BIOASSAY-GUIDED ISOLATION OF BIO-ACTIVE ANTIBACTERIAL COMPOUNDS FROM THE LEAVES OF *Wedelia trilobata*

By

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ABSTRACT

BIOASSAY-GUIDED ISOLATION OF BIO-ACTIVE ANTIBACTERIAL COMPOUNDS FROM THE LEAVES OF *Wedelia trilobata*

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Wedelia trilobata is a member of sunflower family Asteraceae. It had been used as traditional medicine to treat various ailments. The objectives of this study were to evaluate the antibacterial activity of the solvent extracts of *W. trilobata* leaves and to isolate the antibacterial compounds from *W. trilobata* leaves. The leaves of *W. trilobata* were sequentially extracted with hexane, chloroform, ethyl acetate, methanol and 70 % acetone. Each solvent extract was determined for their total phenolic and flavonoid contents via Folin-Ciocalteu and aluminium chloride assays, respectively. Disc diffusion, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays were used to screen the antibacterial activity of each solvent extract against six bacteria strains. The 70 % acetone extract showed the highest antibacterial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis* was further subjected to liquid-liquid partition, yielded chloroform, ethyl acetate, and aqueous fractions. The antibacterial activity for each partitioned fraction was then screened again using

the same antibacterial assays. The ethyl acetate fraction which had exhibited the highest antibacterial activity was further subjected to normal phase column chromatography, led to isolation of 80 fractions. These fractions were then combined based on their thin layer chromatography (TLC) profiles and yielded a total of 20 combined fractions. The antibacterial efficiency of all combined fractions against *S. aureus* was determined by MIC and MBC assays. Based on the results obtained, the lowest MIC value of 0.21 mg/ml was exhibited by CF2. To conclude, the bio-active antibacterial compounds in *W. trilobata* leaves were partially isolated (CF2) in the present study. Further researches are needed to fully purify and chemically identify the bio-active antibacterial compounds from the leaves of *W. trilobata*. Moreover, the overall findings in this study suggest the potential of *W. trilobata* leaves for the development of new antibacterial drugs.

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Last but not least, a million thanks to my family for giving me supports and encouragements in completing my final year research project.

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.

_____

CHAN WEI YANG

APPROVAL SHEET

This project report entitled **"BIOASSAY-GUIDED ISOLATION OF BIO-ACTIVE ANTIBACTERIAL COMPOUNDS FROM THE LEAVES OF *Wedelia trilobata*"** was prepared by CHAN WEI YANG and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biotechnology at Universiti Tunku Abdul Rahman.

Approved by:

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UNIVERSITI TUNKU ABDUL RAHMAN

Date: _____

PERMISSION SHEET

It is hereby certified that **CHAN WEI YANG** (ID No: **12ADB07189**) has completed this final year project entitled "BIOASSAY-GUIDED ISOLATION OF BIO-ACTIVE ANTIBACTERIAL COMPOUNDS FROM THE LEAVES OF *Wedelia trilobata*" under the supervision of Dr Tong Kim Suan from the Department of Biological 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,

_____

(CHAN WEI YANG)

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LIST OF ABBREVIATIONS

$AlCl_3$	Aluminium chloride
AmpC	Class C β-lactamse
ATCC	American type culture collection number
CAP	Covalently attached protein
CF	Combined Fraction
CFU	Colony forming unit
CVC	Central venous catheters
DAEC	Diffusely adherent *Escherichia coli*
EAEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
Etc.	So forth
F	Fraction
GAE	Gallic acid equivalent
GC	Guanine – Cytosine
HPLC	High performance liquid chromatography
IMP	Integral membrane protein
LB	Luria Bertani agar

LP	Lipoprotein
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
NA	Nutrient agar
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
NMR	Nuclear magnetic resonance
OMP	Outer membrane protein
QE	Quercetin equivalent
spp.	Species
TFC	Total flavonoid contents
TLC	Thin layer chromatography
TMV	Tobacco mosaic virus
TPC	Total phenolic contents
UV	Ultraviolet
WTA	Wall teichoic acid

CHAPTER 1

INTRODUCTION

For several decades, plants have been used as traditional medicine to combat different diseases caused by bacterial infection. This is due to the presence of phytochemical constituents in the plant such as alkaloid, anthraquinones, anthocyanin, saponins, sterols, phenolic compounds, tannins, flavonoids and terpenoids (Edeoga, Okwu and Mbaebie, 2005). These phytochemical constituents are initially the secondary metabolites produced by plants to defense themselves against pathogens and herbivores or to attract the pollinators (Brusotti, et al., 2014). Surprisingly, some of these phytochemical constituents are found to exhibit various biological activities such as antibacterial, antioxidant, anti-inflammatory, antitumor and so on.

In recent years, numerous drugs have lost their effectiveness due to the expression of resistance gene in bacteria (Govindappa, et al., 2011). This has encouraged researchers to look into higher plants for potential antibacterial compounds in order to discover alternative sources for the replacement of existing antibiotic. Furthermore, synthetic drugs were found to be associated with side effects such as hypersensitivity, immune-suppression and allergic reactions (Sadeghian, et al.,

2012). Thus, the discovery of plant-derived bio-active compounds has become necessary due to they are cheaper, less toxic and having no side effect (Jethinlalkhosh and Lathika, 2012).

Other than pharmaceutical purposes, plant-derived antibacterial compounds are also important in agriculture field. Nowadays, the usage of chemical pesticide has caused some adverse effects including environmental pollution, health hazard, high cost and the development of pesticide-resistance pathogens (Sehajpal, Arora and Kaur, 2009). Therefore, the production of ecofriendly pesticide using plant-derived antibacterial compounds has become an alternative way to solve the problems.

Knowing the fact that bio-active antibacterial compounds extracted from plants have been used to generate various drugs, pesticides and other antibacterial agents used in our daily life, therefore the objectives of this project were to:

I. To evaluate the total phenolic, total flavonoid contents and antibacterial activity of the sequentially solvent extracts of *W. trilobata*, and

II. To isolate the antibacterial compounds from the most bio-active solvent extract through bioassay-guided technique.

CHAPTER 2

LITERATURE REVIEW

2.1 Background of *Wedelia trilobata* (L.) Hitchc

Figure 2.1 shows the leaves and flower of *W. trilobata*. *W. trilobata* is a creeping, perennial herb that native to Central America, Caribbean and Mexico. This fast-growing, mat-forming herb usually form a dense growth cover that crowd out the growth of other plant species. Hence, it has been listed as one of the top 100 world's worst invasive species. The scientific name of *W. trilobata* is *Sphagneticola trilobata* (L.) Pruski. Some of its common names are Singapore daisy, yellow dots, di jin hua, kra dum tong and wedelia kuning (Singh, Sharma and Goswami, 2013; Qi, et al., 2014).

Figure 2.1: The leaves and flower of *Wedelia trilobata*.

2.1.1 Botanical Description

The coarsely hairy, green stems of *W. trilobata* can grow up to 40 cm or longer. Its roots are developed from the nodes on its stems. The leaves are 2 to 9 cm long and 2 to 5 cm in width. These leaves are winged at the base and acute at apex. Furthermore, these leaves are shiny green, blade obovate, oppositely arranged, irregular toothed on margins and usually have three lobes (hence the name *trilobata*). Single yellow flower with 8 to 13 petals are grow on the axillary stalks and the end of terminal (Thaman, 1999).

2.1.2 Taxanomy

The taxanomy classification of *W. trilobata* is shown in Table 2.1.

Table 2.1: Taxanomy classification of *W.trilobata*.

Kingdom	Plantae – Plants
Subkingdom	Tracheobionta – Vascular plants
Superdivision	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Subclass	Asteridae
Order	Asterales
Family	Asteraceae – Aster family
Genus	*Sphagneticola* O. Hoffmann – Creeping oxeye
Species	*Sphagneticola trilobata* (L.) Pruski

(Balekar, Nakpheng and Srichana, 2014)

2.1.3 Traditional Usages

W. trilobata has been historically used in the treatment of kidney dysfunction, hepatitis, cold inflammation, sores, fever and the removal of placenta after the childbirth (Chethan, et al., 2012; Husain and Kumar, 2015). In Central America and Caribbean, the leaves and aerial parts of *W. trilobata* are used to treat muscular cramp, arthritic painful joints, rheumatism and swelling (Balekar, et al., 2012). Furthermore, it was also reported to be used to treat insect bites, stings, amenorrhea and dysmenorrhea (Toppo, et al., 2013).

2.2 Phytochemical compounds of *W. trilobata*

The phytochemical constituents of *W. trilobata* are mainly consisting of terpenes, sesquiterpene lactones, flavonoids, and steroids (Li, et al., 2012; Balekar, Nakpheng and Srichana, 2014).

2.2.1 Terpenes

Terpenes are natural lipid that commonly used as perfume and flavor in food additives (Schwab, Davidovich-Rikanati and Lewinsohn, 2008). They are derived from a 5-carbon isoprene units, $CH_2=C(CH_3)-CH=CH_2$ (Saxena, et al., 2013). Hence, all terpenes have a molecular formula of $(C_5H_8)_n$. Furthermore, terpenes are found to exhibit various medicinal properties such as anticarcinogenic, antimalarial, antimicrobial and so on (Camargo, et al., 2014).

Diterpenes are terpenes which composed of four isoprene units. Studies showed that a lot of ent-kaurane diterpenoids have been isolated from *W. trilobata***.** For example, wedelobatin A, wedelobatin B, kaurenoic acid, grandiflorenic acid, (3α)-3-(angeloyloxy)-ent-kaur-16-en-19-oic acid, 3α-(angeloyloxy)-9β-hydroxy-ent-kaur-16-en-19-oic acid, grandifloric acid, 12α-methoxygrandiflorenic acid, 12α-hydroxygrandiflorenic acid, (3α)-3-(tiglinoyloxy)- ent-kaur-16-en-19-oic acid, 3α-3-(cinnamoyloxy)-ent-kaur-16-en-19-oic acid, 3α-(cinnamoyloxy)-9β-hydroxy-ent-kaur-16-en-19-oic acid, Wedelidin A and B (Qiang, et al., 2011; Ma, et al., 2013).

According to Balekar, Nakpheng and Srichana (2013), grandiflorenic acid exhibit wound-healing properties due to its ability to stimulate fibroblast and macrophage. Besides, it was reported that the antimicrobial properties of grandiflorenic acid also contribute to its wound-healing properties (Balekar, et al., 2012). On the other hand, kaurenoic acid was found to exhibit anti-leishmaniasis properties due to its lethal effect on axenic amastigotes and promastigotes of *Leishmania (V) braziliensis* (Balekar, Nakpheng and Srichana, 2014). The chemical structures for grandiflorenic acid and kaurenoic acid are shown in Figure 2.2.

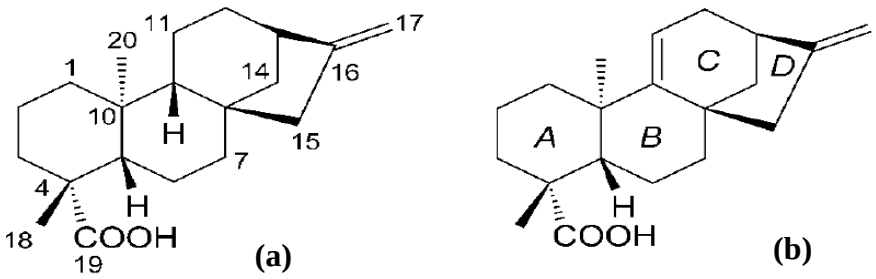


Figure 2.2: The chemical structures of (a) kaurenoic acid and (b) grandiflorenic acid (Qiang, et al., 2011).

Triterpenes are terpenes that composed of 6 isoprene units. Some triterpenes that have been isolated from *W. trilobata* are friedelan-3β-ol, β-amyrine acetate and friedelin as shown in Figure 2.3 (Hoang, et al., 2006; Qiang, et al., 2011).

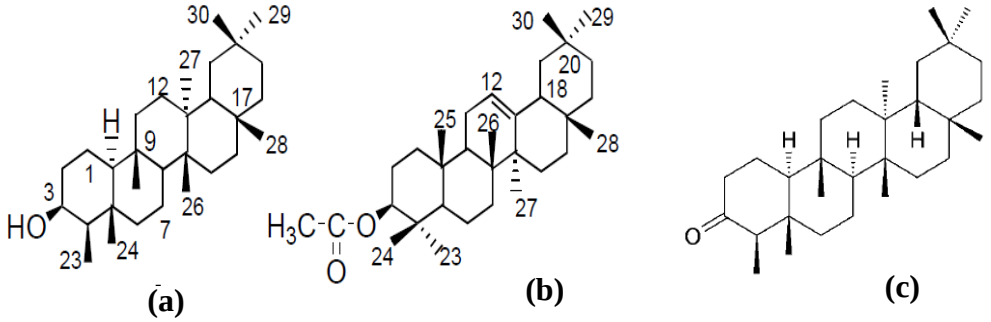


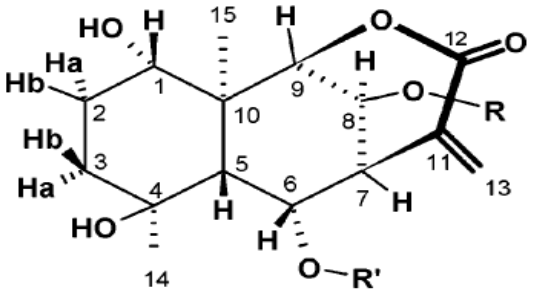
Figure 2.3: The chemical structures of (a) friedelan-3β-ol, (b) β-amyrine acetate and (c) friedelin (Hoang, et al., 2006; Qiang, et al., 2011).

2.2.2 Sesquiterpene Lactones

Sesquiterpene lactones are compounds that consist of one sesquiterpene (build up from three isoprene units) and one lactone ring. The sesquiterpene lactones that

have been discovered from *W. trilobata* are invalin, wedeliatrilolactobe B, wedelolides A and B (Qiang, et al., 2011).

Wedelolides A and B have been reported as the bio-active antimalarial compounds in *W. trilobata* which have been used as traditional medicine against malaria disease in Vietnam (That, et al., 2007). In addition, some other sesquiterpene lactones that isolated from *W. trilobata* were found to exhibit anti-virus effect against tobacco mosaic virus (TMV) (Li, et al., 2013). Thus, the fast growing *W. trilobata* has become potential sources as anti-TMV agents. The chemical structures of wedelolides A and B are shown in Figure 2.4.



(a) R = tigloyl, R' = isobutyroyl

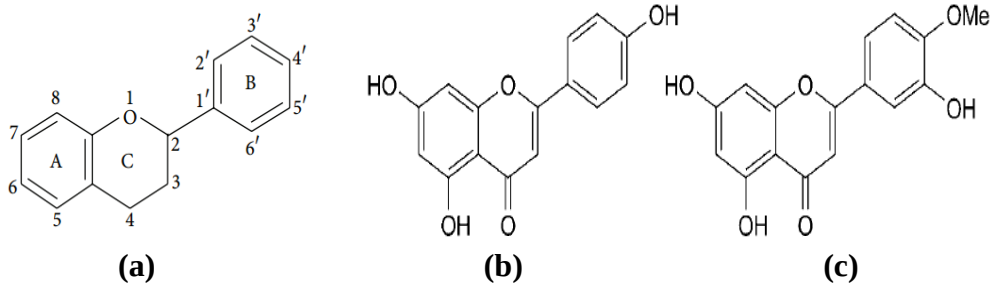
(b) R = tigloyl, R' = methacryloyl

Figure 2.4: The chemical structures of (a) wedelolides A and (b) B (That, et al., 2007).

2.2.3 Flavonoids

Flavonoids are chemical compounds that having basic structure of a 15-carbons

skeleton, consisting of two phenyl rings (A and B) linked together by a heterocyclic ring (C) as shown in Figure 2.5 (a). Flavonoids can be categorized into different classes such as flavones, flavonols, flavanones, isoflavones and anthocyanidins based on the level of oxidation and pattern of substitution on ring C (Sandhar, et al., 2011). Meanwhile, the compounds within a same class can be distinguished from each other based on the pattern of hydroxyl substitution on ring A and B. Flavonoids have been reported to exhibit various biological activities such as antioxidant, antiviral, anti-inflammatory, antibacterial, antitumour, antiallergic, cytotoxic etc. (Patil and Jadhav, 2013). The flavonoids that have been isolated from *W. trilobata* are apigenin and diosmetin which are shown in Figure 2.5 (b, c) (Qiang, et al., 2011).



(a) **(b)** **(c)**

Figure 2.5: The chemical structures of (a) flavonoid skeleton, (b) apigenin and (c) diosmetin (Qiang, et al., 2011; Kumar and Pandey, 2013).

2.2.4 Steroid

Plant steroids are 4 rings structure compounds, where first three rings are composed of 6 carbons (rings A, B and C) followed by a 5-carbons ring (ring D). They consist of a steroid skeleton (Figure 2.6a) with a hydroxyl group attached to

the carbon-3 of ring A and an aliphatic side chain attached to the carbon-17 of ring D. In addition, plant steroids possess a double bond between carbon-5 and carbon-6 which makes them becomes unsaturated compounds (Raju, et al., 2013). They are natural constituents in plant that regulate the biological processes and permeability of the cell membrane (Dufourc, 2008). Besides that, they also act as precursors of some steroidal hormones, steryl glycosides and steryl esters (Silvestro, et al., 2013). Some steroids that have been found in *W. trilobata* are stigmasterol, (7a)-7-hydroxystigmasterol, and (3b)-3-hydroxystigmasta-5, 22-dien-7-one which are shown in Figure 2.6 (Qiang, et al., 2011).

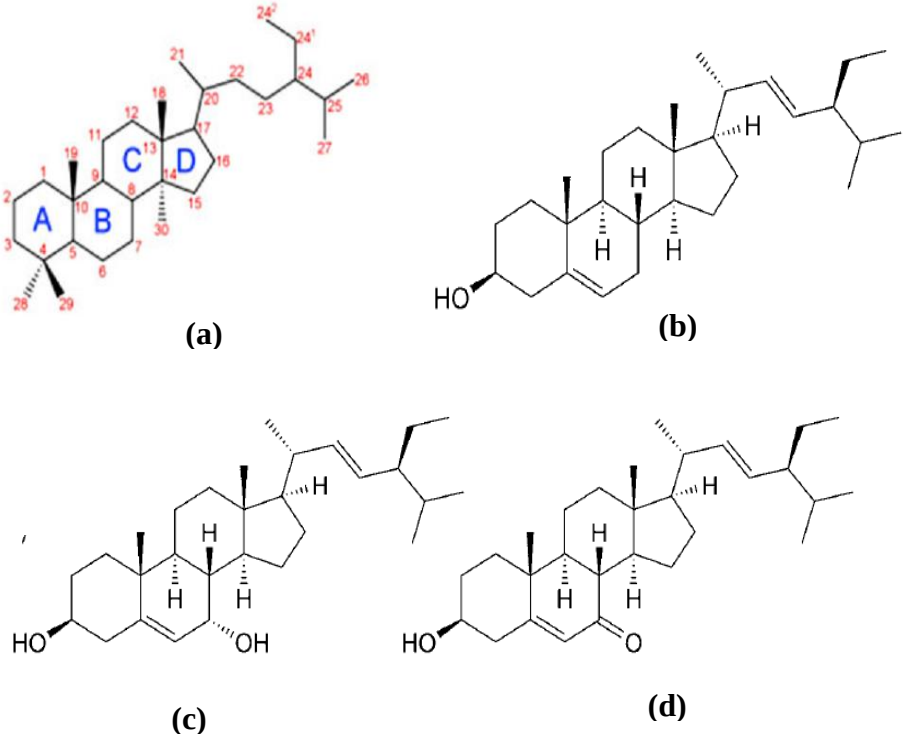


Figure 2.6: The chemical structures of (a) steroid skeleton, (b) stigmasterol, (c) (7a)-7-hydroxystigmasterol and (d) (3b)-3-hydroxystigmasta-5, 22-dien-7-one (Qiang, et al., 2011).

