11** and **23** with substituents attached at the C-3', C-4' and C-5' has found to display strong cytotoxic effects to against both cell lines than compounds **21** and **22**.

According to Su, et al. (2003), one new flavanone namely as (2*R*,3*R*)-7-methoxy-3,5,8-trihydroxyflavanone **[26]** had been successfully isolated from the leaves of *Muntingia calabura* via quinone reductase (QR) induction assay along with

thirteen known compounds which including (2*S*)-7-hydroxyflavanone **[27]**, (2*S*)-5,7-dihydroxyflavanone **[28]**, (2*R*,3*R*)-3,5,7-trihydroxyflavanone **[29]**, (2*S*)-5-hydroxy-7-methoxyflavanone **[30]**, compound **18**, 3,5-dihydroxy-7,8-dimethoxyflavone **[31]**, 5,4'-dihydroxy-3,7,8-trimethoxyflavone **[32]**, 5-hydroxy-3,7,8,4'-tetramethoxyflavone **[33]**, compound **2**, 4,2',4'-trihydroxychalcone **[34]**, 7-hydroxyisoflavone **[35]**, 7,3',4'-trimethoxyisoflavone **[36]**, compound **25** as shown in Appendix A Figure A1. The method employed in isolating the phytochemical compounds with effective induce QR activity was activity-guided isolation. Ethyl acetate soluble extract has found to be the most effective fraction that displayed notable QR inducing activity with the lowest CD (concentration required to double the specific activity of quinone reductase) value of less than 2.5 μg/mL and IC_{50} > 10 μg/mL. Quinone reductase , an induction of Phase 2 drug-metabolizing enzymes is well known for its cancer chemoprevention ability. Cultured mouse Hepa lclc7 cells were used as test object while sulforaphane as positive control. From the results of QR inducing test, new compound **26** isolated gave strong activity with CD value of 4.77 μg/mL. Nevertheless, compound **30** exhibited the most significant QR induction activity with the lowest CD value of <0.15 μg/mL which is considered as effective as to sulforaphane. Besides, compound **30** with CI (chemoprevention index) >132 had found to be greater than sulforaphane with CI value of 25.0.

2.3 Isolation of Secondary Metabolites from Stem Bark and Wood of *Muntingia calabura* and Study on their Biological Activities

According to Chen, et al. (2004), the plant *Muntingia calabura* is rich in plant secondary metabolites flavonoids including flavones, flavanones, flavans, and biflavans as the major components. Chloroform soluble fraction of the stem bark of this plant had been subjected to isolation and purification by column chromatography and preparative TLC which resulted in reporting two new flavones, namely as 8-hydroxy-7,3',4',5'-tetramethoxyflavone **[37]** (light yellow needles) and 8,4'-dihydroxy-7,3',5'-trimethoxyflavone **[38]** (light yellow needles) together with thirteen known compounds such as three known flavones, compound **15**, 5,7-dimethoxyflavone **[39]** and 3,5-dihydroxy-6,7-dimethoxyflavone **[40]**, one known flavan, (2*S*)-5'-hydroxy-7,8,3',4'-tetramethoxyflavan **[41]**, three steroids, β-sitostenone **[42]**, 6β-hydroxystigmast-4-en-3-one **[43]** and β-sitosterol **[44]**, four benzoic acid, syringic acid **[45]**, vanillic acid **[46]**, 3-hydroxy-1-(3,5-dimethoxy-4-hydroxyphenyl)propan-1-one **[47]** and tetracosyl ferulate **[48]**, mixture of long chain alcohols, 1-tetracosanol **[49]**, and 1-hexacosanol **[50]** as shown in Appendix A Figure A1. All together 15 isolates from the stem bark of this plant doesn't show cytotoxicity characteristic against A549 and HT-29 cell lines. On the contrary, compounds **37**, **38** and **47** showed the most significant cytotoxic activities against P-388 cell line *in vitro* with ED_{50} values of 3.56, 3.71, and 3.27 μg/mL respectively.

According to the study conducted by Kuo, Liao and Chen (2014), isolation and purification of dichloromethane soluble fraction of methanolic extract from stem wood of *Muntingia calabura* by techniques of column chromatography and preparative TLC had afforded one new biflavan, namely as (*M),(*2S),(2"*S),(P),(*2S),(2"*S)*-7,8,3',4',5',7",8",3"',4"',5"'-decamethoxy-5,5"-biflavan **[51]** (colourless prism), one new flavone, 4'-hydroxy-7,8,3',5'-tetramethoxyflavone **[52]** (yellowish needles), and one new dihydrochalcone, (*R*)-2',β-dihydroxy-3',4'-dimethoxydihydrochalcone **[53]** (amourphous powder) along with twelfth known compounds including one known biflavan, (*M*),(2*S*),(2"*S*)-,(*P*),(2*S*),(2"*S*)-8,5',8"-trihydroxy-7,3',4',7",3"',4"',5"'-heptamethoxy-5,5"-biflavan **[54]**, three known flavones, compound **13**, quercetin **[55]**, and 7,8,3',4',5'-pentamethoxyflavone **[56]**, two known flavans, (2*S*)-8,5'-dihydroxy-7,3',4'-trimethoxyflavan **[57]** and compound **41**, two known flavanones, compound **27** and (2*S*)-7-hydroxy-8-methoxyflavanone **[58]**, two benzenoids, (*E*)-ferulic acid **[59]** and gallic acid **[60]**, and two steroids, compound **42** and compound **44** as shown in Appendix A Figure A1. Human neutrophils play an essential role in providing immune defense against microorganisms and pathogens. In this study, peptide receptor, *N*-Formylmethionyl-leucyl-phenylalanine (fMLP) has been used to induce the generation of a numbers of reactive oxygen species (ROS) including superoxide anion ($O2^{\bullet-}$) and hydrogen peroxide by human neutrophils and ibuprofen, was used as a reference compound which is a clinically used anti-inflammatory agent. Production of these cytotoxins in human body will lead to the inflammatory diseases which will cause serious damaged. From the results of

inhibitory activities on neutrophile pro-inflammatory responses, compounds **13**, **55** and **27** gave the most effective inhibitory effects on fMLP-induced superoxide anion generation with IC_{50} values of 1.77 μM, 3.82 μM and 4.92 μM respectively.