2.3 Bioassay-Guided Isolation

Bioassay-guided isolation is a step-by-step separation of extracted components based on the assessment of particular biological activity until a pure bio-active compound is isolated (Weller, 2012). This method has been widely used to isolate antibacterial compounds from different plant species for drug discovery purpose in recent years.

In 2008 Gupta et al. reported that glabridin which exhibit antibacterial effect against *Mycobacterium tuberculosis* was successfully isolated from the root of *Glycyrrhiza glabra* through the isolation process guided by Minimum Inhibitory Concentration (MIC) assay. A similar study has been published by Chakarborty and Chakarborti in 2010 showed that catechin was successfully identified as the antibacterial compound in *Camellia sinensis* using the same method. In the year 2011, Sule et al. reported that 3-O-β-D-glucosyl-14 deoxyandrographolide and 14-deoxyandrographolide isolated from *Andrographis paniculata* through MIC assay-guided isolation possessed antibacterial activities. In 2013, Schrader et al. reported that the antibacterial compound which isolated from the roots of *Peganum harmala* through MIC assay-guided isolation was harmine. Ernawati, Yusnelti and Afrida presented a study in 2014 showed that α-mangostine, β-mangostin and epicatechine were the antibacterial compounds isolated from *Garcinia cf cymosa* via disc diffusion assay-guided isolation technique.

2.4 Pathogenic Bacteria

2.4.1 Gram Negative and Gram Positive Bacteria

The main difference between Gram negative and Gram positive bacteria is their cell wall structures that shown in Figure 2.7 (Denyer and Maillard, 2002).

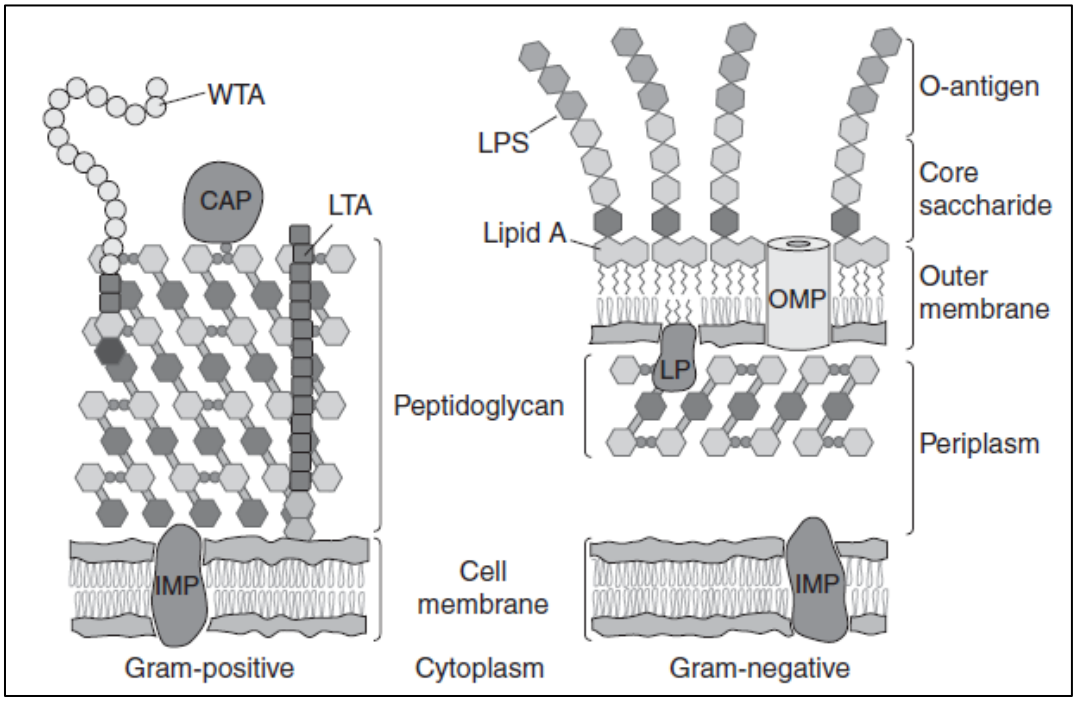


Figure 2.7: The cell envelope of Gram positive and Gram negative bacteria. CAP = covalently attached protein; IMP, integral membrane protein; LP, lipoprotein; LPS, lipopolysaccharide; LTA, lipoteichoic acid; OMP, outer membrane protein; WTA, wall teichoic acid (Silhavy, Kahne and Walker, 2010).

The cell wall of Gram negative bacteria consist of an outer membrane and a very thin layer of peptidoglycan. The outer membrane is a lipid-protein bilayer that composed of lipopolysaccharides, lipoproteins, phospholipid and porins. Meanwhile, lipopolysaccharide is a complex that contains Lipid A, core polysaccharide and O-polysaccharide. Lipid A is an endotoxin that only will be

released during the cell death of Gram negative bacteria. Thus, the endotoxin shock is used as an indicator for the infection caused by Gram negative bacteria. Besides that, the O-polysaccharide is function as the antigen that used to distinguish between different species of Gram negative bacteria (Beveridge, 1999; Denyer and Maillard, 2002).

In contrast, the cell wall of Gram positive bacteria only consists of a thick layer of peptidoglycan. Teichoic acid which only found in Gram positive bacteria is responsible for the rigidity of the cell wall and the regulation of cation across the cell (Silhavy, Kahne, and Walker, 2010).

Due to the presence of extra barrier in Gram negative bacteria, it is reported to have higher resistance to antimicrobial agent compared to Gram positive bacteria that only possess permeable cell wall (Lambert, 2002).

2.4.2 *Staphylococcus aureus*

S. aureus is a Gram positive bacterium that spherical in shape and aggregate to form irregular grape-like cluster (Tortora, Funke and Case, 2013). The species named *aureus*, refers to the fact that *S. sureus* commonly form golden colonies on the medium agar plate. *S. aureus* is non-motile, non-spore forming facultative anaerobe that able to survive under stressed environment such as human nose and

skin (Harris, Foster and Richards, 2002). This is due to the ability of *S. aureus* to survive within a wide range of temperature (7 ℃ to 48.5 ℃), pH (4.2 to 9.3) and sodium chloride concentration up to 15 % (Kadariya, Smith, and Thapaliya, 2014).

S. aureus is a pathogen that responsible for the infections of skin, soft tissue, respiratory system, bone, joints and endovascular tissues (Ray, Gautam and Singh, 2011). Furthermore, studies revealed that forming of chronic wound is associated with the establishment of *S. aureus* biofilm within the wound (Tankersley, et al., 2014). The formation of *S. aureus* biofilm within the wound affects the gene regulation in keratinocytes to produce cytokine which play a role to mediate the host immune response. Thus, the healing of wound is affected (Secor, et al., 2011).

In addition, *S. aureus* is a significant cause of food-borne diseases, causing approximately 240000 cases per year in the United States (Kadariya, Smith, and Thapaliya, 2014). Staphylococcal food poisoning occurs because of the consumption of foods that contain enterotoxin that released by death *S. aureus*. The main symptoms of Staphylococcal food poisoning include nausea, violent vomiting, and abdominal cramping. *S. aureus* is normally transmitted through direct contact between individuals that carrying *S. aureus* or via respiratory secretions (Argudin, Mendoza and Rodicio, 2010).

2.4.3 *Staphylococcus epidermidis*

S. epidermidis is a spherical Gram positive bacterium that forms white colonies on the medium agar plate. It is classified as coagulase-negative staphylococci which can be distinguished from its close relative, coagulase-positive *S. aureus* (Wieser and Busse, 2000).

S. epidermidis is the main colonizer of human skin and mucous membrane (Blum-Menezes, et al., 2000). In the past, it was known as non-pathogenic microbiota and it is pathogenic only when the skin barrier is broken. However, *S. epidermidis* is now the major causative agent of nosocomial bloodstream infections due to the increased use of indwelling prosthetic devices such as intra cardiac devices, central venous catheters (CVC), artificial heart valves, prosthetic joints, vascular grafts and cerebrospinal fluid shunts (Buttner, Mack and Rohde, 2015). This is due to the ability of *S. epidermidis* to adhere and proliferate on the polymer surface of devices (Rupp, et al., 2001). After the insertion of infected devices into patient's body, biofilm is formed to provide protection for bacteria against the host immune response.

On the other hand, recent studies suggested that the serine protease produced by *S. epidermidis* is able to inhibit the colonization and biofilm formation of pathogenic *S. aureus* (Sugimoto, et al., 2013). This shows the potential important of *S. epidermidis* to be used as strategy in controlling the infection and colonization of

pathogens in the future (Sugimoto, et al., 2013). However, the mechanism of inhibition is still unknown.

2.4.4 *Micrococcus luteus*

M. luteus is a Gram positive bacterium that form creamy yellow colony on medium agar plate. It is spherical in shape, having diameter between 0.9 μm – 1.8 μm and can be arranged in tetrad or cluster. It is non-motile, obligate aerobic, does not form any spore, and can survive in medium with sodium chloride concentration up to 7.5 % (Kocur, Pacova, and Martinec, 1972). Besides that, *M. luteus* is able to produce catalase and oxidase (Public Health England, 2014). It reduces nitrate and does not produce acid from the carbohydrate (Fox, 1976). Furthermore, it contains high guanine and cytosine contents (70 - 75 %) in its genome. This causes *M. luteus* has been used to investigate the effects of GC pressure on codon usage and the promoter selectivity for DNA dependent RNA polymerase (Haga, et al., 2003).

M. luteus is normally non-pathogenic colonizer on human skin, oropharynx and permucosae. It rarely causes some infectious diseases such as meningitis, septic arthritis, and valve endocarditis involving prosthetic valve (Peces, et al., 1997). However, *M. luteus* was found to be opportunistic toward immune-compromised patients. An immunosuppressed woman has been diagnosed with endocarditis involving native valve that caused by *M. luteus* (Miltiadous and Elisaf, 2011).

2.4.5 *Proteus vulgaris*

P. vulgaris is a rod-shaped Gram negative bacterium that can be found in polluted water, soil, and gastrointestinal tract of animal and human. Besides that, it is a facultative anaerobic microorganism which able to produce indole. It is an opportunistic pathogen that causes nosocomial urinary tract infection due to the presence of numerous virulence factors (Mohammed, Wang, and Hindi, 2013).

P. vulgaris possesses fimbriae which enhance its adherence to the epithelial tissues during infection. Moreover, it also possesses peritrichous flagella which make it actively motile (swarming phenomena) and thus can cause infection in different anatomical sites of the host. In addition, it produces protease which able to disrupt Immunoglobin A of the host (Rozalski, Sidorczyk and Kotelko, 1997; Mohammed, Wang, and Hindi, 2013).

P. vulgaris is found to secret hemolytic that lead to cell invasiveness and cytotoxicity (Peerbooms, Verweij and Maclaren, 1985). Urease is produced by *P. vulgaris* to hydrolyze urea into carbon dioxide and ammonia which will increase the pH of urine (Rozalski, Sidorczyk and Kotelko, 1997). Meanwhile, the urease also precipitates polyvalent cations out from urea to form stone that causing the obstruction of urinary tract and catheters (Pal, et al., 2014).

2.4.6 *Enterobacter aerogenes*

E. aerogenes is a Gram negative bacterium that belongs to the family of *Enterobacteriaceae.* It is a non-spore forming, rod-shaped and facultative anaerobic microorganism (Davin-Regli and Pages, 2015). Besides that, it is catalase-positive, citrate-positive but unable to produce urease, oxidase and indole in these biochemical test (Mordi and Hugbo, 2011).

Enterobacter spp. have been known as causative agent of nosocomial infection such as urinary tract infections, prosthetic devices infections, wound infections meningitis, and pneumonia (Chang, et al., 2009). In recent years, *E. aerogenes* has emerged as serious clinical challenge due to its multidrug-resistance development. It is found to be resistance to cephalosporin due to its ability to produce AmpC enzymes and extended-spectrum β-lactamases (Khajuria, et al., 2014). Thus, carbapenem has become the most efficiency way to treat *Enterobacter* infection. Unfortunately, recent studies showed that the carbapenem-resistance *Enterobacteriaceae* spp. have been developed (Qin, et al., 2014; Tuon, et al., 2015). Furthermore, alteration of membrane protein composition including absence of its major porin Omp36, alteration of lipopolysaccharide structure and the expression of efflux pump also led to its resistance to multi-drugs (Bosi, et al., 1999; Chevalier, et al., 2008).

2.4.7 *Eschericia coli*

E. coli is a Gram negative bacterium that belongs to the family of *Enterobacteriaceae*. It is a facultative anaerobic microorganism that bacillus in shape and does not form any spore. Most of the *E. coli* are harmless inhabitants that colonize lower intestine of human or animal. However, some of them are pathogenic. The pathogenic *E. coli* are generally divided into six categories which are enterotoxigenic *E. coli*, enteropathogenic *E. coli*, enteroinvasive *E. coli*, enterohemorrhagic *E. coli*, enteroaggregative *E. coli* and diffusely adherent *E. coli* (Ibrahim, Al-Shwaikh, and Ismaeil, 2014). The virulence factors and diseases caused by different categories of pathogenic *E. coli* are shown in the Table 2.2.

Table 2.2: The virulence factors and diseases caused by different categories of pathogenic *E. coli.*

Categories	Diseases	Virulence factors	Citation
Enterotoxigenic *E. coli* (ETEC)	Diarrhea without fever	- Bind to intestinal cells using fimbrial adhesin. - Produce heat-labile and heat stable enterotoxin	(Odonkor and Ampofo, 2013)
Enteropathogenic *E. coli* (EPEC)	Inflammatory human infantile diarrhea	- Attaching and effacing (A/E) lesion	(Sousa, 2006)
Enteroinvasive *E. coli* (EIEC)	Inflammatory colitis and dysentery	- Cell invasiveness	(Mainil, 2013; Odonkor and Ampofo, 2013)
Enterohemorrhagic *E. coli* (EHEC)	Hemorrhagic colitis, thrombotic thrombocytopenic purpura, bloody diarrhea, hemolytic uremic syndrome (HUS), food-borne diseases.	- Produce Shiga-like toxin - Attaching and effacing lesion	(Chen, et al., 2014; Goldwater and Bettelheim, 2012; Herrera-Luna, et al., 2009)
Enteroaggregative *E. coli* (EAEC)	Watery diarrhea without fever	- "stacked brick" adherence pattern on cultured HEp-2 cells - Plasmid–encoded aggregative adherence fimbriae (AAF/I and AAF/II) - plasmid-encoded toxin (Pet) - produce heat stable enterotoxin (EAST-1)	(Weintraub, 2007; Vila, et al., 2000; Zamboni, et al., 2004)
Diffusely adherent *E. coli* (DAEC)	Watery diarrhea	- Distinctive adherent pattern on cultured HEp-2 cells using fimbrial F1845	(Sousa, 2006)

2.5 Antibacterial Drugs

Antibacterial drugs are antibacterial agents that used to treat infectious diseases caused by bacteria. Some major sources of potential antimicrobial agents include *Streptomyces*, *Actinomycetes*, *Penicilliums*, and *Bacilli* (Bbosa, et al., 2014). Antibacterial drugs can be either bactericidal (kill bacteria directly) or bacteriostatic (inhibit the growth of bacteria). They are generally classified into five categories based on their mechanism of metabolic action. Table 2.3 shows the classes of antibacterial drugs with their examples (Jayaraman, et al., 2010; Kohanski, Dwyer, and Collins, 2012; Soares, et al., 2012; Monte., 2013).

Table 2.3: Different categories of antibacterial drugs and their examples.

Mechanisms of action	Examples
Inhibit the synthesis of bacterial cell wall	-Catechin and tannin inhibit the chitin synthase (fungus)
Affect the permeability of cell membrane	-Essential oils disrupt cell membrane through their lipophilic structures -Alkaloid such as berberine and piperine
Inhibit the synthesis of protein	-Phenolic acid such as gallic acid, ellagic acid and protocatechuic acid interact with protein non-specifically
Inhibit the synthesis of essential metabolites	-Phenolic acid such as gallic acid, ellagic acid and protocatechuic acid can cause enzyme inhibition
Inhibit the synthesis of nucleic acid	-Flavonoids such as myricetin and rutin -Alkaloid such as berberine and piperine may intercalate with DNA

(Jayaraman, et al., 2010; Kohanski, Dwyer, and Collins, 2012; Soares, et al., 2012; Monte., 2013)

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental Design

The experimental design for the project is shown in Figure 3.1.

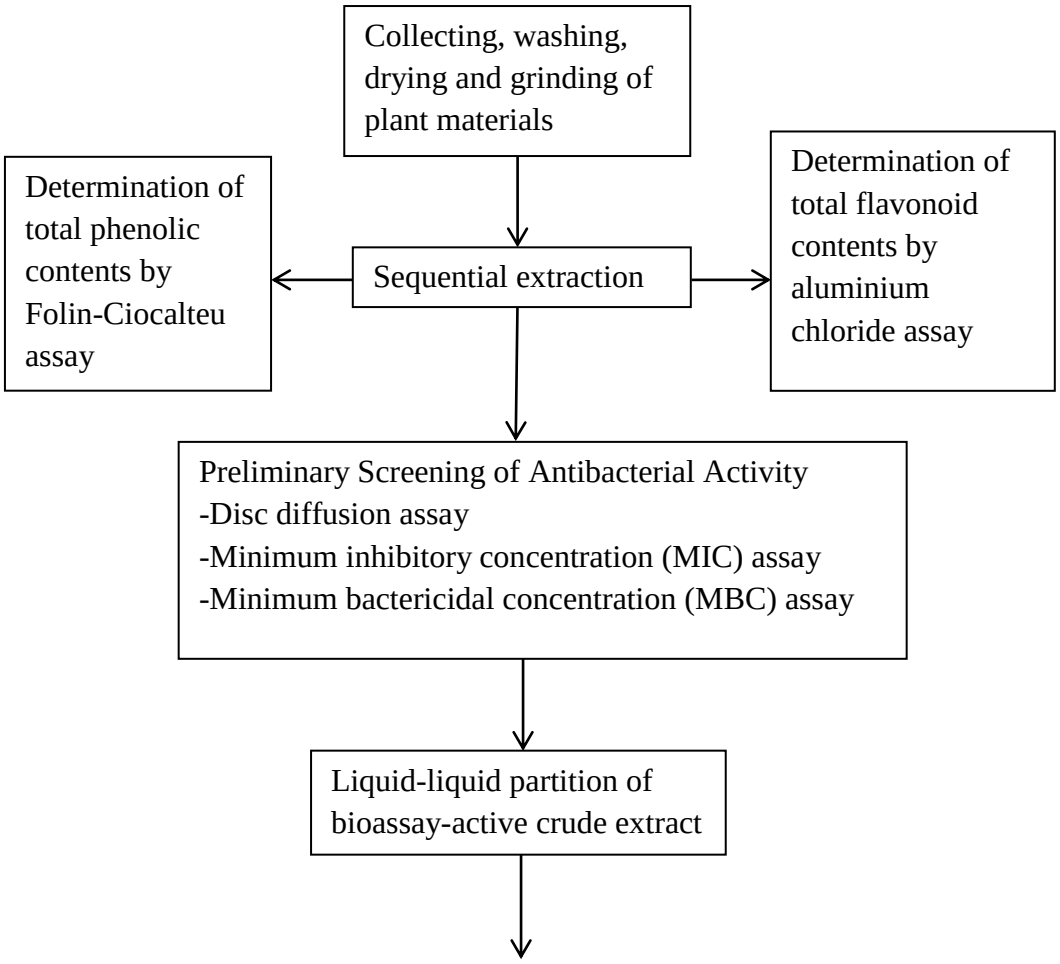


Figure 3.1: The experimental design for the project (To be continued).