2.4 Isolation of Secondary Metabolites from Root of *Muntingia calabura*

Isolation of phytochemical constituents from root of *Muntingia calabura* collected from Thailand had been done by Kaneda, et al. (1991). From the study, this plant is rich with flavans, biflavans and flavones. Ethyl acetate soluble fraction of methanolic extract from root of *Muntingia calabura* has led to the discovery of total eight known flavonoids compounds including five known flavans, (2S)-8-hydroxy-7,3',4',5'-tetramethoxyflavan **[61]**, (2S)-7,8,3',4',5'-pentamethoxyflavan **[62]**, (2S)-8,2'-hydroxy-7,3',4',5'-tetramethoxyflavan **[63]**, (2S)-2'-hydroxy-7,8,3',4',5'-pentamethoxyflavan **[64]** and (2S)-5'-hydroxy-7,3',4'-trimethoxyflavan **[65]**, two known flavones, 8,5'-dihydroxy-7,3',4'-trimethoxyflavone **[66]** and 5'-hydroxy-7,8,3',4'-tetramethoxyflavone **[67]** and one known biflavan, (M),(2S),(2"S),(P),(2S),(2"S)-8,5',5",8"-tetrahydroxy-7,3',4',7",3"',4"'-hexamethoxy-5,5"-biflavan **[68]** as shown in Appendix A Figure A1.

2.5 Antimicrobial Activity of Extracts of *Muntingia calabura*

In year 2016, William, et al. (2016) had conducted antimicrobial study of ethanolic extract from leaves and stems of *Muntingia calabura* by disc diffusion

assay. The extract showed the most significant activity against *P. aeruginosa* and *S. aureus* with largest average inhibition zone diameter and lowest MIC values of 20.0 mm; 2.500 ppm and 37.7 mm; 1.250 ppm respectively. The MIC was greater than 10.000 ppm against both *S.* Typhimurium and *B. subtilis* with moderate inhibition zone diameter of 19.0 mm and 17.0 mm respectively and with minimal activity against *E. coli.* Secondary metabolites responsible to antimicrobial activity have included polyphenols, tannins and flavonoids were detected in higher concentration under phytochemical screening revealed the fact that *Muntingia calabura* could be a potential alternative source antibacterial agent against both *P. aeruginosa* and *S. aureus.* Zakaria et al. (2010) had employed micro-dilution assay to examine the antibacterial activity on various extracts, partitions and fractions of *Muntingia calabura* leaves. The methanolic extract exhibited the strongest activity than chloroform and aqueous extracts against MSSA, ATCC 25923 (MIC = 1250 ppm; MBC = 1250 ppm) and MRSA, ATCC 33591 (MIC = 2500 ppm; MBC = 2500 ppm). The methanolic extract was subjected to sequential partitioning process with aqueous, petroleum ether and ethyl acetate with ethyl acetate partition possess the most significant activities against MSSA (MIC = 156 ppm; MBC = 156 ppm) and MRSA (MIC = 313 ppm; MBC = 313 ppm). The ethyl acetate partition was then further fractionated to obtain total 15 fractions labeled as A1 to A15 where fractions A9 to A15 were found to show potent antibacterial activity against both the MSSA and MRSA with both MIC and MBC values in the range from 78 to 2500 ppm.

2.6 Antimicrobial Activity of Flavonoids

Xie, et al. (2015) had summarized the structure-antibacterial activity relationship of flavonoids and proposed mechanism. The study had revealed that hydroxylation of flavonoids at specific sites may increase the activity but effect diminished when methylation of hydroxyl groups occurred. Antimicrobial activity of chalcones and others flavonoids also improve when the lipopholicity of ring A increased. The hydrophobic substituents responsible for the increased lipopholicity include prenyl groups, alkyl chains, alkylamino chains and heterocyclic moieties contained nitrogen or oxygen. Table 2.1 summarizes the structure-activity relationship of some important classes of flavonoids.

Table 2.1: Summarization of structure-activity relationship of some important classes of flavonoids (Xie, et al., 2015).

Flavonoid (s)	Structure-Activity Relationship
Flavone	- Highly prenylated flavone showed significant antibacterial activity but intra-chelation of prenyl group with adjacent hydroxyl group will decrease the activity. - Acyl group at C-7 position in Oroxylin has improved the activity and even further when contain long chains alkyls.
Flavanone	- Saturated C3-C4 bond affect the affinities towards proteins and showed stonger activity than flavone. - Prenyl groups at C-6 or C-8 positions improved activity against gram-positive bacteria but less effective than monoprenylated flavanone. - Extending the hydrocarbon chains length substituents at C-7 position of hesperetin also increase the effect.

Table 2.1: Continued.

Flavonol	-	Has better antibacterial effect than flavone due to additional 3-hydroxyl group.
Dihydroflavonol	-	Hydrocarbonylated and prenylated flavonol improved the activity but less effective than dihydroflavonol.
	-	Heterocyclic substitutions on ring A of flavonol even possess strong activity than oxacillin.
Chalcones	-	Hydroxyl groups at C-4' and C-4 positions, substitution of prenyl, hexyl and electron withdrawing groups (chloro) increase the activity but with 4' and 4 dihydroxyl groups will reduce the effect against *E.coli*.
Isoflavonoid	-	Prenyl, hydroxyl and aryl groups enhanced the activity but cyclization between hydroxyl and prenyl groups and methoxylation will decrease the effect.

In year 2005, Cushnie and Lamb stated that there are several factors may contribute to inconsistencies results of antibacterial activity of flavonoids from several published reports. For instance, different assays chosen with varying degree of variations, type and volume of broth or agar, sizes of bacteria suspensions, sizes of wells, sizes of paper disks, strains of particular bacteria pathogens chosen, incubation period, solvent chosen to dissolve the test samples, etc. Formation of precipitate after the tested flavonoids compounds dissolve in broth medium may severely affect the accuracy of MIC assay due to the inhibition of contact between bacterial cells and tested compounds. The study also suggested three possible antibacterial mechanisms of flavonoids which involve (1) inhibition of nucleic acid synthesis: for instance, robinetin inhibits the DNA and RNA

synthesis as caused by intercalation or hydrogen bonding of ring B with nucleic acid bases, (2) inhibition of cytoplasmic membrane function: for example, galangin destroy the cytoplasmic membrane and induce potassium loss from bacteria cells and (3) inhibition of energy metabolism: for instance, licochalcones inhibits the uptake of oxygen in *M.luteus* and *S.aureus* and thus cutoff the energy supplement for the bacteria cells.

2.7 Bacterial Pathogens: Gram Positive and Gram Negative Bacteria

Multilayered cell envelop of bacteria is complex which serves to protect them against hostile environment and can be divided into two categories which are Gram positive and Gram negative as shown in Figure 2.1 and their comparative characteristics were summarized in Table 2.2 below (Gerard, Berdell and Christine, 2010).

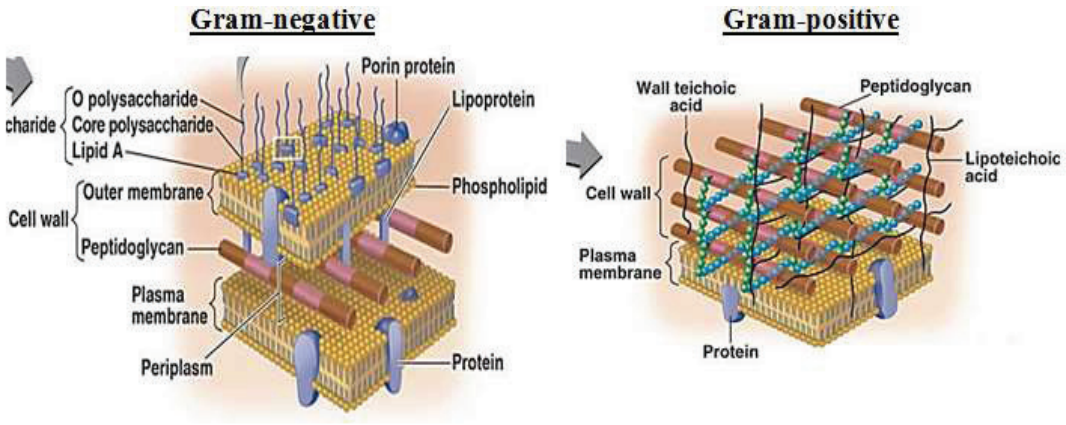


Figure 2.1: Membrane structures of Gram negative and Gram positive bacteria (Gerard, Berdell and Christine, 2010).

Table 2.2: Comparative characteristics of gram positive and gram negative bacteria (Gerard, Berdell and Christine, 2010).

Gram-negative	Gram-positive
Not be able to retain the stain which are counterstain and stain pink or red	Able to retain colored which are crystal violet dye and stain blue or purple
Presence of outer membrane	Absence of outer membrane
Single Peptidoglycan layer	Multi Peptidoglycan layers
High lipopolysaccharide (LPS) content	Low lipopolysaccharide (LPS) content
Produce endotoxin and exotoxins	Produce exotoxins
Absence of teichoic acids	Cell wall contain many teichoic acids
Presence of periplasmic space	Absence of periplasmic space
Flagellum's basal body contain four rings	Flagellum's basal body contain two rings
Cell wall with thickness of 8-12 nm and low disruption by lysozyme	Cell wall with thickness of 20-30 nm and high disruption by lysozyme

2.7.1 *Bacillus cereus*

Bacillus cereus is a Gram-positive bacterium, rod shaped, facultative aerobe, motile and widely distributed environmentally such as soil, dust, plants and food through spore forming due to adhesive nature of its endospores and as a vegetative cell when colonizing in human body. *B. cereus* belongs to the genus of *Bacillus* and family of Bacillaceae and has high resistance towards extreme conditions including heat, freezing, drying and even radiation and thus possess

problems particularly to food industry as it will becomes part of transitory human intestinal flora when the bacterium is ingested in small amount. Besides, *B. cereus* can convert from single cell to multicellular when it grows in soil for translocation. It can presents as straight or lightly curled slender bacilli with the square ends singly or appearing in short chains through blood culture (Figure 2.2a) and can also appears in opaque and dull gray rough matted surface spread irregularly on 5% sheep blood agar at 37°C under aerobic conditions (Figure 2.2b). *B. cereus* also grows as smooth colonies surrounding by uniform zone of beta-hemolysis on 5% sheep blood agar when grown apart from the initial inoculum (Figure 2.2c) (Bottone, 2010).