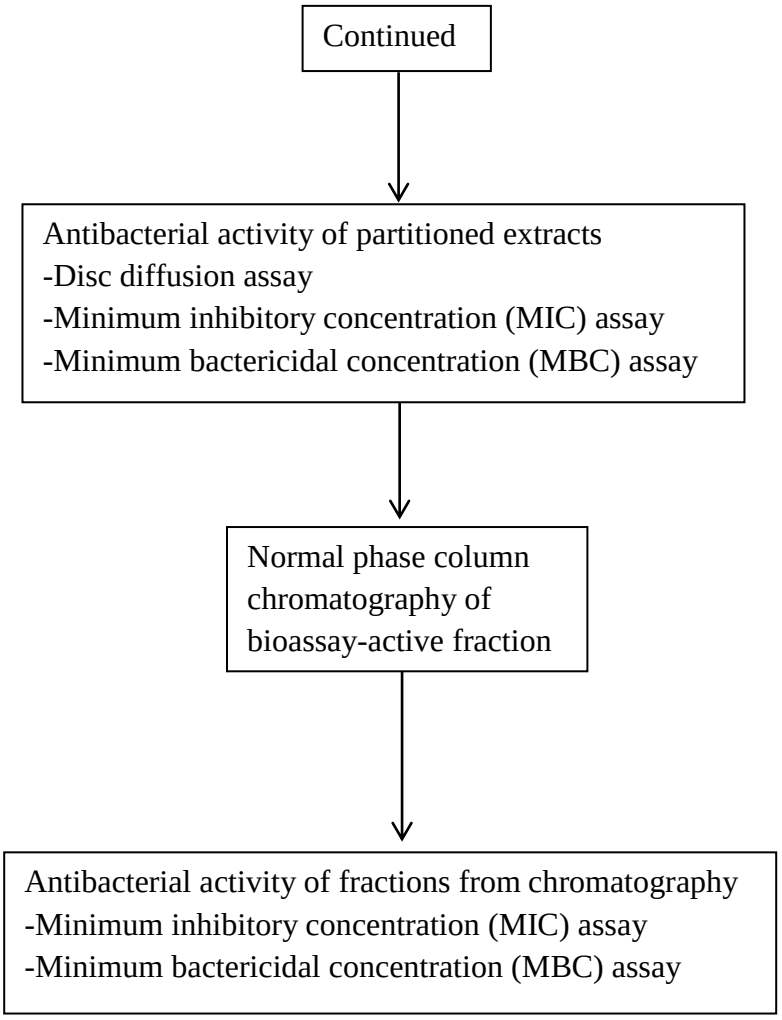


Figure 3.1 (continued): The experimental design for the project.

3.2 Materials

3.2.1 Plant Materials

Fresh leaves of *W. trilobata* were harvested from Block B and C of University Tunku Abdul Rahman (UTAR), Perak campus.

3.2.2 Bacteria Strains

The Gram positive and negative bacteria that used in this project are listed in Table 3.1. All bacteria species were cultured on Nutrient Agar (NA) except *Escherichia coli* was cultured on Luria Bertani (LB) agar.

Table 3.1: The bacteria strains used in antibacterial assay.

Bacteria Strains	American Type Culture Collection (ATCC) Number
Gram positive	
Staphylococcus aureus	25923
Staphylococcus epidermidis	12228
Micrococcus luteus	4698
Gram negative	
Escherichia coli	35218
Proteus vulgaris	29905
Enterobacter aerogenes	13048

3.2.3 Apparatus, Chemicals and Consumables

The apparatus, chemicals and consumables used are listed in Appendix A.

3.3 Methods

3.3.1 Sequential Extraction

The collected leaves were washed thoroughly under running tap water, dried in oven at 40 ℃ for 4 days and grinded into fine powder using a food processing blender. A 20 g of the grinded plant sample was sequentially agitated (200 rpm) with 200 ml of hexane, chloroform, ethyl acetate, methanol and 70 % acetone for 24 hours with each solvent. Each mixture was then vacuum filtered through Whatman filter paper No. 1 and each filtrate was concentrated using rotary evaporator under reduced pressure at 40 ℃. The crude extracts obtained were weighed to calculate the percentage yield of extraction based on equation 3.1. After that, each extract was kept in 4 ℃ for further studies (Ayoola, et al., 2008). Figure 3.2 shows the flowchart of sequential extraction and Table 3.2 shows the polarity indexes of each solvent used. All extraction was performed in triplicate.

$$\text{Percentage yield of extraction (\%)} = \frac{\text{dry weight of crude extract (g)}}{\text{original weight of powdered plant material used (g)}} \times 100\ \%$$

(Equation 3.1)

Table 3.2: The polarity indexes of solvents used in sequential extraction.

Solvent	Polarity index
Hexane	0.00
Chloroform	4.10
Ethyl acetate	4.40
Methanol	5.10
70 % of acetone	6.27

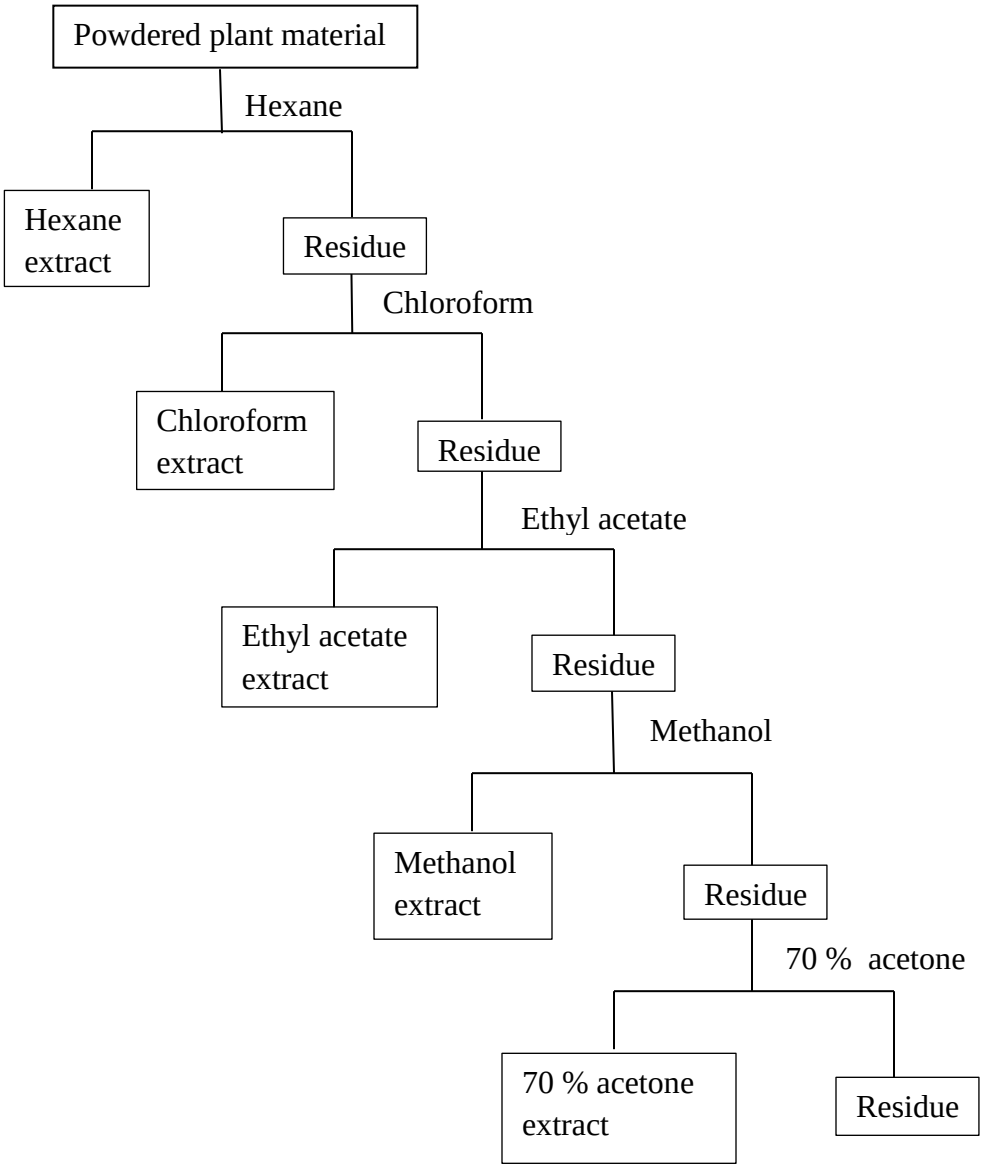


Figure 3.2: Flowchart of sequential extraction.

3.3.2 Determination of Total Phenolic Contents (TPC)

The TPC of *W. trilobata* crude extracts were determined via Folin-Ciocalteu method as described by Amzad Hossain and Shah (2015) with slight modifications. A concentration of 0.2 mg/ml of hexane, chloroform, and ethyl acetate extracts were prepared in their solvent respectively. Meanwhile, 0.7 mg/ml and 0.1 mg/ml of methanol and 70 % acetone extracts were prepared in methanol and distilled water, respectively. A 0.5 ml of each extract was mixed with 1.5 ml of Folin-Ciocalteu reagent (10 fold dilution) and 1.2 ml of 5 % (w/v) sodium carbonate into the test tubes. The test tubes were then incubated in dark condition for 30 minutes and the absorbance of mixture was read at 760 nm using spectrophotometer. Gallic acid was used as the standard solution for this assay and the standard calibration curve of gallic acid (0.01 - 0.08 mg/ml) is shown in Figure B1 of Appendix B. The phenolic contents were expressed as mg of gallic acid equivalent (GAE)/g of dried extract. All tests were performed in triplicate and the mean and standard deviation values were taken.

3.3.3 Determination of Total Flavonoid Contents (TFC)

Total flavonoid contents of *W.trilobata* were determined via aluminium chloride colorimetric assay as described by Kaur and Mondal (2014) with slight modifications. A 6.0 mg/ml of hexane and 70 % acetone extracts were prepared in hexane and distilled water, respectively. Meanwhile, various concentration including 3.0, 4.0, and 8.0 mg/ml of chloroform, ethyl acetate, and methanol

extracts were prepared in their solvent respectively. A 0.5 ml of each extract was added with 2.0 ml of distilled water followed by 0.15 ml of 1 % (w/v) $NaNO_2$ solution into the test tubes and incubated for 5 minutes at room temperature. After that, 0.15 ml of 10 % (w/v) $AlCl_3$ solution was added into the mixture. Subsequently, 1.0 ml of 1 % (w/v) NaOH was added and immediately the absorbance of mixture was read at 510 nm using spectrophotometer. Quercetin was used as the standard unit for this assay and the standard calibration curve of quercetin (2 - 10 mg/ml) is shown in Figure B2 of Appendix B. The flavonoid contents were expressed in mg of quercetin equivalent (QE)/g of dried extract. All tests were performed in triplicate and the mean and standard deviation values were taken.

3.3.4 Antibacterial Activity Screening— Disc Diffusion Assay

3.3.4.1 Preparation of the Test Sample

The hexane, chloroform, ethyl acetate and methanol extracts were dissolved in their solvent respectively whereas the 70 % acetone extract was dissolved in distilled water to give a final concentration of 100 mg/ml. The extract solutions were then filtered through a 0.45 µm nylon membrane filter in laminar air flow.

3.3.4.2 Preparation of Bacterial Suspension

The bacteria used were cultured on NA and incubated at 37 ℃ for 24 hours prior to the disc diffusion assay. A few bacterial colonies from the one day old plates were inoculated into 0.85 % (w/v) of sterile saline water and the turbidity of saline water was adjusted to 0.5 McFarland standard (OD_{625} = 0.08 - 0.10) in which the cell concentration was equivalent to approximately 1.5×10^8 CFU/ml (Chuah, et al., 2014).

3.3.4.3 Disc Diffusion Procedures

The bacterial suspension was swabbed onto sterile Mueller-Hinton agar (MHA). A 20 µL of prepared extract solution (100 mg/ml) was loaded into the 6 mm diameter sterile disc to give a final concentration of 2 mg/ disc. The disc was then allowed to dry for few minutes in the laminar air flow. After that, the impregnated disc was transferred onto the MHA which had inoculated with test bacteria using a sterile forcep. All plates were sealed, inverted, and incubated at 37 ℃ for 24 hours (Chuah, et al., 2014).

After 24 hours, the diameter of inhibition zone was measured using a ruler. All experiment was performed in triplicate and the mean and standard deviation values were taken. Hexane, chloroform, ethyl acetate, methanol and distilled water were used as negative controls in this assay. Streptomycin was used as positive control

for *S. aureus, M. luteus, P. vulgaris, E. coli* and *E. aerogenes.* Besides, penicillin was used as positive control for *S. epidermidis* in this assay.

3.3.5 Antibacterial Activity (Quantitative) — Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Assay

Based on the disc diffusion assay, methanol and 70 % acetone extracts exhibited the highest inhibition zone values against *S. aureus* and *S. epidermidis.* Thus, methanol and 70 % acetone extracts were subjected to the MIC assay against *S. aureus* and MBC assay.

3.3.5.1 Preparation of the Test Sample

Methanol and 70 % acetone extracts were dissolved in methanol and distilled water, respectively to give the final concentration of 100 mg/ml. The extract solutions were then filtered through a 0.45 µm nylon membrane filter in laminar air flow.

3.3.5.2 Preparation of Bacteria Suspension

S. aureus was cultured on NA and incubated at 37 ℃ for 24 hours. A few bacteria colonies were transferred from the one day old plates into sterile Mueller-Hinton Broth (MHB) and the turbidity of MHB was adjusted to 0.5 McFarland standard (OD_{625} = 0.08 - 0.10). Then, the 0.5 McFarland bacteria suspension was further

diluted 200 times with sterile MHB to give the bacteria suspension with the concentration that equivalent to approximately 5×10^5 CFU/ml.

3.3.5.3 Minimum Inhibitory Concentration (MIC) Procedures

The MIC assay was carried out in a 96-wells microplate based on the method described by Mbaveng, et al. (2008) with slight modifications. Firstly, a 100 µL of MHB containing 10 % (w/v) of glucose and 0.05 % (w/v) of phenol red was dispensed into each well at the first column. Meanwhile, 75 µL of MHB containing 10 % (w/v) of glucose and 0.05 % (w/v) of phenol red was added into the remaining wells. After that, 50 µL of methanol and 70 % acetone extract solutions were added into the wells at the first column. A serial of two-fold dilution was made by transferring 75 µL of suspension from the wells at the first column to the subsequent wells up till the wells at the 12^{th} column. The excessive 75 µL of suspension was discarded from the wells at the 12^{th} column. Then, 75 µL of inoculum suspension was added to each well on the plate. Lastly, the plate was covered, sealed and incubated at 37 ℃ for 24 hours. The testing concentration range was between 16.67 mg/ml and 0.01 mg/ml. The colour of phenol red changes from red to yellow indicates the growth of bacteria due to the conversion of the carbohydrate into carboxylic acid by the living bacteria. Thus, the well with the lowest concentration that appears to prevent the change of colour is taken as the MIC value of the extract. Ampicillin was used as positive control in this assay.

3.3.5.4 Minimum Bactericidal Concentration (MBC) Procedures

A loopful of suspension from each well that showed no bacteria growth in the MIC assay was streaked on the NA and incubated at 37 ℃ for 24 hours. The plate with the lowest concentration that showed no bacteria growth after an overnight incubation is taken as the MBC value (Balekar, et al., 2012).

3.3.6 Liquid-liquid Partition

70 % acetone extract that exhibited the highest antibacterial activity was subsequently partitioned with chloroform (4 ×15 ml) and ethyl acetate (4 × 15 ml). The 70 % acetone extract (13.56 g) was firstly dissolved in 15 ml of distilled water and stirred vigorously with equal volume of chloroform in a beaker using magnetic stirrer. The mixture was poured into a separating funnel and allowed to stand for 10 minutes in the fume hood. After 10 minutes, two distinct layers were formed as shown in Figure 3.3. The layers formed were then separated into different beakers. The aqueous layer was re-partitioned with equal volume of chloroform for another three times. The combined chloroform layer was concentrated using rotary evaporator at 40 ℃ under reduced pressure and the dry weight of chloroform fraction extract was recorded.

Then, the aqueous layer was further partitioned with equal volume of ethyl acetate for four times and the dry weight of ethyl acetate fraction extract was recorded.

Lastly, the aqueous layer was freezed in -20 ℃ freezer for overnight. The water residue in the aqueous layer was subsequently removed using freeze dryer for five days and the dry weight of the aqueous fraction extract was recorded. Figure 3.4 shows the flowchart of liquid-liquid partition of 70 % acetone extract. The chloroform fraction was assigned as Fraction A, ethyl acetate fraction as Fraction B and aqueous fraction as Fraction C in further experiment.

Fraction A and B were dissolved in methanol whereas Fraction C was dissolved in distilled water to give the final concentration of 60 mg/ml. The fraction solutions were then filtered through a 0.45 µm nylon filter. After that, each prepared fraction solutions were subjected to disc diffusion, MIC and MBC assay as described in Section 3.3.5.

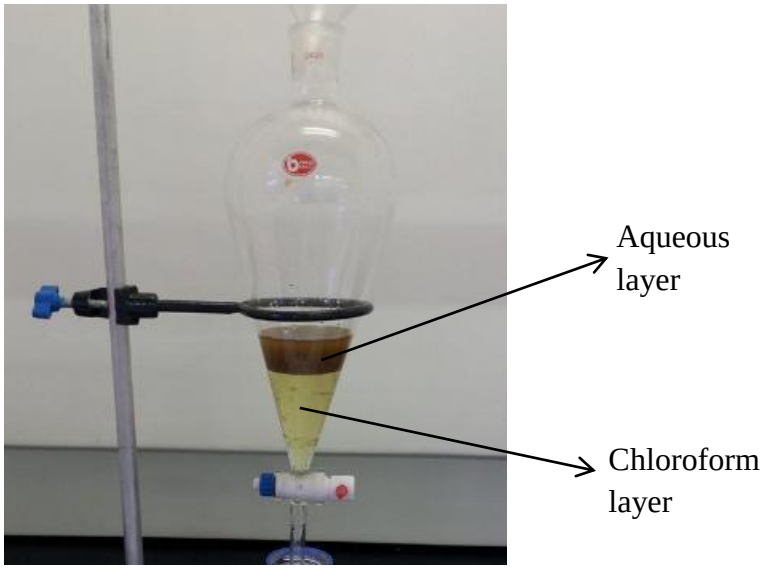


Figure 3.3: Separating funnel containing distinct chloroform and aqueous layers.

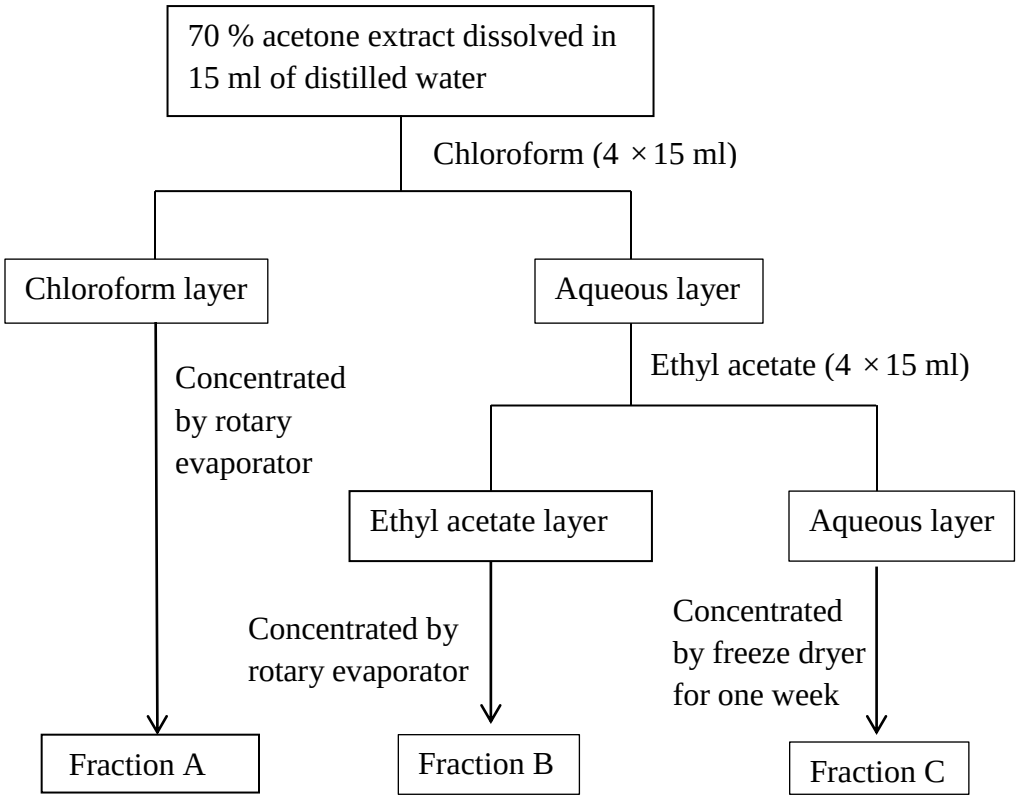


Figure 3.4: Flowchart of liquid-liquid partition of 70 % acetone extract.