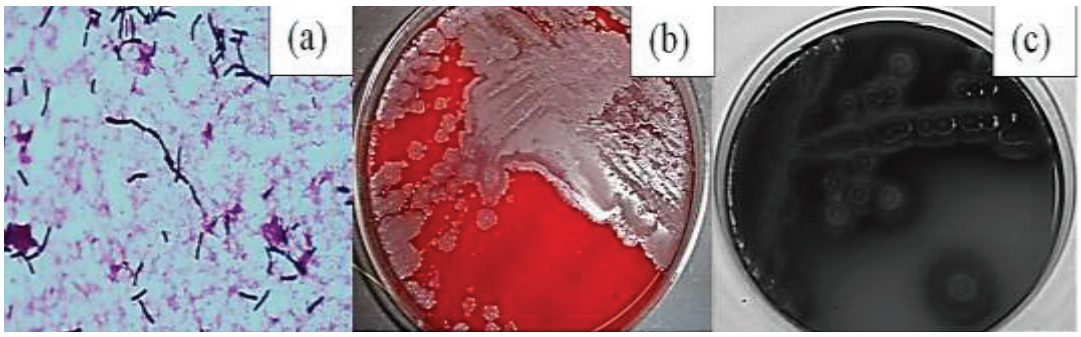


Figure 2.2: *B. cereus* on (a) blood culture, (b) 5% sheep blood agar, (c) 5% sheep blood agar when grown apart from the initial inoculum (Bottone, 2010).

B. cereus causes foodborne diseases due to growing of endospores and induces nausea, vomiting and diarrhea syndromes. Pathogenicity of *B. cereus* is associated to the production of tissue-destructive exoenzyme. For instance, secretion of protein enterotoxin induces diarrheal syndrome and emetic toxin induces vomiting syndrome. It is also responsible to numbers of infections to individuals who are both immunodeficiency and immunocompetent which including

pneumonia, fulminant bacteremia, endophthalmitis, central nervous system (CNS) involvement (meningitis and brain abscesses), skin-lesions and gas gangrene-like cutaneous infections and were susceptible to chloramphenicol, tetracycline ciprofloxacin, gentamicin, streptomycin, etc. The common environmental reservoirs for *B. cereus* are usually from contaminated air filtration, hospital linen, gloves, ventilation equipment, hands of nursing staff and intravenous catheters (Bottone, 2010).

2.7.2 *Salmonella* Typhimurium

Salmonella Typhimurium is a Gram-negative, rod-shaped, facultative anaerobic and motile by peritrichous flagella. It is belongs to genus of *Salmonella* and family of Enterobacteriaceae and has emerged as medically important pathogen for both humans and animal as it can spread locally and worldwide by the absence of effective antimicrobial and human travelling respectively. At the state of extreme dryness, *S.* Typhimurium able to exert cross-tolerance effect that resists to commonly used disinfectants, dry heat and UV irradiation. Besides, it can also survive in estuarine environments below 10°C (Andino and Hanning, 2015). It can grows as spherical smooth colonies (2-4 mm in diameter) on agar but appeared in bluish green with black centres on Hektoen enteric agar which allow it to be distinguished from others species as shown in Figure 2.3 (The Journal of Undergraduate Biological Studies, 2010).

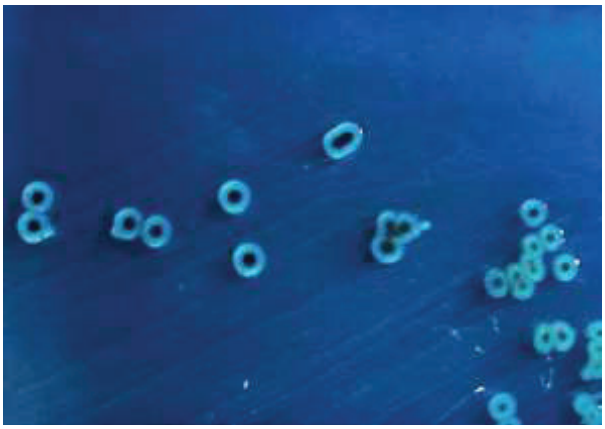


Figure 2.3: *Salmonella* Typhimurium on Hektoen enteric agar.

S. Typhimurium causes typhoid fever, food poisoning and diarrheal infections such as gastroenteritis and mainly passes from one person to another via fecal-oral pathway. When small amount of this pathogen is ingested, it will attack the host by penetrating the intestinal mucosa and spreading to spleen and liver to cause diseases as the outer membrane consist of lipopolysaccharide (LPS) that will release the lipid A endotoxin to produce shock that can be fatal. Production of chelating agent, enterochelin cause iron sequestered from host body which in turn result in enhancing its harmfulness. It was susceptible to gentamicin, norfloxacin, gemifloxacin, ciprofloxacin, etc and the common symptoms of infections will only arise after 12 to 24 hours were involved diarrhea, vomiting, anorexia, headache, myalgias, constipation and fever (Ralph, 1996).

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Materials

3.1.1 Plant Material

The plant*, Muntingia calabura* was chosen to be studied in this project. The leaves of *Muntingia calabura,* approximately 5 kg was collected from roadside of Eastlake in Kampar, Perak.

3.1.2 Chemicals

The chemical solvents, materials, consumables employed throughout this project were listed in Appendix B.

3.1.3 Bacterial Strains

The Gram-positive bacterial strain; *Bacillus cereus* (ATCC 13061) and Gram-negative bacterial strain; *Salmonella* Typhimurium (ATCC 14028) were used as test bacteria. All of the bacteria were grown in Mueller Hinton broth at 37°C and maintained on Mueller Hinton agar at 4 °C until use.

3.2 Methodology

The methodologies can be mainly divided into four steps, including extraction, isolation, purification and elucidation. Approximately 5 kg of leaves of *Muntingia*

calabura were air dried in shaded area and finely grinded into powder form to increase the surface area of plant material so as to maximize the extraction of phytochemical constituents. Maceration technique had been chosen as a simple extraction method in which the dried leaves powder was soaked in 95 % ethanol solvent. Duration of soaking process was 1 week at room temperature to afford dark green solution. Next, the ethanolic crude extract was filtered through the cotton wool and the filtrate was evaporated under the reduced pressure to remove the ethanol solvent via rotatory evaporator at around 50 °C. Temperature setting for solvent evaporation should be in the range of 40 °C to 50°C to prevent natural compounds decomposition at high temperature. The ethanol solvent was recovered and used in further soaking to minimize wastage. The extraction was repeated 4 times, three days once to ensure maximum extraction of bioactive compounds. The extracts were combined and partition with water and ethyl acetate to obtain ethyl acetate extract. It was then subjected to gravity column chromatography to obtain a total of 300 series of fractions. The purity and effectiveness of compounds separation were monitored by TLC analysis from time to time. For instance, a fraction could be considered as impure if more than one spot were visualized on developed TLC plate. This fraction would further subjected to isolation and purification by chromatographic techniques until pure chemical compounds of interest can be isolated successfully. Basically, pure compounds that show only a single spot on the developed TLC plate would be sent for spectroscopic analysis for further purity confirmation.

3.3 Chromatographic Techniques

3.3.1 Column Chromatography

Column chromatography is a common isolation and purification techniques widely used in organic field to isolate pure compounds from sample mixtures even though they are available in small quantity. Increasing popularity of column chromatographic application is mainly due to its effectiveness in separating mixture components that have similar physical and chemical properties and hardly separate by other separation methods. There are two phases involved in chromatography separation such as stationary phase and mobile phase. Generally, stationary phase is referred as solid adsorbent. Porous silica gel (SiO_2) or alumina (Al_2O_3) can be held in a vertical glass column as stationary phase. The mobile phase known as eluent, can be a liquid or a gas will allow to flow past the column through the stationary phase by either gravity force (gravity column chromatography), centrifugal force (centrifugal column chromatography) or with the aid of external pressure (flash column chromatography). The separation of sample components is achieved based on the principle as the mobile phase flows through the stationary phase, it will bring along the mixture components which will move at the different rate due to their different affinity or absorption toward on both the stationary and mobile phases. Hence, pure components can be collected separately according to the separated color bands along the column as shown in Figure 3.1 or specific volumes when the band has the same color.

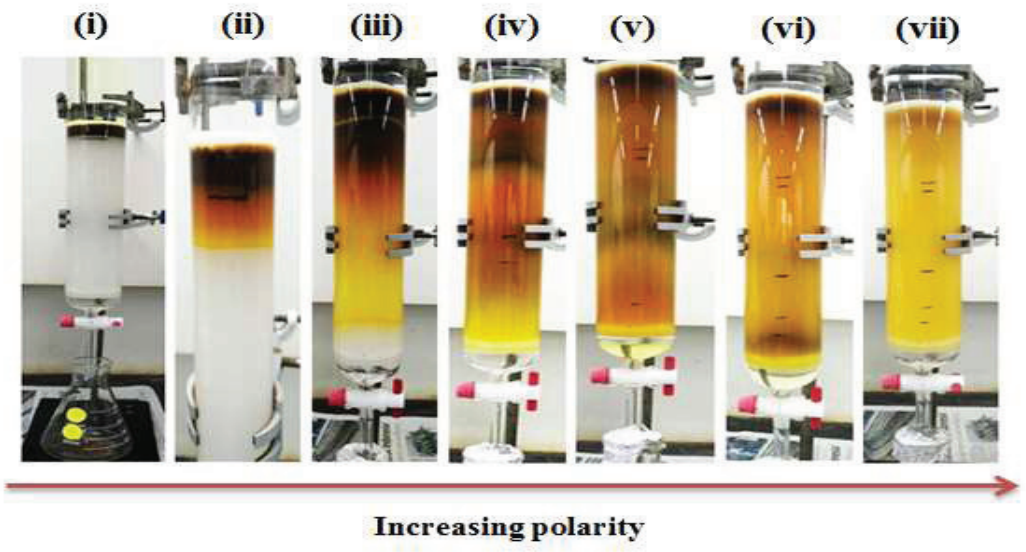


Figure 3.1: Elution process of ethyl acetate crude extract.