3.3.7 Normal Phase Column Chromatography

In this experiment, Fraction B exhibited the highest antibacterial activity was further subjected to normal phase column chromatography. Silica gel which used as stationary phase was kept in oven for overnight before the chromatography in order to remove the moisture completely. The progress fractionation of bio-active compounds from 70 % acetone extract is shown in Figure 3.5.

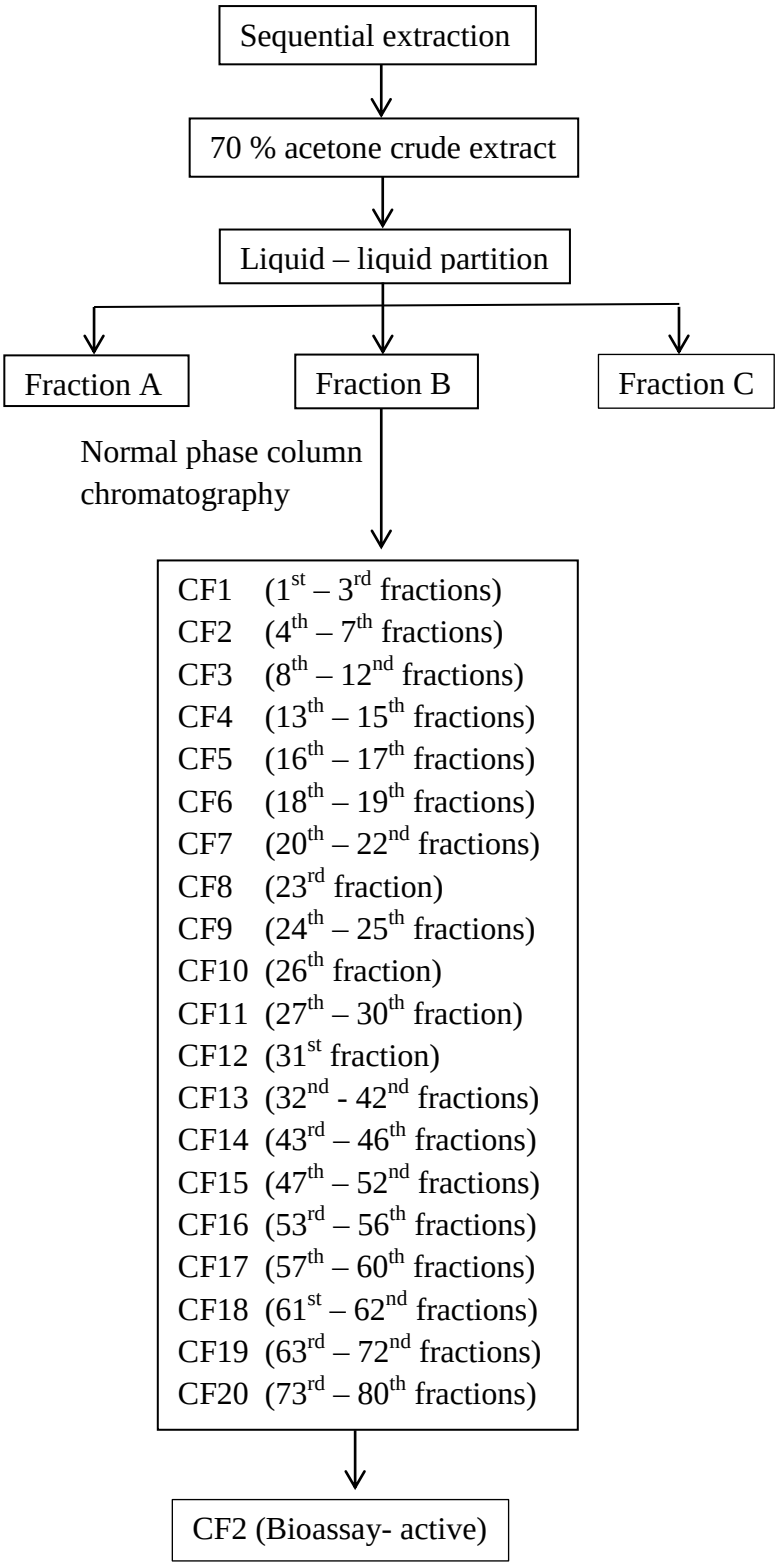


Figure 3.5: Progress fractionation of bioactive compounds from 70 % acetone crude extract of *W.trilobata*

Firstly, a layer of sea sand with the thickness of 0.5 cm - 1.0 cm was introduced onto the bottom of a glass column (3 cm × 90 cm) to prevent the run off of silica gel in the subsequent column packing step. Then, approximately 50 g of dried silica gel (Merck Kieselgel 60, 40 µm-63 µm, 230 - 400 mesh) was dissolved in minimum amount of hexane and the mixture was stirred vigorously to form slurry. The silica slurry was poured into the glass column and allowed to settle down in the column. Meanwhile, the column was tapped gently with a rubber band and the stop cork was opened to drain out the hexane in order to pack the silica gel compactly. After that, another layer of sea sand (thickness = 0.5 cm – 1.0 cm) was added onto the top of stationary phase which serve as a protective layer (Kavya, Harish and Channarayappa, 2014). Figure 3.6 shows the glass column containing the packed silica gel and sea sand layers.

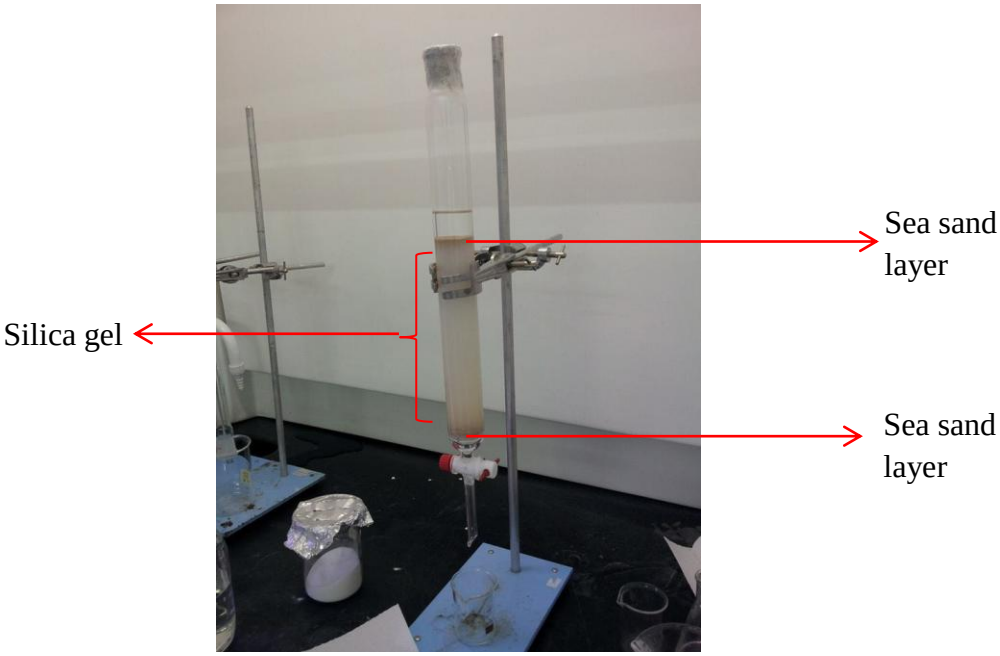


Figure 3.6: Glass column containing packed silica gel and sea sand layers.

The sample was prepared via wet packing method in which 0.5 g of Fraction B was dissolved in 3.0 ml of ethyl acetate and loaded into the column dropwisely. The excess solvent in the column was drained out to allow the sample move downward across the sea sand layer and merged into the stationary phase.

A gradient elution system (hexane - ethyl acetate - methanol) with increasing polarity index was used as mobile phase to separate the bioactive compounds in Fraction B. The combination of mobile phase and the volume of each mobile phase used are shown in Table 3.3. Total of eighty fractions with approximately 50 ml of each elute were collected and labeled accordingly from F1 to F80. Each fraction was then concentrated using rotary evaporator under reduced pressure at 40 ℃.

Table 3.3: Mobile phases used in column chromatography.

Sequence of mobile phases used	Elution system	Volume of mobile phase (ml)
1	40 % Hexane : 60 % Ethyl acetate	400
2	20 % Hexane : 80 % Ethyl acetate	400
3	100 % Ethyl acetate	400
4	90 % Ethyl acetate: 10 % Methanol	400
5	80 % Ethyl acetate: 20 % Methanol	400
6	60 % Ethyl acetate: 40 % Methanol	400
7	40 % Ethyl acetate: 60 % Methanol	400
8	20 % Ethyl acetate: 80 % Methanol	400
9	10 % Ethyl acetate: 90 % Methanol	400
10	100 % Methanol	400

3.3.8 Thin Layer Chromatography (TLC)

All collected fractions from the normal phase chromatography were subjected to TLC analysis on the aluminum plate that has been coated with silica gel (Merck TLC Silicagel 60 F_{254}, 2 cm × 8 cm). The mobile phase used for F1 to F26 was hexane : ethyl acetate (8:2 v/v). The mobile phase used for F27 to F52 was ethyl acetate : methanol (5:5 v/v) and the mobile phase used for F53 to F80 was ethyl acetate : methanol (2:8 v/v). The spots that developed on the TLC plates were viewed under Ultraviolet (UV) at long wavelength (365 nm) and short wavelength (254 nm). The TLC profiles of each collected fractions at 254 nm and 365 nm are shown in Appendix E and F, respectively. Those fractions with similar TLC profiles were combined and their new codes assigned were shown in Figure 3.5. A total of 20 combined fractions (CF) were obtained. Each combined fraction was then concentrated using rotary evaporator under reduced pressure at 40 ℃.

All extracts were dissolved in methanol to give the final concentration of 10 mg/ml and the extract solutions were filtered through a 0.45 µm nylon membrane filter. All prepared extract solutions were then subjected to MIC and MBC assays as described in section 3.3.5.

CHAPTER 4

RESULTS

4.1 Sequential Extraction

The leaves of *W. trilobata* were sequentially extracted with hexane, chloroform, ethyl acetate, methanol and 70 % acetone. The percentage yields of extraction are shown in Figure 4.1. As can be seen from Figure 4.1, 70 % acetone extract showed the highest extraction yield of 10.32 ± 0.01 %. This was followed by methanol, hexane and chloroform extracts with the extraction yields of 9.88 ± 0.14 %, 5.55 ± 0.05 %, and 2.72 ± 0.02 %, respectively. Ethyl acetate extract showed the lowest extraction yield of 0.87 ± 0.06 %.

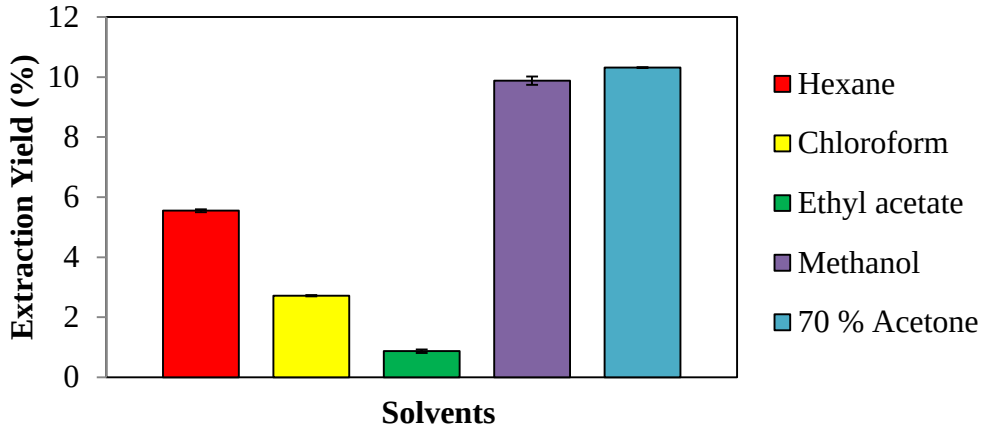


Figure 4.1: The extraction yields of solvent extracts of *W. trilobata* leaves. The data are expressed as mean value ± standard deviation of three replicates.

4.2 Total Phenolic Contents (TPC)

The TPC present in solvent extracts of *W. trilobata* were determined via Folin - Ciocalteu assay and presented in Table 4.1. Based on Table 4.1, the lowest TPC was found in hexane extract (15.66 ± 0.82 mg of GAE /g of dried extract). TPC in different extracts were found to increase with the increasing polarity of extracting solvents. The TPC in chloroform, ethyl acetate and methanol extracts were ranged from 25.25 ± 2.29 to 106.70 ± 4.95 mg of GAE /g of dried extract. The highest TPC of 192.29 ± 8.57 mg of GAE /g of dried extract was observed in the 70 % acetone extract which have the highest polarity.

Table 4.1: Total phenolic contents present in the solvent extracts of *W. trilobata.*

Solvent Extracts	Total Phenolic Contents (mg of GAE /g of dried extract)
Hexane	15.66 ± 0.82
Chloroform	25.25 ± 2.29
Ethyl acetate	41.75 ± 0.64
Methanol	106.70 ± 4.95
70 % acetone	192.29 ± 8.57

Note: The data are expressed as mean value ± standard deviation of three replicates.

4.3 Total Flavonoid Contents (TFC)

The TFC present in the solvent extracts of *W. trilobata* were estimated using aluminium chloride colorimetric assay and the findings are presented in Table 4.2. According to Table 4.2, ethyl acetate extract showed the highest TFC of 2013.18 ± 91.87 mg of QE /g of dried extract. This was followed by chloroform, hexane and 70 % acetone extracts with the TFC of 1899.05 ± 39.57, 620.96 ± 12.78, and 404.12 ± 21.42 mg of QE /g of dried extract, respectively. The lowest TFC of 277.92 ± 8.49 mg of QE /g of dried extract was observed in the methanol extract.

Table 4.2: Total flavonoid contents present in the solvent extracts of *W. trilobata*.

Solvent Extracts	Total Flavonoid Contents (mg of QE /g of dried extract
Hexane	620.96 ± 12.78
Chloroform	1899.05 ± 39.57
Ethyl acetate	2013.18 ± 91.87
Methanol	277.92 ± 8.49
70 % acetone	404.12 ± 21.42

Note: The data are expressed as mean value ± standard deviation of three replicates.

4.4 Antibacterial Activity of Crude Extracts of *W. trilobata*

The solvent extracts of *W. trilobata* were screened for their antibacterial activities against six bacteria strains through the disc diffusion, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays.

4.4.1 Disc Diffusion Assay (Preliminary Screening)

Figure 4.2 shows the inhibition zones exhibited by various solvent extracts of *W. trilobata* against *S. epidermidis* and *S. aureus*, respectively. The inhibition zones exhibited by various solvent extracts of *W. trilobata* against other bacteria strains are shown in Figure C3 of Appendix C. The diameter of inhibition zones exhibited were measured and shown in Table 4.3. The larger the inhibition zone value, the higher the antibacterial activity of the solvent extract

Based on Table 4.3, 70 % acetone extract exhibited the highest antibacterial activities against *S. epidermidis* and *S. aureus* with the inhibition zone values of 15.00 ± 1.73 mm and 10.33 ± 1.53 mm, respectively. This was followed by methanol extract which exhibited antibacterial activities against *S. epidermidis* and *S. aureus* with the inhibition zone values of 14.33 ± 1.53 mm and 9.00 ± 1.00 mm, respectively. All solvent extracts were unable to perform antibacterial activity against selected Gram negative bacteria except 70 % acetone extract which

showed positive result against *P. vulgaris* with the inhibition zone value of 9.33 ± 1.15 mm.

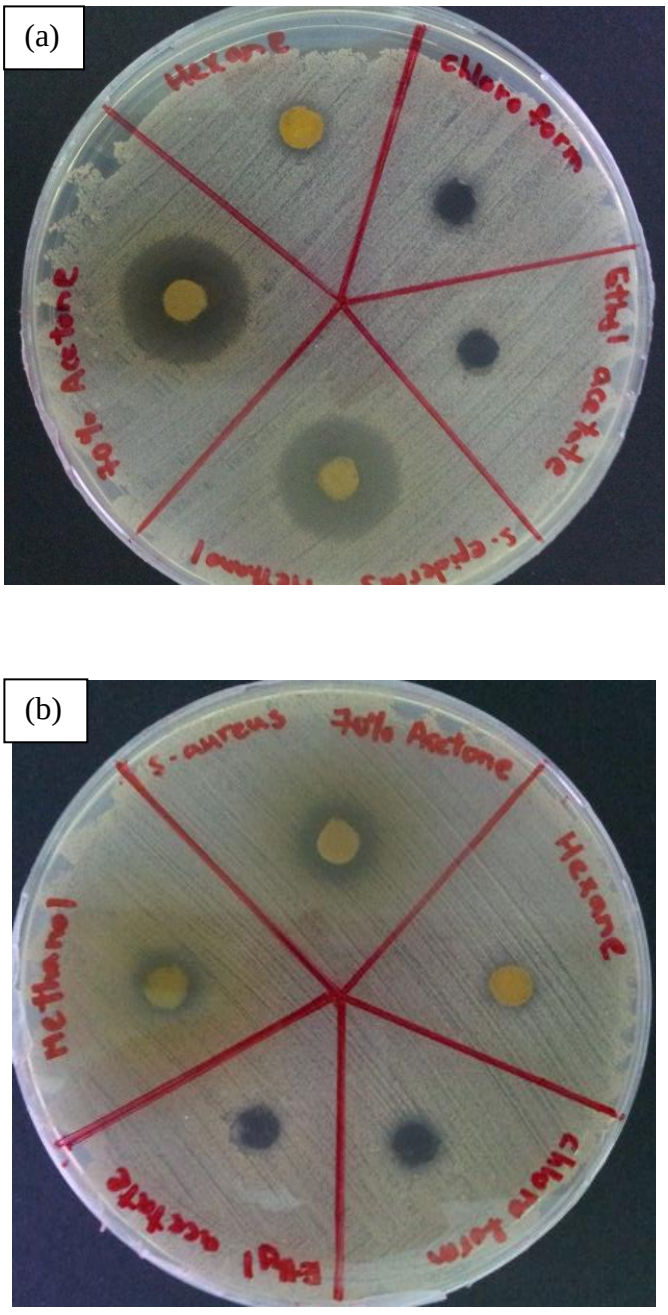


Figure 4.2: The inhibition zones exhibited by solvent extracts of *W. trilobata* against (a) *S. epidermidis* and (b) *S. aureus.*

Table 4.3: Inhibition zone values exhibited by the solvent extracts of *W. trilobata* .