The theory behind the different migration rate of chemical compounds is mainly controlled by both the stationary phase and mobile phase. The stationary phase, silica gel consists of polar groupings such as hydroxyl group and ether linkage. Based on "like dissolve like" principle, these polar groups will hold the higher polarity molecule tightly whereas holding the lower polarity molecule loosely. So, lower polarity molecule tends to migrate faster than higher polarity molecule by the solvent, mobile phase. Hence, to promote a high speed elution of all compounds, the polarity of the solvent system can be increased as polar solvent molecule will compete with the silica absorption site with higher polarity molecule resulting in increasing the migration rate of compounds. Gravity column chromatography is a simple, low cost and convenient method chosen to isolate the natural compounds by gravity flow without the need to be driven by external vacuum or pressure lines. Figure 3.2 show the apparatus setup of column chromatography.

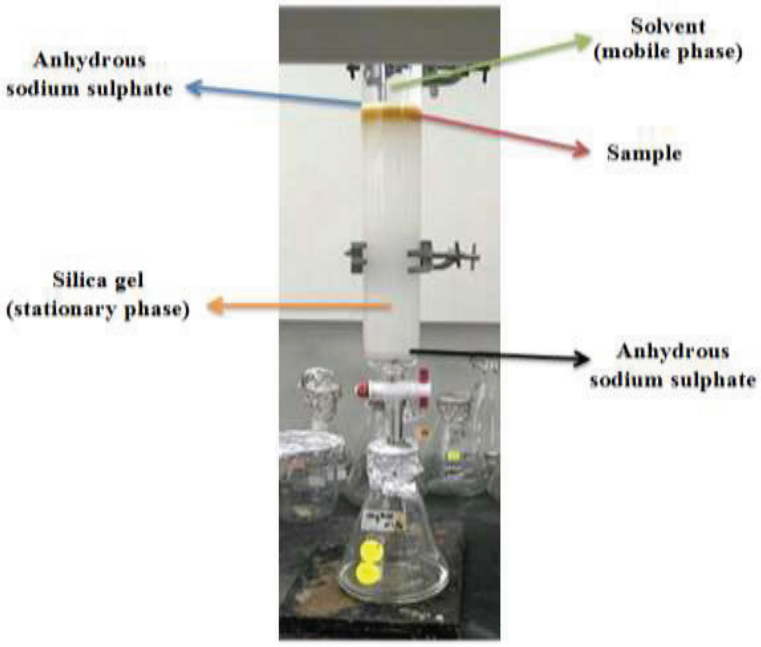


Figure 3.2: Apparatus set up for column chromatography.

Correct packing of a column is the most important experimental factor to achieve the best separation of mixture components. Slurry method has been chosen in this project in which the first step in column packing was to select the glass column with suitable internal diameter which depends on the amount of the sample will be loaded. Next, a 0.5 cm layer of anhydrous sodium sulphate was added. The column was then filled up with hexane and slurry that was made by mixing silica gel with hexane in separated beaker. At this stage, the column was gently tapped by rubber tubing to remove the air bubbles and to allow compact packing of column. The solvent was allowed to drain off but do not allow the solvent level below the silica gel to avoid cracking of packed material that will result in poor compound separation. Once the column was densely packed, the sample was transferred to the top of the silica gel follow by adding 0.5 cm layer of anhydrous sodium sulphate which serves as a protection layer and also to absorb moisture

from the mobile phase. Gradient elution technique will be used in purifying the ethyl acetate extract of *Muntingia calabura* by gradually increase the polarity of solvent system from low to high eluting strength throughout the entire course of column running to obtain a series of fractions. The polarity of solvent system can be increased once there was no movement observed for colored bands in the column or when the color intensity of sample solution collected getting weaker. There are two ways available to load sample onto column such as wet loading and dry loading. Dry loading method provides best separation and purification of mixture components even it is time consuming in preparing the sample as compared to wet loading method. Hence, dry loading method has been chosen to prepare the sample in which the sample was dissolved in minimum amount of solvent that able to dissolve the sample to avoid dilution. Next, the sample was mixed homogenously with minimal amount of silica gel to remove the solvent until a fine powder form sample was obtained. The solid sample was covered with aluminium foil with several numbers of small holes to allow the solvent to evaporate and left it aside for one night at room temperature for drying purpose.

3.3.2 Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) is a simple and widely used method in the field of natural product research due to its usefulness to identify the purity of a component and also to determine the proper solvent system for effective isolation of pure compounds by column chromatography. Working principle of TLC is

more or less same with the column chromatography. Different components will move different distances up the plate, so with different retention factors, R_f. High polarity solvents, usually acetone, can be used to speed up the movement of components on the plate and thus resulting in larger R_f value whereas smaller R_f value would be obtained when low polarity solvent is used such as dichloromethane. Figure 3.3 show the process of developing the TLC plate.

Figure 3.3: Developing TLC plate.

The retention factor, R_f value is used to monitor the compounds separation. The larger the difference of R_f value of mixture components, the better the separation. The developed TLC plate is shown in Figure 3.4. R_f can be determined by measuring the distance travel up by a component from the base line on the plate divided with the distance travel by the solvent from the base line. This number always ranges in between zero and one and equation is shown as below:

$$R_f = \frac{\text{Distance travelled by compound (cm)}}{\text{Distance travelled by solvent (cm)}}$$