Solvent Extracts / Positive control	Zone of Inhibition (mm)					
	Gram positive			Gram negative		
	S. aureus	***S. epidermidis***	***M. luteus***	***P. vulgaris***	***E. coli***	***E. aerogenes***
Hexane	7.00 ± 0.00	7.00 ± 0.00	8.67 ± 0.58	—	—	—
Chloroform	8.33 ± 1.15	—	9.67 ± 0.58	—	—	—
Ethyl acetate	—	—	—	—	—	—
Methanol	9.00 ± 1.00	14.33 ± 1.53	—	—	—	—
70 % acetone	10.33 ± 1.53	15.00 ± 1.73	—	9.33 ± 1.15	—	—
Streptomycin (0.5 mg/disc)	36.33 ± 0.58	—	46.67 ± 8.33	34.33 ± 0.58	18.00 ± 0.00	29.33 ± 1.15
Penicilin (0.2 mg/disc)	×	27.33 ± 0.58	×	×	×	×

Note: The inhibition zone values are expressed as mean ± standard deviation of three replicates.
The symbol '—' indicates that no antibacterial activity is observed.
The symbol '×' indicates that the disc diffusion assays of penicillin against *S. aureus, M. luteus, P. vulgaris, E. coli* and *E. aerogenes* are not included in this study.

4.4.2 Minimum Inhibitory Concentration (MIC) Assay

The methanol and 70 % acetone crude extracts which exhibited the highest antibacterial activities in the disc diffusion assay were tested for their MIC values against *S. aureus* in 96-wells plate. The lowest concentration that remains as red colour after 24 hours of incubation was taken as the MIC value of the extract. The MIC assays of methanol and 70 % acetone extracts are shown in Figure 4.3. Based on Figure 4.3, 70 % acetone extract exhibited a lower MIC value of 8.34 mg/ml as compared with the methanol extract which exhibited a higher MIC value between 8.34 mg/ml and 16.67 mg/ml against *S. aureus*. Ampicillin which used as positive control showed a MIC value of < 0.01 mg/ml. The positive, negative, and solvents control of the MIC assay are shown in Figure D1 of Appendix D.

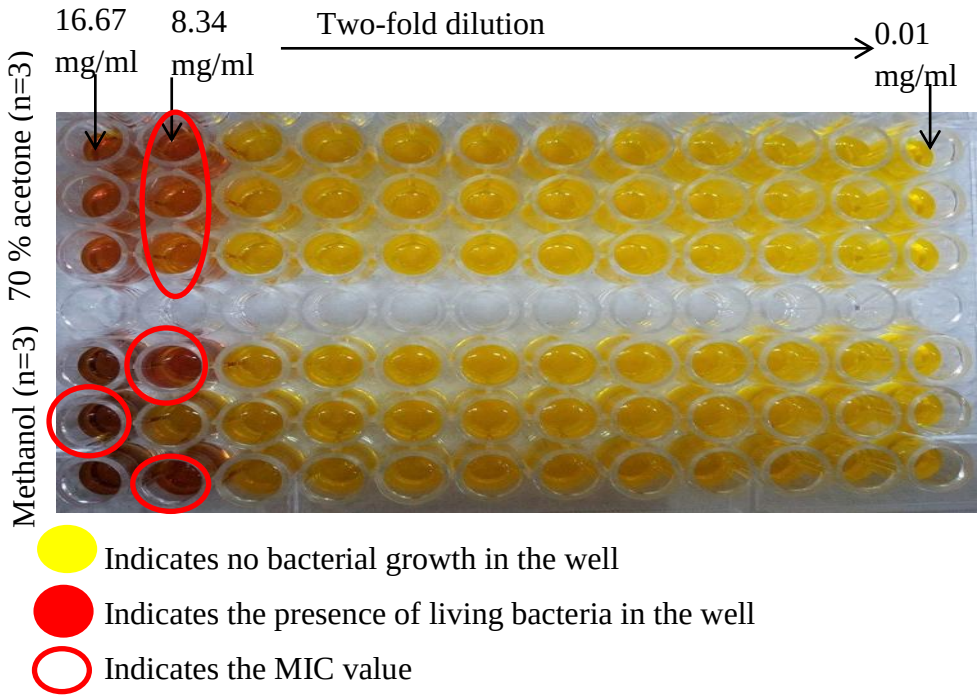


Figure 4.3: The MIC assays of methanol and 70 % acetone extract against *S. aureus.*

4.4.3 Minimum Bactericidal Concentration (MBC) Assay

Figure 4.4 shows the MBC assays of methanol and 70 % acetone extracts. *S. aureus* was subcultured from the positive MIC assay of both extracts onto the MHA and incubated for 24 hours. Based on Figure 4.4, methanol extract of *W. trilobata* was found to perform bacteriostatic activities against *S. aureus* as the golden colonies of *S. aureus* were observed on the MHA after 24 hours of incubation. On the other hand, 70 % acetone extract of *W. trilobata* exhibited bactericidal activities against *S. aureus* due to no bacterial growth was observed on the MHA after 24 hours of incubation.

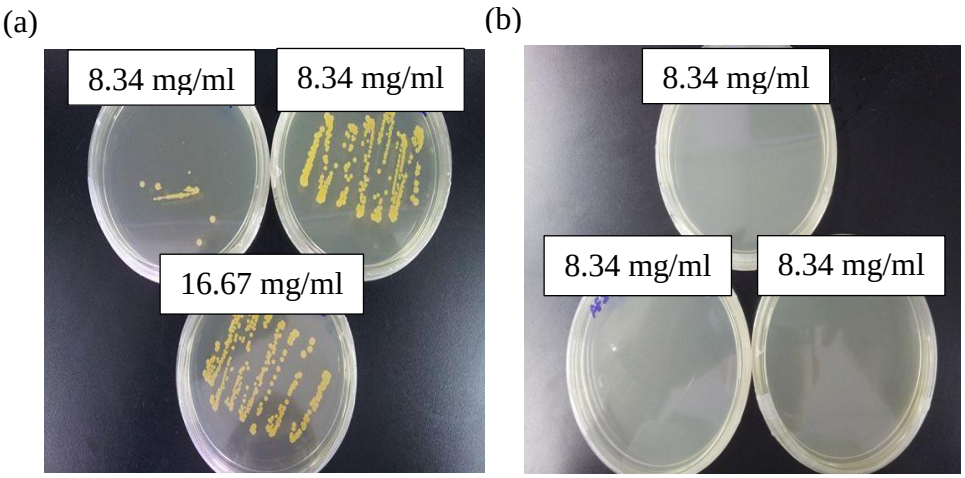


Figure 4.4: The MBC assays of (a) methanol and (b) 70 % acetone extracts in triplicate. Golden colonies of *S. aureus* were observed in MBC assays of methanol extract but absent in MBC assays of 70 % acetone extract after 24 hours of incubation.

4.5 Antibacterial Activity of Partitioned Fractions

The 70 % acetone crude extract which exhibited the highest antibacterial activity against *S. aureus* in preliminary screening was further subjected to liquid-liquid partition using chloroform and ethyl acetate. Three fractions were yielded which are chloroform (Fraction A), ethyl acetate (Fraction B) and aqueous fraction (Fraction C). Each partitioned fraction yielded was then screened again for their antibacterial activities against six bacteria strains via disc diffusion assay.

4.5.1 Disc Diffusion Assay (Partitioned Fractions)

Figure 4.5 shows the inhibition zones exhibited by each partitioned fraction against *S. aureus*. The inhibition zones exhibited against other bacteria strains are shown in Figure C4 - C8 of Appendix C. All inhibition zones values was measured and presented in Table 4.4. Based on Table 4.4, Fraction B exhibited the highest antibacterial activities against *S. aureus* and *S. epidermidis* with the inhibition zone values of 14.33 ± 0.58 and 12.00 ± 1.00 mm, respectively. In addition, Fraction B also performed antibacterial activities against *M. luteus* and *P. vulgaris* with the inhibition zone values of 11.33 ± 0.58 and 7.00 ± 0.00 mm, respectively. Nevertheless, Fraction B was failed to show any antibacterial activity against *E. coli* and *E. aerogenes*.

Out of the three Gram positive bacteria tested, Fraction A only showed antibacterial activity against *M. luteus* with the inhibition zone value of 8.00 ± 0.00 mm. Meanwhile, Fraction C only showed antibacterial activity against *S. epidermidis* with the inhibition zone value of 8.00 ± 0.00 mm. Both Fraction A and C were unable to inhibit the growth of all Gram negative bacteria tested.

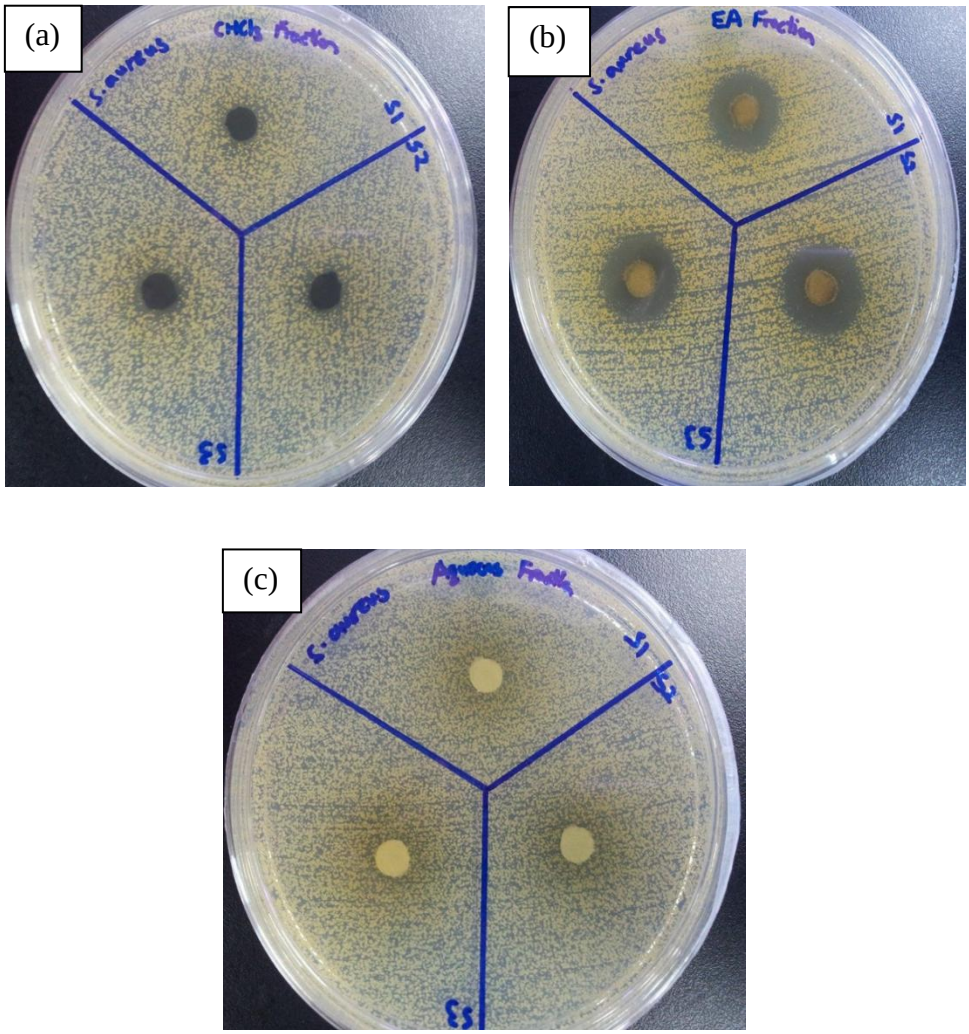


Figure 4.5: Disc diffusion assays of (a) Fraction A, (b) Fraction B and (c) Fraction C against *S. aureus.* Only Fraction B exhibited inhibition zones against *S. aureus.*

Table 4.4: Inhibition zone values exhibited by each partitioned fraction.

Partitioned Fractions	Zone of Inhibition (mm)					
	Gram positive			Gram negative		
	S. aureus	***S. epidermidis***	***M. luteus***	***P. vulgaris***	***E. coli***	***E. aerogenes***
Fraction A	—	—	8.00 ± 0.00	—	—	—
Fraction B	14.33 ± 0.58	12.00 ± 1.00	11.33 ± 0.58	7 .00 ± 0.00	—	—
Fraction C	—	8.00 ± 0.00	—	—	—	—
Streptomycin (0.5 mg/disc)	36.33 ± 0.58	—	46.67 ± 8.33	34.33 ± 0.58	18.00 ± 0.00	29.33 ± 1.15
Penicilin (0.2 mg/disc)	×	27.33 ± 0.58	×	×	×	×

Note: The inhibition zone values are expressed as mean ± standard deviation of three replicates.
The symbol '—' indicates that no antibacterial activity is observed.
The symbol '×' indicates that the disc diffusion assays of penicillin against *S. aureus*, *M. luteus*, *P. vulgaris*, *E. coli* and *E. aerogenes* are not included in this study.

4.5.2 Minimum Inhibitory Concentration Assay (Partitioned Fractions)

Each partitioned fraction was subjected to MIC assay against *S. aureus* in 96-wells plate and the findings are shown in Figure 4.6. Based on Figure 4.6, Fraction B gave the highest antibacterial activity against *S. aureus* with the lowest MIC value of 0.31 mg/ml. On the other hand, Fraction A and C showed higher MIC values of 5.0000 mg/ml and 10.0000 mg/ml against *S. aureus*, respectively. Ampicillin which used as positive control showed a MIC value of < 0.01 mg/ml. The positive, negative, and solvents controls of MIC assay are shown in Figure D1 of Appendix D.

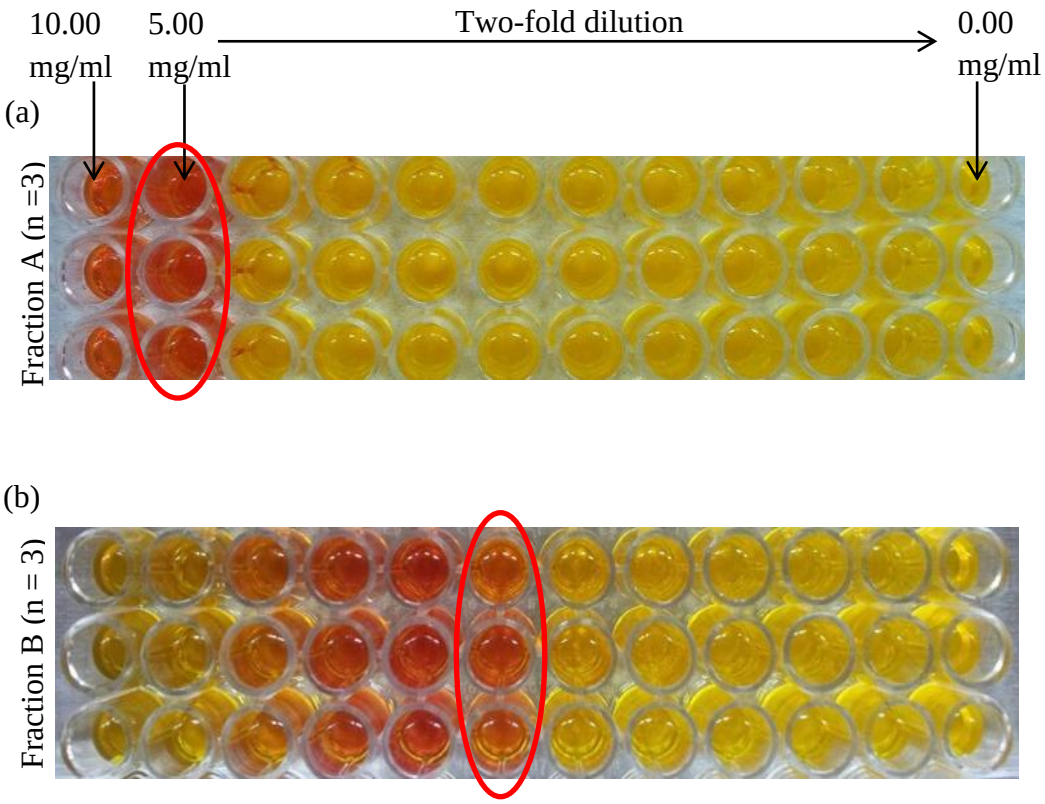
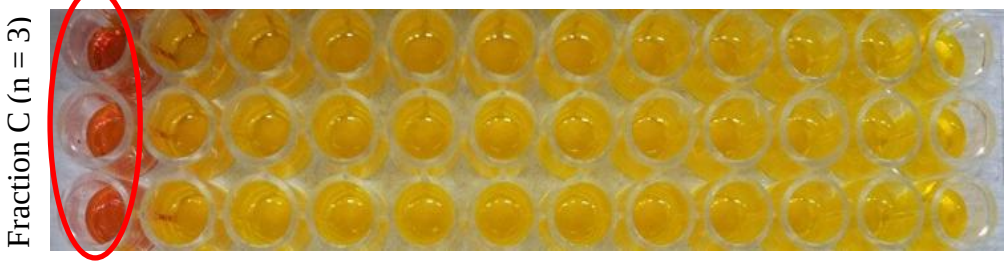


Figure 4.6: The MIC assays of (a) Fraction A, (b) Fraction B and (c) Fraction C against *S. aureus.*

(c)



Indicates no bacterial growth in the well

Indicates the presence of living bacteria in the well

Indicates the MIC value

Figure 4.6 (Continued): The MIC assays of (a) Fraction A, (b) Fraction B and (c) Fraction C against *S. aureus*.

4.5.3 Minimum Bactericidal Concentration Assay (Partitioned Fractions)

Figure 4.7 shows the MBC assays of Fraction A, B and C. *S. aureus* was subcultured from the positive MIC assay of each partitioned fraction onto MHA and incubated for 24 hours. The growth of golden colonies on MHA after 24 hours of incubation indicates that the antibacterial activity exhibited by the extract against *S. aureus* is bacteriostatic. In contrast, the absence of bacteria colonies after 24 hours of incubation indicates that the antibacterial activity exhibited by the extract is bactericidal. According to Figure 4.7, Fraction A and B was found to exhibit bacteriostatic activities against *S. aureus* while Fraction C exhibited bactericidal activity against *S. aureus*.

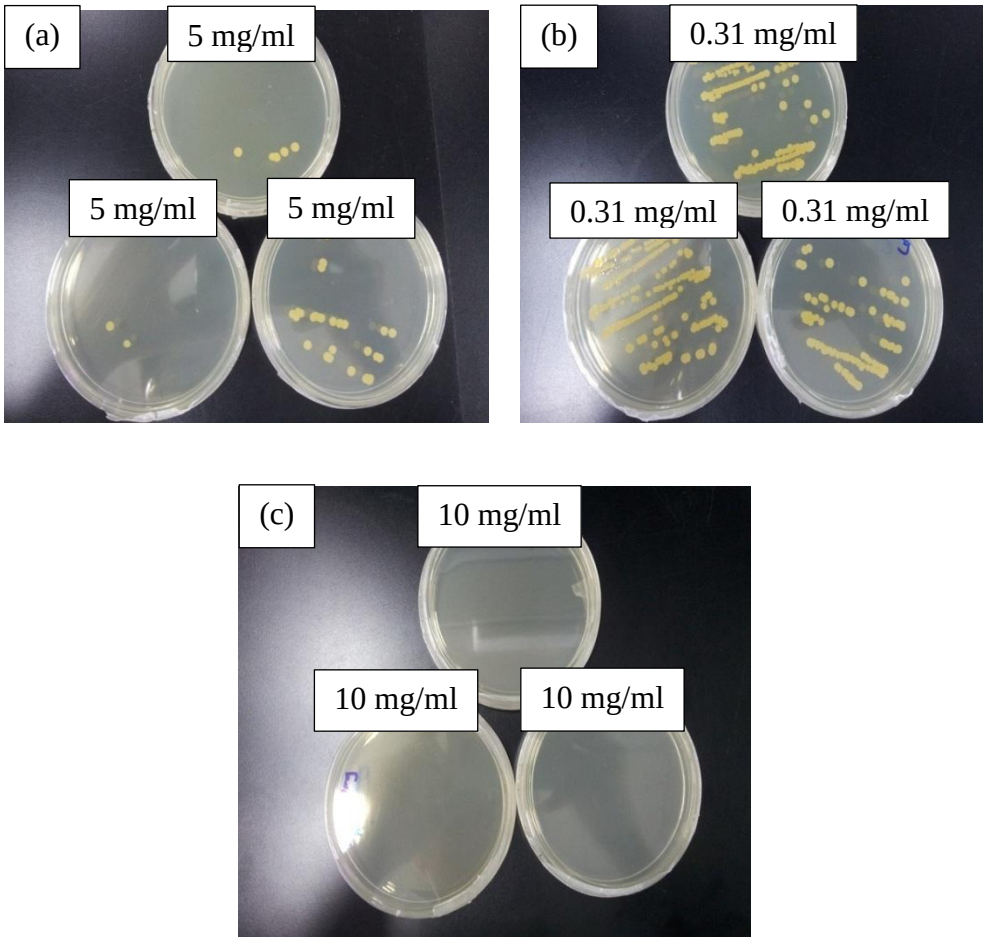


Figure 4.7: The MBC assays of (a) Fraction A, (b) Fraction B in triplicate and (c) Fraction C in triplicate. Golden colonies of *S. aureus* were observed in MBC assays of Fraction A and B after 24 hours of incubation but was absent in the MBC assays of Fraction C.