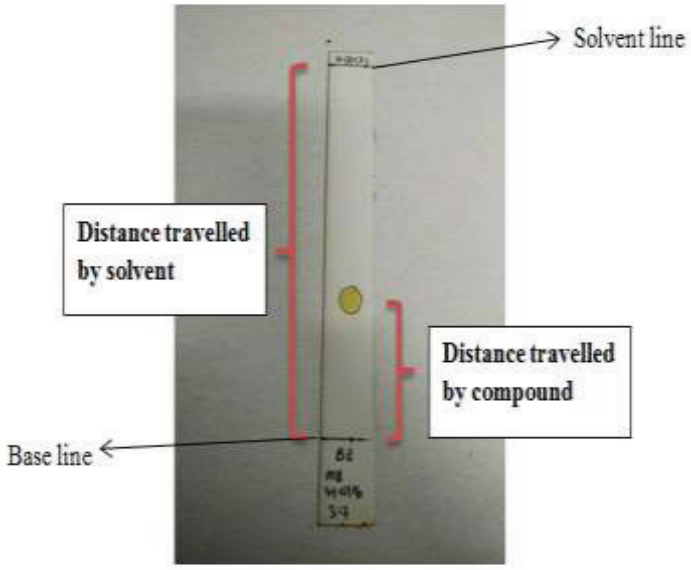


Figure 3.4: Developed TLC plate.

3.4 TLC Detection Methods

3.4.1 Ultraviolet (UV) Detector Lamp

UV detection method is a non-destructive method, in which two different wavelength of UV light are available for visualization, including 254 nm (short wavelength) and 365 nm (long wavelength). The thin layer of silica gel on TLC plate is usually impregnated with zinc sulfide (ZnS), a fluorescent material that glows in bright green light and purple blue light under the short wave and long wave of UV light respectively. UV active compounds with aromatic rings and conjugation system will displayed as dark spots on glowing background of developed TLC plate due to the interference with fluorescence material. UV light is extremely damaging and direct contact on skin and eyes should be avoided**.**

3.4.2 Iodine Vapor Detection

Iodine vapor detection is an extremely useful detection method for visualization of non UV active compounds on developed TLC plate. Appearance of dark brown spots on developed TLC plate indicated the presence of terpenoid and non-conjugated compounds as most of organic compounds form a dark-brown complex with iodine vapor. The dark brown colored spots appeared must be circled with pencil immediately as the staining effect of iodine is just temporary since the iodine will evaporate.

3.5 Instruments

3.5.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

The working principle of Nuclear Magnetic Resonance (NMR) spectroscopy is based on the magnetic behavior of an atom's nucleus. NMR is worked as not all the atoms in molecule resonate at the same frequency due to their existence in different electronic environment. It is an extremely useful modern spectroscopic device to provide valuable physical, chemical, electronic and structural information of a compound. The model of NMR spectrometer employed in this project to conduct both one time dimension (1D NMR): ^{1}H and ^{13}C NMR and two time dimension analyses (2D NMR): COSY, HMQC and HMBC is JOEL JNM-ECX 400 MHz spectrometer. The operating frequencies of the NMR

spectrometer for ^{1}H NMR and ^{13}C NMR experiments are 400 MHz and 100 MHz respectively. In preparing NMR sample, about 20.0 mg sample to be analyzed were measured and dissolved into minimum amount of proper deuterated solvents to avoid any interfering protons exists which depends on the extent of solubility of sample compound in a particular solvent. The sample solvent prepared was transferred into a cleaned and dried NMR tube to about 4 cm of the height. The tube was labeled and capped properly to prevent the deuterated solvent from being evaporated during the experiment. Tetramethylsilane (TMS) was added in small amount to serve as an internal reference.

3.5.2 Infrared (IR) Spectroscopy

Infrared (IR) Spectroscopy allows the determination of valuable structural information of a compound based on the characteristic fingerprint pattern shown at various frequencies indicated different types of functional groups may be presence in a compound. The model of IR spectrometer used to carry out an IR analysis is Perkin Elmer 2000-FTIR spectrometer. KBr pellet method has been chosen to prepare the solid sample by mixing solid sample homogenously with potassium bromide powder in ratio of 1:10 (solid sample: KBr powder). The mixture was compressed under high pressure to melt the KBr and seals the solid sample in a matrix form. The scanning range setting for IR analysis of a sample was usually from 4000 cm^{-1} to 400 cm^{-1}.

3.5.3 Ultraviolet-Visible (UV-Vis) Spectroscopy

The model of UV-Vis Double Beam Spectrophotometer used to conduct a structural analysis on chemical compound isolated was Shimadzu UV-1700 UV-VIS Spectrophotometer. It is a helpful advance spectroscopic instrument that provides useful information on the presence of chromophore, a part of compound responsible for its colour due to electronic transition. Other valuable structural information of a compound such as conjugated π bonds system can also be detected through the presence of significant absorption band occurred in the range of wavelengths from 400 to 700 nm. The sample to be analyzed was prepared in the concentration of 100 ppm (dissolve in analytical grade methanol) initially. The concentration of sample prepared can be adjusted through dilution when peak fluctuation is observed.

3.5.4 Melting Point Apparatus

The purity of isolated compounds can be examined by Stuart Melting point SMP 10 apparatus based on the measurement of melting point range (the point where the compound starts to melt until it is completely melted). A compound is considered pure if it melts over narrow range of two degrees or less. Contrary, a compound with wider range of melting point can be assumed to be impure.

3.6 Antibacterial Assay

3.6.1 Medium Preparation

Mueller Hinton Agar: 34 g of the MH agar powder was dissolved in 1 L of purified water. The medium was sterilized by autoclaving at 121 °C for 15 minutes. Freshly prepared MH agar was poured onto the sterilized petri dish and allowed to cool to room temperature.

Mueller Hinton Broth: 21 g of the MH broth powder was suspended in 1 L of purified water. The medium was sterilized by autoclaving at 121 °C for 15 minutes.

3.6.2 Growth of Bacteria Cultures

Bacillus cereus and *Salmonella* Typhimurium were cultured by streaking the respective bacteria strains from master plates on the MH agar plate to obtain single colonies and incubated at 37 °C for 16 to 18 hours.

3.6.3 Bacteria Suspension Preparation

Direct Colony Suspension Method was chosen as it is simple and convenient by direct making of broth of isolated colonies selected**.** Few colonies of bacteria was inoculated into MH broth medium and the turbidity was adjusted and measured by UV-Vis spectrophotometer at 625 nm until the absorbance unit fall between 0.80 to 1.00 to meet 0.5 McFarland standard where the concentration of bacteria is 1 x 10^8 CFU/mL (Lalitha, 2004).

3.6.4 Growth Indicator Preparation

P-iodonitrotetrazolium violet (INT) was used as growth indicator. INT stock solution of 0.4 mg/ml was prepared by dissolving 40 mg of INT in 100 mL of sterilized deionized water. The tube was wrapped with aluminium foil as INT is light sensitive material and it was kept at 4 °C until used.

3.6.5 Antibiotic Preparation

Gentamicin is an antibiotic useful for the treatment of a number of bacterial infections that can serve as positive control in this antimicrobial susceptibility testing. It was dissolved in sterilized deionized water to concentration of 10 mg/ml as stock solution. Working solution for antibiotic, gentamicin of 512 mg/L was prepared by diluting the stock solution in MH broth medium. Concentration of positive control was tested in the range from 128 mg/L to 1 mg/L.

3.6.6 Solvent Control Preparation

32 % (v/v) of DMSO was prepared by diluting 100 % of DMSO with MH Broth medium. Concentration of solvent control was tested in the range from 8 % to 0.0625 %.

3.6.7 Stock and Working Solutions Preparation

Stock solutions of 5 mg/ml of each isolated compounds in DMSO were prepared. The working solutions of 1600 mg/L for each isolated compounds were prepared by diluting the stock solution in MH broth medium. Concentration of isolates was tested in the range from 400 mg/L to 3.125 mg/L.

3.6.8 Minimum Inhibitory Concentration (MIC) Determination

In this study, MIC defined as minimal concentration of isolates that are able to inhibit the visible growth of microorganism. The sterile and non-treated 96-well U-shaped microplate was used to perform the test. Each of the isolates was tested in triplicate for each bacterium to ensure the reproducibility of result. The design of the 96 well microplates was shown in Figure 3.5. First and foremost, 50 µl of MH broth was added into each well except the well for sterility control which was 100 µl. Next, 50 µl of working solution of 512 mg/L gentamicin (positive control), and 32 % DMSO (solvent control) and 1600 mg/L of samples were added into the respective well of first row. A serial two-fold dilution was performed by removing 50µL of contents from wells in the first row and transferring to the wells in the second row and was repeated until the row H was reached. The excess 50 µl of contents in row H was discarded. After that, 50 µl of 1 x 10^8 CFU/mL bacteria suspension was added into each well except the well for sterility control. The microplate was incubated for 16 to 18 hours at 37 °C. Next, 20 µl of INT growth indicator was added to the well and further incubated for 20 minutes at 37 °C. Visualization of red color was indicated as microbial growth due to staining effect while lack of color change represents no viability of microorganism.

3.6.9 Minimum Bactericidal Concentration (MBC) Determination

A loopful of broth from the well of MIC assay with no visible bacteria growth was inoculated onto MH agar by streaking with inoculating loop. The plate was incubated at 37 °C for 24 hours. MBC in this study was defined as the lowest concentration of isolates showing no bacterial growth on the MH agar plates or less than three colonies observed (Sufian, et al., 2013).

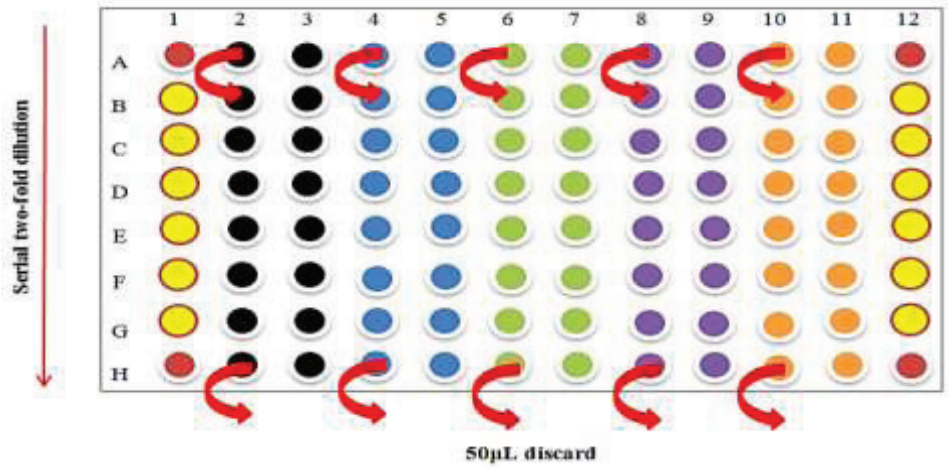


Figure 3.5: Design of 96 well U-shaped microplates for MIC determination.

	Purpose
Sterility Control	To ascertain the sterility of broth medium
Growth Control	To ensure the viability of bacteria
Solvent Control	To determine DMSO solvent inhibitory effect
Positive Control	To compare the effectiveness of bacteria growth inhibition between the antibiotic with isolates.
Sample 1	
Sample 2	
Sample 3	