4.6 Antibacterial Activity of Isolated Fractions from Normal Phase Column Chromatography

Fraction B with highest antibacterial activity was further subjected to normal phase column chromatography and a total of eighty isolated fractions were collected. These fractions were then subjected to thin layer chromatography (TLC) and the TLC profiles of each isolated fractions at 254 nm and 365 nm are shown in

Appendix E and F, respectively. Those isolated fractions which showed the similar TLC profiles were combined together. For example, Fraction 33 (F33) to Fraction 42 (F42) which gave the similar TLC profiles as shown as in Figure 4.8 were pooled together and named as Combined Fraction 13 (CF13). A total of twenty combined fractions were obtained.

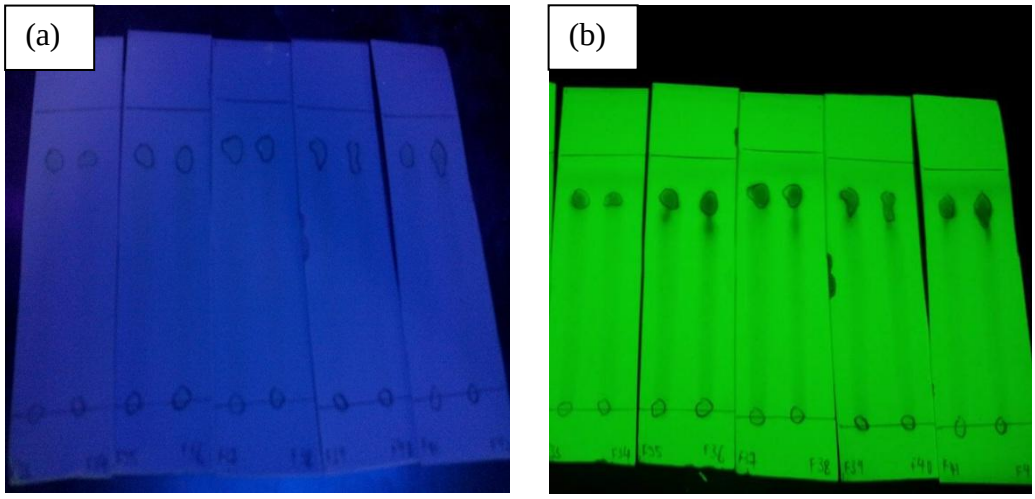


Figure 4.8: The TLC profiles of F33 to F42 at (a) 365 nm and (b) 254 nm.

4.6.1 Minimum Inhibitory Concentration Assay (Combined isolated Fractions)

All combined fractions from normal phase column chromatography were determined for their MIC values against *S. aureus* in 96-wells plate. The MIC assays for CF1 to CF7 are shown in Figure 4.9 while the MIC assays for CF8 to CF20 are shown in Figure D2 and D3 of Appendix D. The MIC values of each combined fraction against *S. aureus* are summarized in Table 4.5. Based on Table 4.5, CF2 exhibited the lowest MIC value of 0.21 mg/ml. Furthermore, the highest MIC value of > 1.67 mg/ml was exhibited by CF20. The MIC values for other

combined fractions were ranged from 0.42 mg/ml to 1.67 mg/ml. Ampicillin which used as positive control in this assay showed a MIC value of < 0.01 mg/ml. The positive, negative, and solvent controls of MIC assay are shown in Figure D1 in Appendix D.

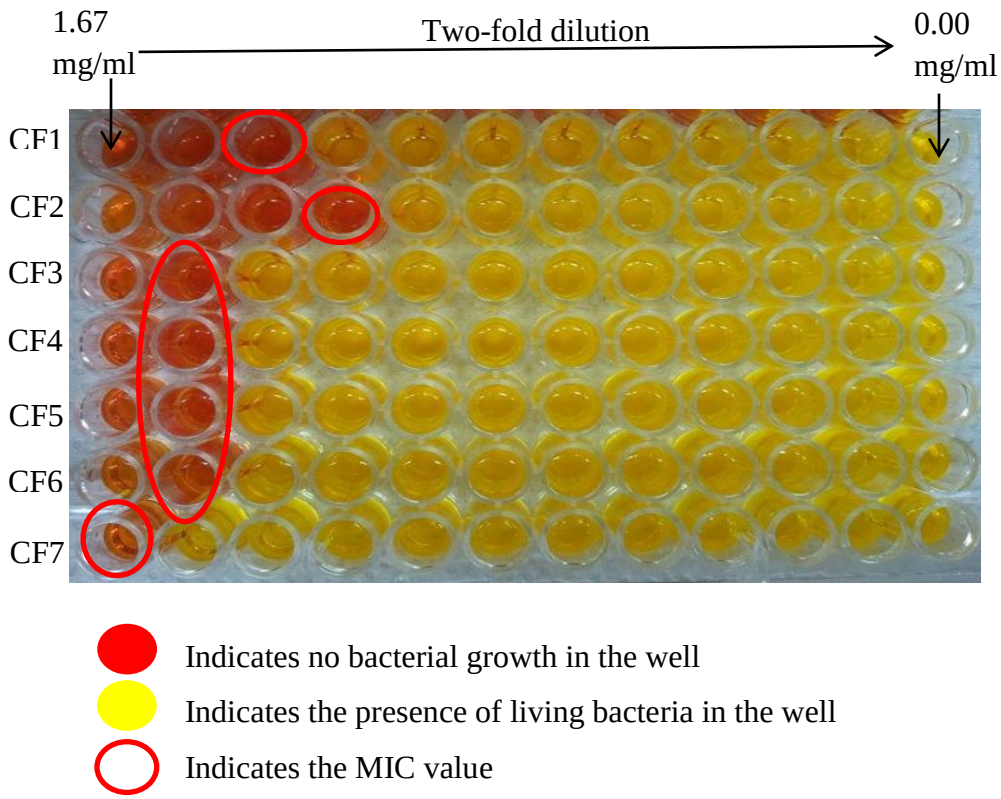


Figure 4.9: The MIC assays of CF1 to CF7

4.6.2 Minimum Bactericidal Concentration Assay (Combined Isolated Fractions)

Figure 4.10 shows the MBC assays of CF1 to CF6. The MBC assays for CF7 to CF20 are shown in Figure D4 of Appendix D. *S. aureus* was subcultured from the

positive MIC assay of each combined fraction onto MHA and incubated for 24 hours. After 24 hours, golden colonies of *S. aureus* were found on all the MHA. This indicates that the antibacterial activities exhibited by all combined fractions against *S. aureus* are bacteriostatic. The MBC values of each combined fraction against *S. aureus* are presented in Table 4.5.

According to Figure 4.10, more golden colonies were observed on Plate B than Plate A. This indicates that the bacteriostatic activity exhibited by CF1 is stronger than CF2 although CF1 gave a higher MIC value than CF2. Furthermore, more golden colonies were found on Plate D as compared to Plate C, E and F. This indicates that the bacteriostatic activities exhibited by CF4 are weaker although CF4 gave the same MIC value with CF3, 5 and 6.

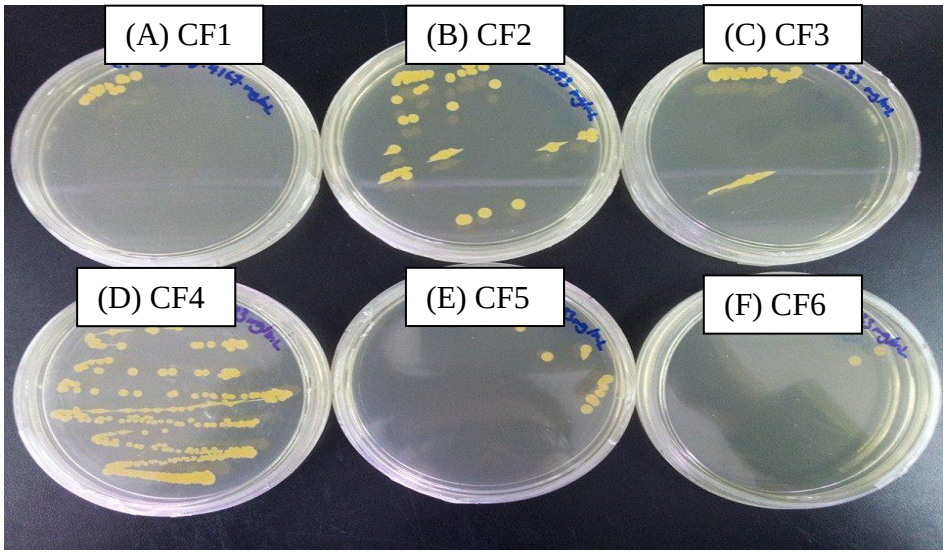


Figure 4.10: The MBC assays of CF1 to CF6. Golden colonies of *S. aureus* were observed on all MHA plate after 24 hours of incubation

Table 4.5: MIC and MBC values of each combined isolated fraction against *S. aureus*

Combined Fraction	MIC values against *S. aureus* (mg/ml)	MBC value against *S. aureus*
CF1	0.42	Bacteriostatic (0.42 mg/ml)
CF2	0.21	Bacteriostatic (0.21 mg/ml)
CF3	0.83	Bacteriostatic (0.83 mg/ml)
CF4	0.83	Bacteriostatic (0.83 mg/ml)
CF5	0.83	Bacteriostatic (0.83 mg/ml)
CF6	0.83	Bacteriostatic (0.83 mg/ml)
CF7	1.67	Bacteriostatic (1.67 mg/ml)
CF8	1.67	Bacteriostatic (1.67 mg/ml)
CF9	1.67	Bacteriostatic (1.67 mg/ml)
CF10	0.42	Bacteriostatic (0.42 mg/ml)
CF11	1.67	Bacteriostatic (1.67 mg/ml)
CF12	1.67	Bacteriostatic (1.67 mg/ml)
CF13	1.67	Bacteriostatic (1.67 mg/ml)
CF14	1.67	Bacteriostatic (1.67 mg/ml)
CF15	1.67	Bacteriostatic (1.67 mg/ml)
CF16	0.83	Bacteriostatic (0.83 mg/ml)
CF17	1.67	Bacteriostatic (1.67 mg/ml)
CF18	1.67	Bacteriostatic (1.67 mg/ml)
CF19	1.67	Bacteriostatic (1.67 mg/ml)
CF20	> 1.67	Bacteriostatic (1.67 mg/ml)
Ampicillin (positive control)	< 0.01	Bacteriostatic

CHAPTER 5

DISCUSSION

5.1 Extraction Yield

The leaves of *W. trilobata* were sequentially extracted with hexane, chloroform, ethyl acetate, methanol and 70 % acetone. According to Figure 4.1, the percentage yields of extraction differed when different extracting solvents were used. This is due to the difference between chemical structures of solvents and their polarities (Hassim, et al., 2015). Furthermore, the chemical components of extractable constituents in plant also influence the extraction efficiency of solvent (Sultana, Anwar and Ashraf, 2009).

Based on the results obtained, higher efficiency of extraction was generally observed in highly polar solvents such as 70 % acetone and methanol extracts as compared to the rest of solvents. This indicates that the phytochemicals present in the leaves of *W. trilobata* are mostly high in polarity. This finding is consistent with the study reported by Govindappa et al. (2011) that *W. trilobata* possess a numerous number of polyphenol compounds such as saponins, terpenoids, coumarins, flavonoids, tannins and phenols.

5.2 Total Phenolic Contents

The extraction of phenolic compounds is depending on the compatibility of phenolic compounds toward the solvent used (Tan, Tan and Ho, 2013). Based on Table 4.1, higher extraction yield solvents such as 70 % acetone and methanol gave higher TPC values. These TPC values are 5 - 10 times higher than other less polar solvents. This high extraction efficiency of TPC is due to the nucleophilic functional groups of high polar solvents enable them to form hydrogen bonds with phenolic compounds (Azrie, et al., 2014). In addition, the present findings also reveals that the phytochemicals extracted by 70 % acetone and methanol consist significant amount of phenolic compounds. Some phenolic compounds that found in *W. trilobata* by other researchers have been discussed in Section 2.2 together with their chemical structures.

5.3 Total Flavonoid Contents

Flavonoids can be categorized into different classes based on their oxidation on middle ring and substitutions (Yang, Lin and Kuo 2008). Hence, solvents with different polarity indexes are used to extract different classes of flavonoids. Hydrophilic flavonoids such as flavonoid glycosides are usually extracted by high polar alcohol solvent or water-alcohol mixture solvent. In contrast, less polar solvents such as chloroform and ethyl acetate are used to extract flavanones, methylated flavones, flavonols and isoflavones (Ferreira and Pinho, 2012).

The present study shows that higher TFC values were found in ethyl acetate and chloroform extracts. Furthermore, higher extraction efficiency of TFC was generally observed in less polar solvents as compared with high polar solvents. These results obtained are compatible to the findings reported by Qiang, et al. (2011) and Balekar, Nakpheng and Srichana (2014) in which flavonoids present in the leaves of *W. trilobata* are apigenin, diosmentin and 3-hydoxyl-6-methoxychromen-4-one. Apigenin is a member of flavones while diosmentin and 3-hydoxyl-6-methoxychromen-4-one are methylated flavones. Both flavones and methylated flavones are classified as lipophilic flavonoids that lack of hydroxyl functional group. Hence, these less polar flavonoids are more likely to be extracted by less polar solvents such as chloroform and ethyl acetate.

5.4 Preliminary Antibacterial Screening

Based on Table 4.3, hexane, chloroform, methanol and 70 % acetone extracts showed antibacterial activities against different bacteria tested. 70 % acetone and methanol extracts exhibited higher antibacterial activities against *S. epidermidis* and *S. aureus* as compared with hexane and chloroform extracts. However, the antibacterial compounds that present in 70 % acetone and methanol extracts were yet identified at this stage. So, the high phenolic contents in 70 % acetone and methanol extracts are presumably responsible for their antibacterial activities. Although the antibacterial mechanisms are not clearly understood, it has been

suggested that the phenolic derivatives are able to disrupt the structure cell membrane (Martino, et al., 2009).

On the other hand, lower antibacterial activities were observed in hexane and chloroform extracts against Gram positive bacteria. This indicates the presence of low polar antibacterial compounds in the leaves of *W. trilobata* which possess lower antibacterial properties. The results obtained are compatible to the findings reported by Taddei and Rosas-Romero (1999) in which the hexane extract of aerial parts (without flowers) of *W. trilobata* exhibited antibacterial activities against different bacteria. In addition, Shankar and Thomas (2014) also reported the antibacterial activity exhibited by the chloroform extract of *W. trilobata* flower heads. Furthermore, ethyl acetate extract showed negative results against all bacteria tested in this study. The results obtained are agree with the findings reported by Taddei and Rosas-Romero (1999) that ethyl acetate extract of aerial parts (without flowers) of *W. trilobata* did not exhibit any antibacterial activity against *S. aureus*, *S. epidermidis*, *P. vulgaris*, and *E. coli*. Although highest TFC value was showed by ethyl acetate extract, the antibacterial activities of flavonoids that present in *W. trilobata* leaves have not been well documented.

In overall, Gram negative bacteria are less susceptible to the solvent extracts of *W. trilobata* leaves as compared with Gram positive bacteria. This is due to the presence of an extra outer layer in the cell wall structure of Gram negative bacteria

as shown in the Section 2.3.1. This extra outer layer consists of phospholipid and lipopolysaccharide act as an additional barrier to hinder the movement of antibacterial compounds into the cell (Darah, Lim and Nithianantham, 2013). Thus, Gram positive bacteria which lack of this extra outer layer are more susceptible to the inhibition of solvent extracts.

The *S. aureus* was inhibited by the bactericidal activity of 70 % acetone extract at MIC value of 8.34 mg/ml. This may due to the inhibition of nucleic acid synthesis by the antibacterial compounds present in the 70 % acetone extract (Kohanski, Dwyer and Collins, 2010). Besides, the antibacterial compounds in the 70 % acetone extract might inhibit the synthesis of peptidoglycan in *S. aureus* and thus weakening its cell wall (Tortura, Funke and Case, 2013). Hence, *S. aureus* undergoes cell lysis and unable to recover their growth after subcultured onto an extract-free media.

On the other hand, the growth of *S. aureus* was inhibited by the bacteriostatic activity of methanol extract at MIC value between 8.34 mg/ml and 16.67 mg/ml. This may due to the antibacterial compounds in methanol extract are able to interfere the 50S or 30S ribosomes in *S. aureus* and thus inhibit its mRNA translation process (Kohanski, Dwyer and Collins, 2010). As a result, the synthesis of certain proteins or enzymes in *S. aureus* is prohibited and the growth of *S.*

aureus is inhibited. However, *S. aureus* was able to recover its growth once the methanol extract was eliminated from its growing environment.

5.5 Antibacterial Activities of Liquid-Liquid Partitioned Fractions

Liquid-liquid partition of 70 % acetone crude extract gave chloroform (Fraction A), ethyl acetate (Fraction B) and aqueous fractions (Fraction C). Based on Table 4.4, Fraction B exhibited antibacterial activities against *S. aureus*, *S. epidermidis* and *P. vulgaris*. This has revealed that the bio-active compounds which responsible for the antibacterial activities in 70 % acetone crude extract were successfully partitioned into Fraction B. Nevertheless, Fraction B demonstrated antibacterial activity against *M. luteus* in which this phenomenon did not shows by 70 % acetone crude extract. This may due to the presence of chemical antagonist in 70 % acetone crude extract that can interfere with the antibacterial activity against *M. luteus* (Mwambete, 2009; Otto, Ameso and Onegi, 2014).

The growth of *S. aureus* was inhibited by Fraction B at MIC value of 0.31 mg/ml. In comparison with the MIC value of 70 % acetone crude extract (8.34 mg/ml), it can be seen that the antibacterial activity exhibited by Fraction B against *S. aureus* was significantly enhanced. This is due to the impurities and chemical antagonist in 70 % acetone crude extract have been partitioned into other fractions, and hence increased the purity of the antibacterial compounds in Fraction B (Mwambete, 2009).

Based on Figure 4.7, the antibacterial activities exhibited by Fraction B and C were bacteriostatic and bactericidal, respectively. This reveals that the antibacterial compounds responsible for the bactericidal activities in 70 % acetone crude extract h partitioned into Fraction C. Furthermore, less *S. aureus* were able to recover their growth on Plate A as compared to Plate B in Figure 4.7. This higher effectiveness of bacteriostatic activity from Fraction A is presumably related to its higher concentration of antibacterial compounds.

5.6 Antibacterial Activity of Combined Isolated Fractions from Normal Phase Column Chromatography

In the current study, a total of eighty isolated fractions were collected from normal phase column chromatography and these fractions were further combined into twenty combined fractions based on their TLC profiles. After that, these combined fractions were subjected to MIC assay against *S. aureus* in 96-wells plate. Based on Table 4.5, the highest antibacterial activity was exhibited by the CF2 with the lowest MIC value of 0.21 mg/ml. Hence, the bio-active antibacterial compounds were expected to present in CF2.

A significant enhancement of antibacterial activity can be seen clearly by comparing the MIC values of CF2, Fraction B and 70 % acetone crude extract. The lowest concentration required to inhibit the growth of *S. aureus* was reduced from 8.34 mg/ml to 0.31 mg/ml after liquid-liquid partition of 70 % acetone crude extract. The MIC value was further descended to 0.21 mg/ml after the isolation

process through normal phase column chromatography. This enhancement of antibacterial activity is due to the removal of impurities and antagonistic metabolites from 70 % acetone crude extract and Fraction B. The bio-active antibacterial compounds that present in CF2 were partially isolated at this stage.

For the normal phase column chromatography, silica gel was used as polar stationary phase while the gradient elution system was used from non-polar to polar. So, the least polar compounds will be eluted first and the more polar compounds will be eluted later. The present study shows that CF2 (Fraction 4-7) were eluted by the mobile phase consists of hexane: ethyl acetate (4:6 v/v). Hence, the antibacterial compounds in the CF2 are predicted to be slightly low polar compounds with polarity index of 2.64 approximately.

Based on the discussion above, an assumption has been made that kaurenoic acid and granliflorenic acid are the suspected bio-active antibacterial compounds that present in CF2. These compounds are low polar terpenes in the leaves of *W. trilobata* which have been reported to exhibit antibacterial activities (Balekar, et al., 2012; Balekar, Nakpheng and Srichana, 2014). The chemical structures of these compounds have been presented in Section 2.2.1.

The assumption made is supported by few studies in which the conditions of bioassay-guided isolation used are similar to the present study. Balekar, Nakpheng and Srichana (2013) reported that the ethanolic extract of *W. trilobata* leaves was sequentially liquid-liquid partitioned with organic solvents. The ethyl acetate fraction was then further subjected to the normal phase column chromatography and grandiflorenic acid was successfully eluted by the combination of hexane and ethyl acetate (Balekar, Nakpheng and Srichana, 2013). Besides, Okoye, et al. (2012) also reported that the methanol-methylene chloride extract of the root bark of *Annona senegalensis* was sequentially liquid-liquid partitioned with hexane, ethyl acetate and methanol. After that, the ethyl acetate fraction was further subjected to normal phase column chromatography and kaurenoic acid was eluted by the mobile phase consists of hexane and ethyl acetate. Therefore, one of the antibacterial compounds in CF2 is predicted to be either kaurenoic acid or grandiflorenic acid due to the condition of bioassay-guided isolation used in current study is similar to the studies discussed.

5.7 Future Perspectives

In the present study, the bio-active antibacterial compounds in the leaves of *W. trilobata* were partially purified. Further studies are required to completely purify the bio-active antibacterial compounds in the CF2. Instrumental analysis such as high performance liquid chromatography (HPLC) and nuclear magnetic resonance

(NMR) can be used to elucidate the chemical structures of the pure antibacterial compounds.

In addition, other microorganisms can be involved to study the antifungal and antiviral activity of the leaves of *W. trilobata*. Future works also can be carried out to evaluate the antioxidant activity of the leaves of *W. trilobata* due to its presence of high phenolic contents. Furthermore, bioassay-guided isolation of antibacterial compounds from the methanol extract which showed high antibacterial activity in this study is suggested.

CHAPTER 6

CONCLUSIONS

In this project, polar solvents showed higher percentage yields of extraction as compared to low polar solvents. This reveal that the phytochemical constituents present in the leaves of *W. trilobata* are mostly high in polarity. The highest total flavonoid contents were observed in ethyl acetate extract of *W. trilobata*. However, no antibacterial activity was exhibited by these flavonoids in this study. On the other hand, 70 % acetone and methanol extracts of the leaves of *W. trilobata* exhibited strong antibacterial activity against *S. aureus* and *S. epidermidis*. This is presumably due to the strong occurrence of phenolic contents in these extracts. Nevertheless, further investigations are necessary to confirm this hypothesis.

To conclude, the present findings provide scientific evidence to support the traditional uses of *W. trilobata* leaves and its potential for the development of new antibacterial drugs. The bio-active antibacterial compounds of *W. trilobata* leaves were partially isolated through the bioassay-guided isolation in the current study. Hence, further studies are suggested to fully purify and identify the active principles present in the leaves of *W. trilobata*.

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Appendix A

Table A1: The list of apparatus and equipment with their respective brand and model.

Apparatus/ Equipments	**Brand/ Model**
Blender	KHIND, Malaysia
Fume cabinet	myLab, Finland
Hotplate Stirrer	LMS, Japan
Incubator	Memmert, Germany
Laboratory oven	Memmert, Germany
Laminar air flow cabinet	Camfil FARR, Malaysia
Micropipettes	Eppendorf, Germany
Multichannel pipette	Thermo Scientific, USA
pH meter	Eutech Instruments, Singapore
Spectrophotometer	Thermo Scientific, USA
UV lamp	UVP, UK
Vacuum rotatory evaporator	BUCHI Rotavapor, Switzerland
Vortex mixer	Stuart, USA
Weighing balance	Kern ABJ, Germany

Table A2: The list of consumables and chemicals with their respective manufacturers.

Consumables/ Chemicals	Manufacturers
n- Hexane	Qrec Asia Sdn Bhd, Malaysia
Chloroform	Qrec Asia Sdn Bhd, Malaysia
Ethyl acetate	Qrec Asia Sdn Bhd, Malaysia
Methanol	Qrec Asia Sdn Bhd, Malaysia
Acetone	Qrec Asia Sdn Bhd, Malaysia
Luria Bertani agar	CONDA, Spain
Nutrient agar	Merck KGaA, Germany
Mueller Hinton agar	Oxoid, England
Mueller Hinton broth	Becton Dickinson and company, USA
D-glucose	SYSTERM, Malaysia
Gallic acid	Merck KGaA, Germany
Folin- Ciocalteu reagent	Merck KGaA, Germany
Phenol red	Nacalai tesque, Japan
Sodium chloride	Merck KGaA, Germany
Sodium hydroxide	Merck KGaA, Germany
Sodium nitrate	Or ë c®
Aluminium chloride	Merck Schuchardt OHG, Germany
Quercetin	Alfa Aesar, England
Portable Bunsen burner	CAMPINGAZ C206, Czech Republic
96 well microplates	TPP, Switzerland
Centrifuge tube	Falcon, USA
Microcentrifuge tube	Axygen, USA
Sterile Cotton swab	Premier Diagnostics, Malaysia
Membrane filter, 0.45 µm	TPP, Switzerland
Syringe 10ml	Cellotron, Malaysia
Silica Gel Plates	Merck, USA
Filter paper	Whatman (1001-150), United Kingdom

Appendix B

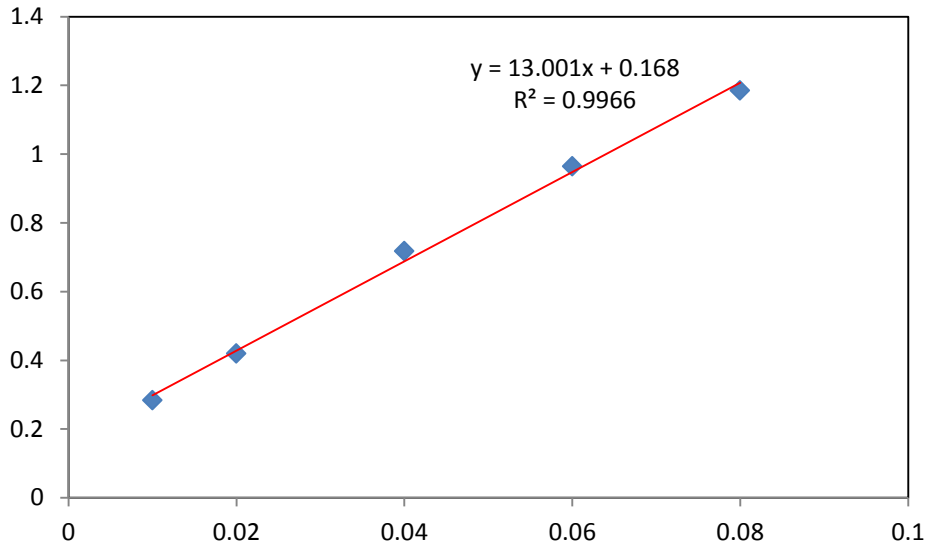

Figure B1: The Garlic acid standard curve for the determination of total phenolic contents.

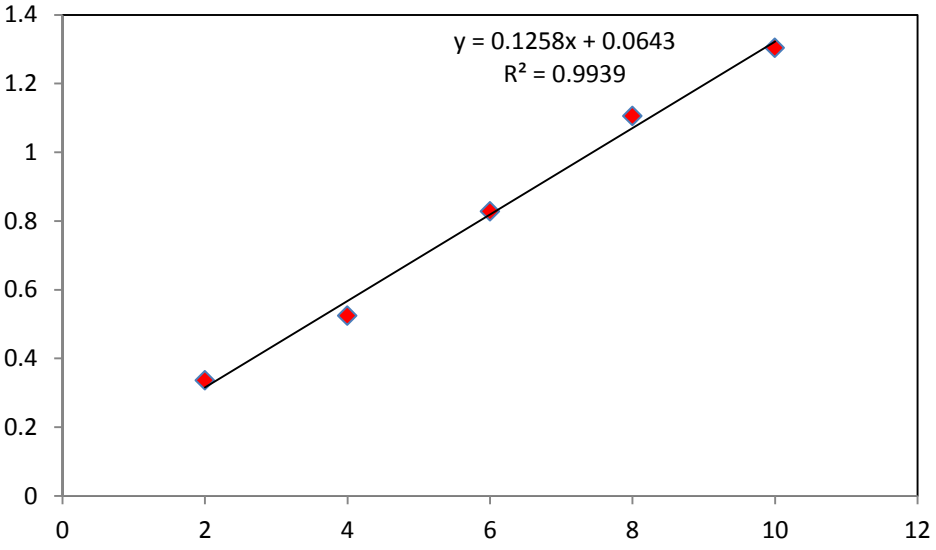

Figure B2: The Quercetin standard curve for the determination of total flavonoids content.

Appendix C

Disc diffusion assays

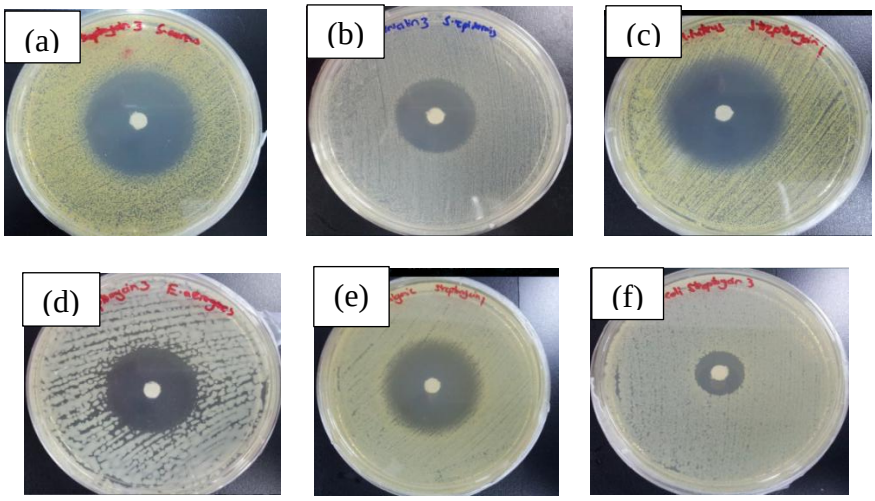

Figure C1: Zone of inhibition exhibited by positive control (a) Streptomycin against *S. aureus*, (b) Penicilin against *S. epidermidis*, (c) Streptomycin against *M. luteus*, (d) Streptomycin against *E. aerogenes*, (e) streptomycin against *P. vulgaris* and (f) streptomycin against *E.coli*.

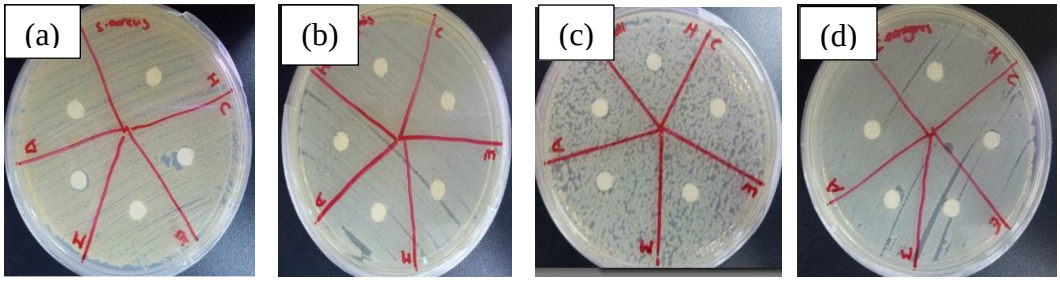

Figure C2: Zone of inhibition exhibited by hexane, chloroform, ethyl acetate, methanol, 70 % acetone against (a) *S. aureus*, (b) *P. vulgaris*, (c) *E. coli* and (d) *E. aerogenes.*

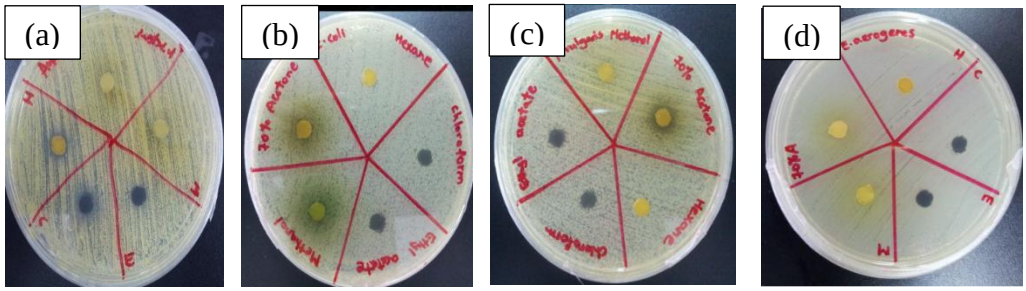

Figure C3: Zone inhibition exhibited by crude extracts of *W. trilobata* against (a) *M. luteus*, (b) *E. coli*, (c) *P. vulgaris* and (d) *E. aerogenes*.

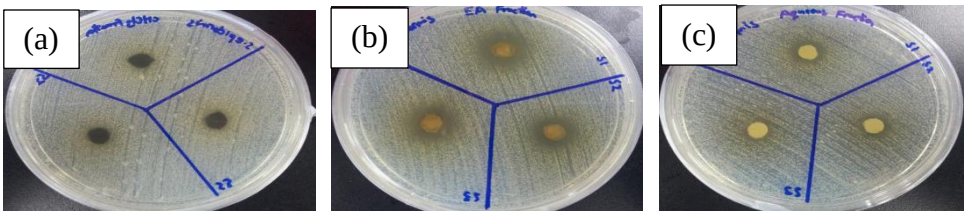

Figure C4: Zone of inhibition exhibited by (a) Fraction A, (b) Fraction B and (c) Fraction C against *S. epidermidis*.

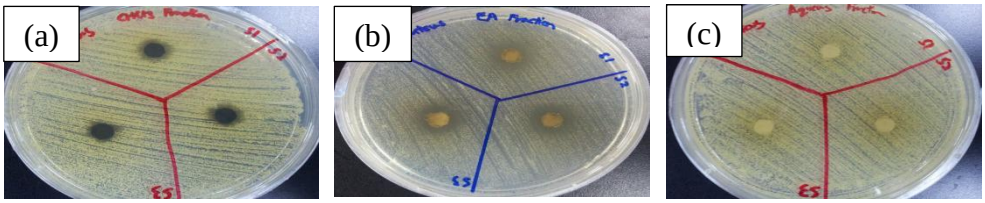

Figure C5: Zone of inhibition exhibited by (a) Fraction A, (b) Fraction B and (c) Fraction C against *M. luteus.*

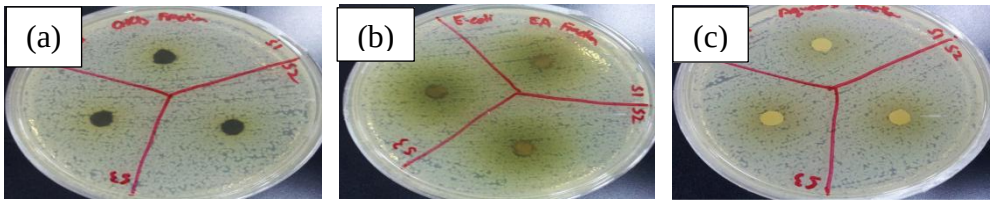

Figure C6: Zone of inhibition exhibited by (a) Fraction A, (b) Fraction B and (c) Fraction C against *E. coli*.

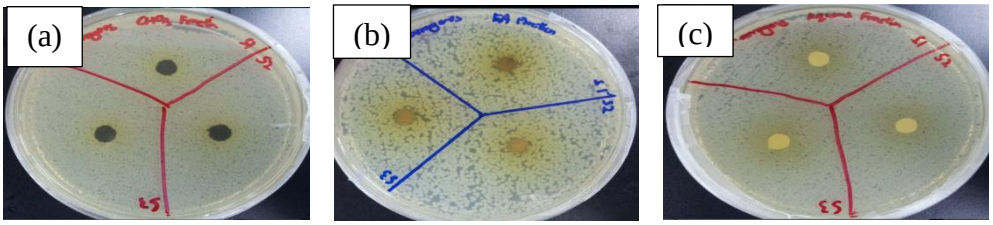

Figure C7: Zone of inhibition exhibited by (a) Fraction A, (b) Fraction B and (c) Fraction C against *E. aerogenes*.

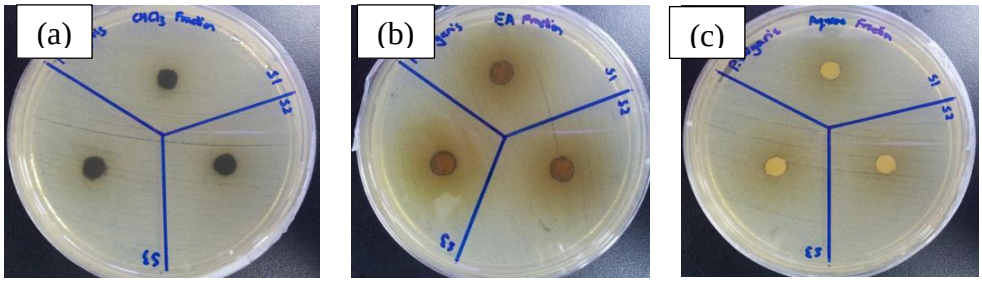

Figure C8: Zone of inhibition exhibited by (a) Fraction A, (b) Fraction B and (c) Fraction C against *P. vulgaris*.

Appendix D

MIC and MBC assays

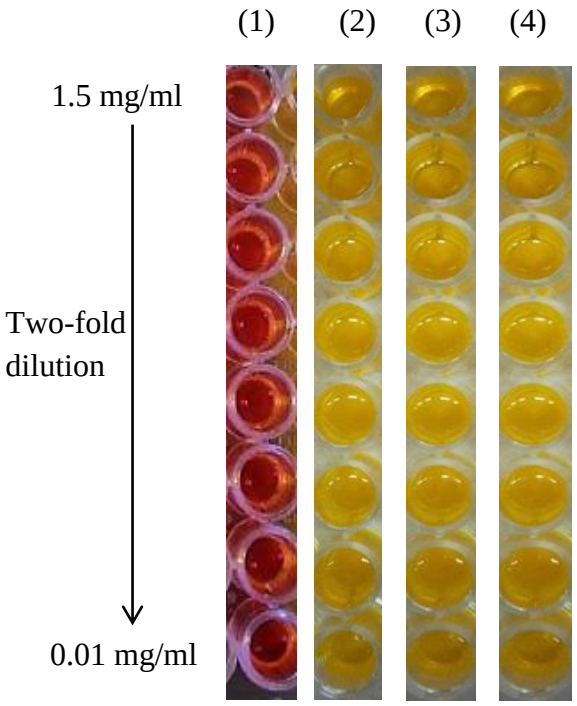

Figure D1: Column 1 and 2 show the positive and negative controls of the MIC assay, respectively. Column 3and 4 show the solvent controls for methanol and distilled water, respectively.

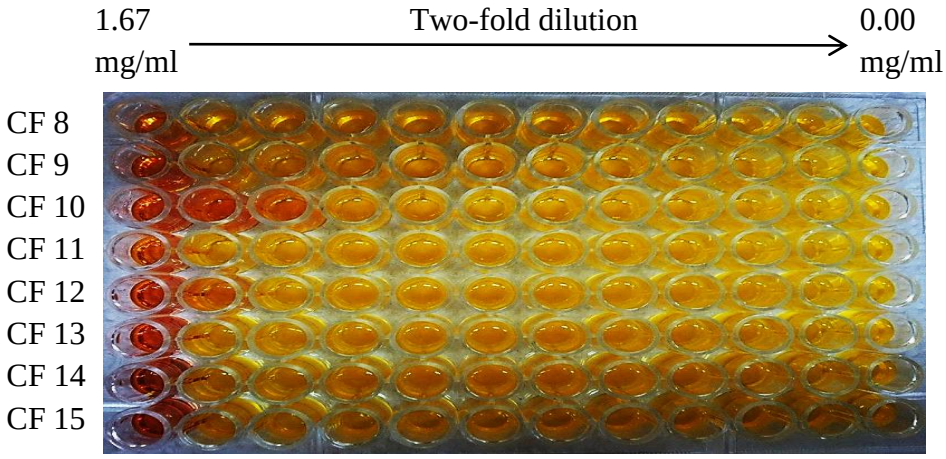

Figure D2: MIC assays of CF8 to CF15.

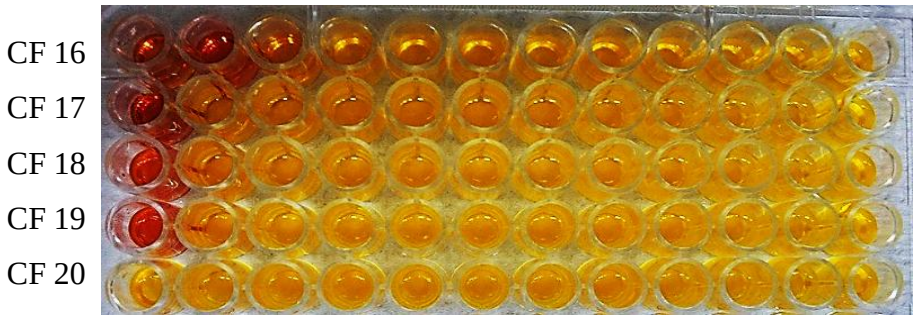

Figure D3: MIC assays of CF16 to CF20.

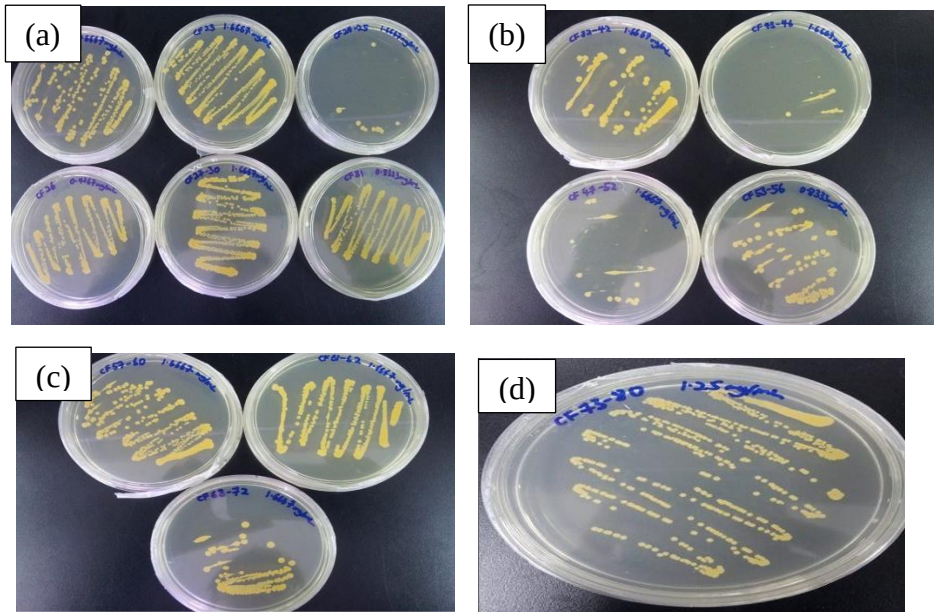

Figure D4: MBC assays of (a) CF7 - CF12, (b) CF13 - CF16, (c) CF17 - CF 19 and (d) CF20.

Appendix E

TLC profiles of each column isolated fraction under UV light at 254 nm

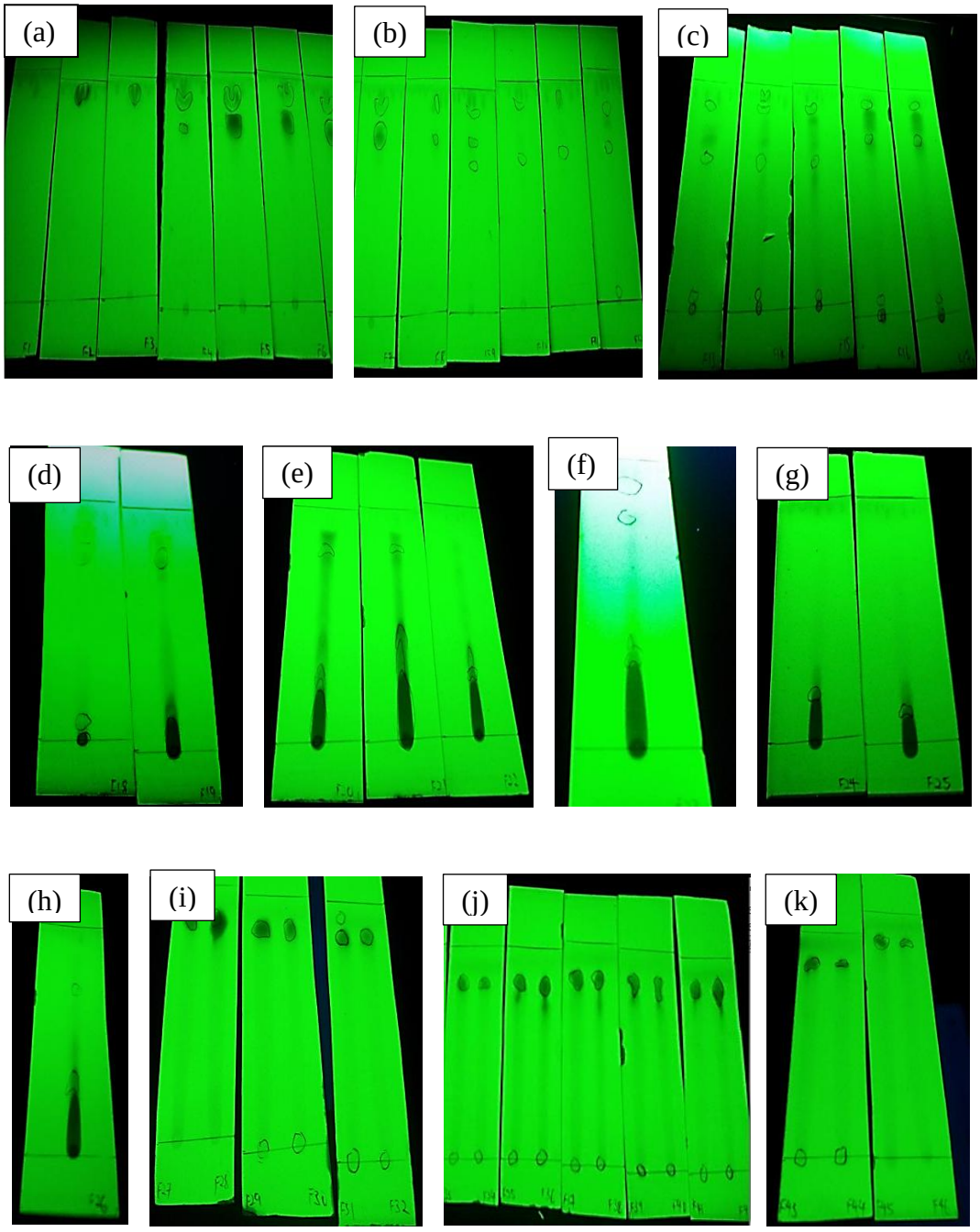

Figure E1: TLC profiles at 254 nm of (a) F1 – F6, (b) F7 – F12, (c) F13 – F17, (d) F18 – F19, (e) F20 – F 22, (f) F23, (g) F24 – F25, (h) F26, (i) F27 – F32, (j) F33 – F42, (k) F43 – F46, (l) F47 – F52, (m) F53 – F56, (n) F57 – F62, (o) F63 – F72 and (p) F73 – F76 and (q) F77 – F80.

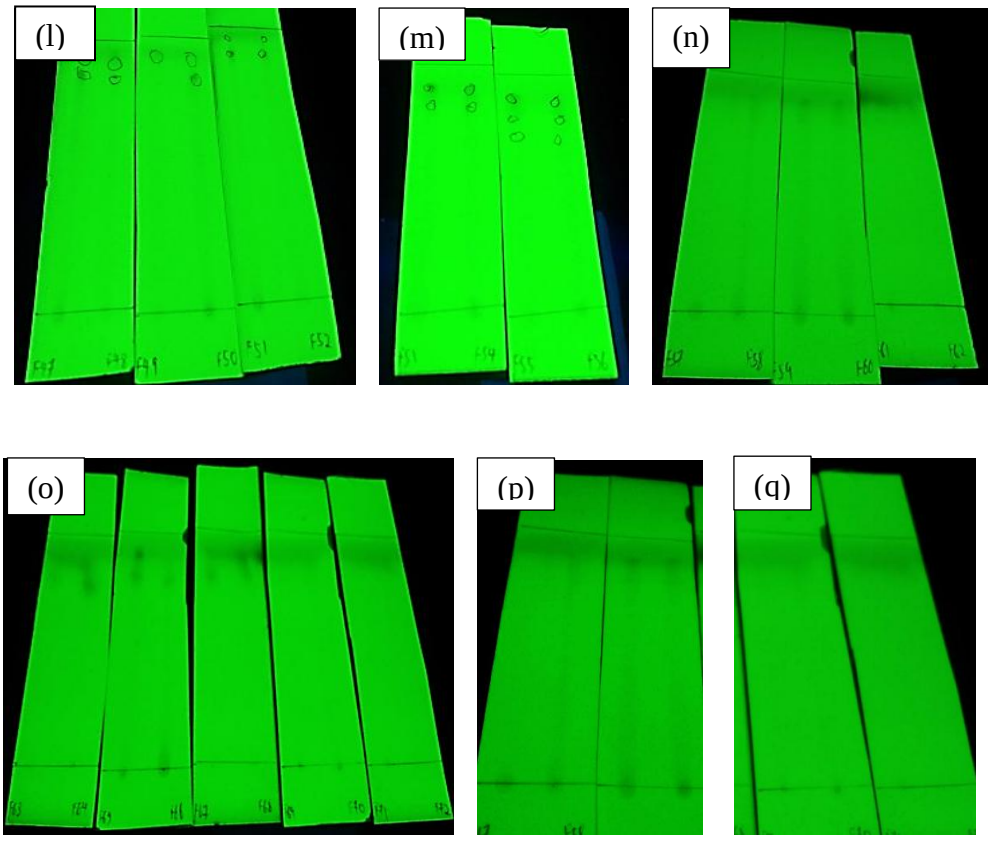

Figure E1 (Continued): TLC profiles at 254 nm of (a) F1 – F6, (b) F7 – F12, (c) F13 – F17, (d) F18 – F19, (e) F20 – F 22, (f) F23, (g) F24 – F25, (h) F26, (i) F27 – F32, (j) F33 – F42, (k) F43 – F46, (l) F47 – F52, (m) F53 – F56, (n) F57 – F62, (o) F63 – F72 and (p) F73 – F76 and (q) F77 – F80.

Appendix F

TLC profiles of each column isolated fraction under UV light at 365 nm

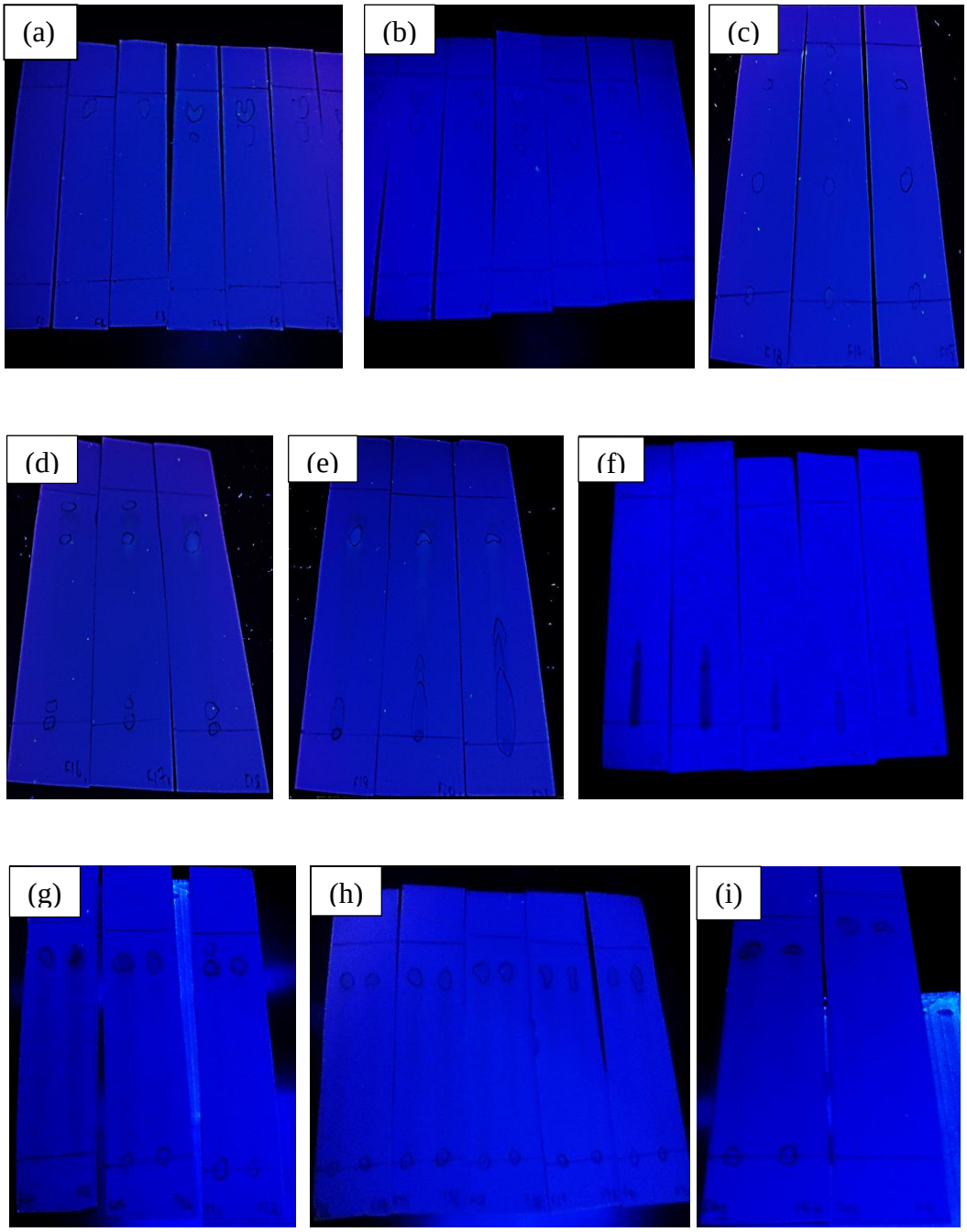

Figure F1: TLC profiles at 365 nm of (a) F1 – F6, (b) F7 – F12, (c) F13 – F15, (d) F16 – F18, (e) F19 – F21, (f) F22 – F26, (g) F27 – F32, (h)F33 – F42, (i) F43 – F46, (j) F53 – F56, (k) F57 – F62, (l) F63 – F72 and (m) F73 – F80.

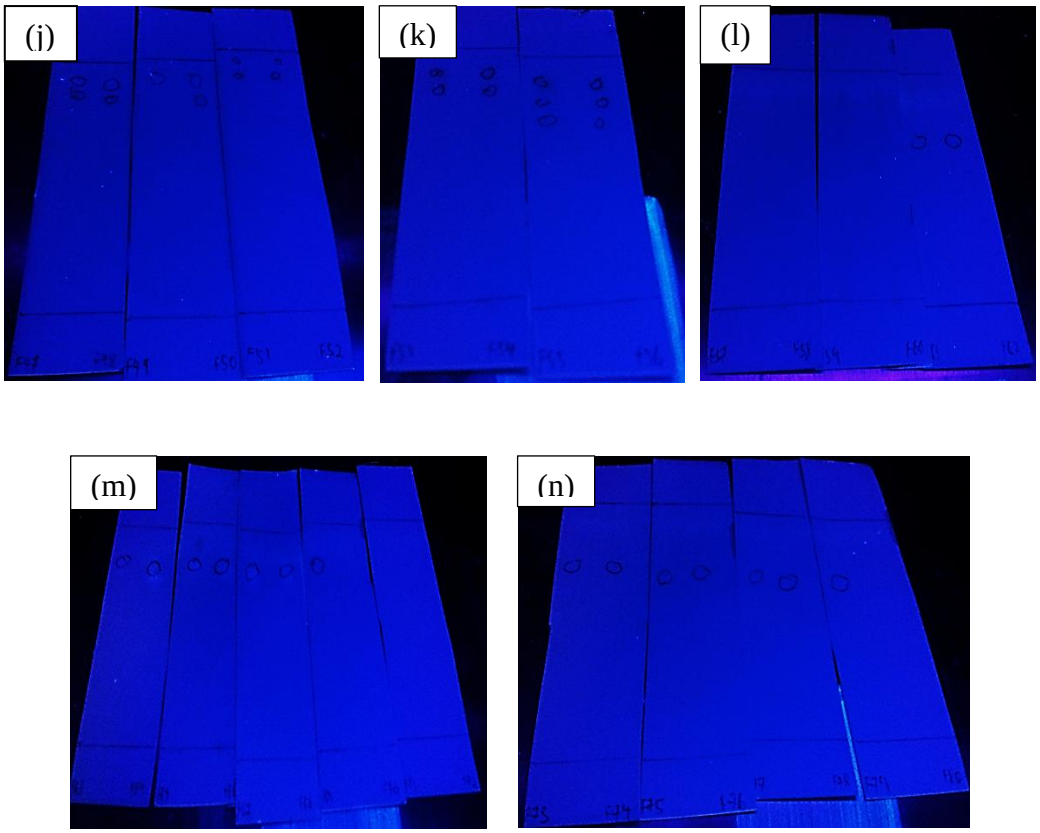

Figure F1 (Continued): TLC profiles at 365 nm of (a) F1 – F6, (b) F7 – F12, (c) F13 – F15, (d) F16 – F18, (e) F19 – F21, (f) F22 – F26, (g) F27 – F32, (h) F33 – F42, (i) F43 – F46, (j) F53 – F56, (k) F57 – F62, (l) F63 – F72 and (m) F73 – F80.