ANTIBACTERIAL AND BIOACTIVITY ANALYSIS OF SELECTED MEDICINAL PLANTS AND THEIR EFFECTS ON BACTERIAL PROTEIN EXPRESSION PROFILES

By

YONG ANN LI

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ABSTRACT

ANTIBACTERIAL AND BIOACTIVITY ANALYSIS OF SELECTED MEDICINAL PLANTS AND THEIR EFFECTS ON BACTERIAL PROTEIN EXPRESSION PROFILES

Yong Ann Li

Medicinal plants have been utilized throughout the centuries as a form of remedies against human diseases. Fifteen medicinal plants were investigated for their antibacterial properties and three plant extracts (*Melastoma candidum, Callicarpa formosana* and *Scutellaria barbata*) showed antibacterial activities. Among these three plants, *M. candidum* extract demonstrated the highest activity, followed by *S. barbata* and *C. formosana*. Time-kill assays were performed to determine the bacteriostatic and bactericidal effects of the plant extracts. *M. candidum* extract possessed bactericidal activity at 1× MIC against *Pseudomonas aeruginosa* and 2× MIC against *Staphylococcus aureus* at 24 hours of incubation. In addition, these three plants were also detected with DPPH and NO_2 radical scavenging activity, as well as ferrous ion chelating activity. The plant extracts were then subjected to thin layer chromatography-bioautography analysis to determine the antibacterial compounds. On the basis of gas chromatography-mass spectrometry, the following compounds were identified: 2,4-bis-(1,1-dimethylethyl)-phenol, hexadecanoic acid, benzenepropanoic acid, 1-butyl 2-(2-

ethylhexyl) phthalate, 1-propanone and 2-heptanamine. Some of these compounds (e.g. 2,4-bis-(1,1-dimethylethyl)-phenol, hexadecanoic acid, benzenepropanoic acid) were previously known to be antibacterial. The antibacterial compounds from the plant extracts should be identified and studied their certain antibacterial mechanism. Further studies were done using the three plant extracts to investigate their effects on bacterial protein expression profiles. Based on the study, seventeen differentially expressed bacterial proteins were identified from four different bacterial strains. These bacterial proteins (e.g. Formate c-acetyltransferase, elongation factors, outer membrane protein A) were found to be essential for the survival and growth of the bacteria. Additionally, scanning electron microscope was utilized to investigate the morphological changes on the bacteria cell surfaces. Upon treatment with antibacterial plant extracts, the bacteria cell surfaces were shown to be damaged, deformed, pitted and shriveled compared to the untreated bacteria. From this study, potential antibacterial protein targets were identified, with further understandings of how antibacterial plant extracts affect bacteria at the molecular and physical levels.

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APPROVAL SHEET

This dissertation entitled **"ANTIBACTERIAL AND BIOACTIVITY ANALYSIS OF SELECTED MEDICINAL PLANTS AND THEIR EFFECTS ON BACTERIAL PROTEIN EXPRESSION PROFILES"** was prepared by YONG ANN LI and submitted as partial fulfillment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

Approved by:

_____

(Assoc. Prof. Dr. WONG FAI CHU) Date:……..
Supervisor
Department of Chemical Science
Faculty of Science
Universiti Tunku Abdul Rahman

_____

(Assoc. Prof. Dr. SIM KOOI MOW) Date:……..
Co-supervisor
Department of Chemical Science
Faculty of Science
Universiti Tunku Abdul Rahman

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date:_____

SUBMISSION OF DISSERTATION

It is hereby certified that YONG ANN LI (ID No: 12ADM01478) has completed this dissertation entitled "ANTIBACTERIAL AND BIOACTIVITY ANALYSIS OF SELECTED MEDICINAL PLANTS AND THEIR EFFECTS ON BACTERIAL PROTEIN EXPRESSION PROFILES" under the supervision of Assoc. Prof. Dr. Wong Fai Chu (Supervisor) from the Department of Chemical Science, Faculty of Science, and Assoc. Prof. Dr. Sim Kooi Mow (Co-supervisor) from Department of Chemical Science, Faculty of Science.

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DECLARATION

I Yong Ann Li hereby declare that the dissertation 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.

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(YONG ANN LI)

Date _____

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
$AlCl_3.6H_2O$	Aluminum chloride hexahydrate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BPB	Bromophenol blue
CFU	Colony forming unit
DHF	Dihydrofolate
DNA	Deoxyribonucleic acid
DPPH	1, 1-diphenyl-2-picrylhydrazyl
DTT	Dithiothreitol
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic acid
EF-G	Elongation factor G
EF-Tu	Elongation factor thermo unstable
EOs	Essential Oils
$FeSO_4$	Iron (II) sulphate
FIC	Ferrous ion chelating
GABA	ɣ-aminobutyric acid
GAD	Glutamate decarboxylase
GC-MS	Gas chromatography-mass spectrometry
GTE	Green tea extract
GTP	Guanosine triphosphate

H-NS	Histone-like nucleoid-structuring
HPLC	High performance liquid chromatography
INT	*p*-iodonitrotetrazolium chloride
MalK	Maltose transporter
MBC	Minimum bactericidal concentration
MDR-TB	Multidrug-resistant *Tuberculosis*
MHB	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
*m*RNA	Messenger ribonucleic acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
NaOH	Sodium hydroxide
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance
NO	Nitric oxide
OmpA	Outer membrane protein A
PABA	*p*-aminobenzoic acid
PBS	Phosphate buffer saline
PLP	Pyridoxal 50-phosphate
PRSP	Penicillin-resistant *Streptococcus pneumoniae*
QE	Quercetin equivalents
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPM	Revolutions per minute

SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
SspA	Stringent starvation protein A
TEM	Transmission electron microscope
TFC	Total flavonoid contents
THF	Tetrahydrofolic acid
TLC	Thin-layer chromatography
tRNA	Transfer ribonucleic acid
VRE	Vancomycin resistant *Enterococcus*
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Plants as an Important Source of Medicine

The utilization of plants as a source of preventive and curative traditional medicine preparation for human beings has been indispensable since ancient time (Lulekal et al., 2008). The earliest recordings of the use of plants as medicine can be found in Sanskrit texts written between 4500 and 1600 BC (Rokaya et al., 2010). Phytochemicals are natural products which are considered an important source of medicine and most of the drugs found today are derived from them (Tringali, 2001).

There has been a wide range of plants discovered containing beneficial and medicinal properties as it represents some of the most abundant reservoirs for drug discovery (Newman and Cragg, 2007). Tropical rainforest such as the one found in Malaysia has been estimated to holds more than half of the world flora collection and many of these plants therapeutic potentials remain unbroached (Taylor, 2004).

More than 60% of the world's population still depends on traditional medicine for treatment of common illness. Drugs derived from plants remain till today as an important source in many developing countries to combat severe

diseases (Hasson et al., 2011). Using plants in developing medicines is a cheaper alternative compared to modern medicine (Otshudi et al., 2000). In present time, a renewed interest in complementary and alternative medicine has encouraged the incorporation of plants origin into the production of modern drugs.

Most aromatic plants have been utilized as spice and condiments to add aroma but only recently that scientific research has paid more attention on their essential oils (EOs) and its extracts as natural sources of antioxidant and antimicrobial compounds. The antioxidant and antibacterial activities of these substances were both demonstrated *in vitro* and *in vivo* studies (Viuda-Martos, 2011).

In this project, the plants were chosen due to its medicinal value and bioactivity. *Melastoma candidum* is a medicinal plant used traditionally to eliminate stasis; toxin cleansing and recent research have also revealed to have broad spectrum antibacterial activities (Wang and Huang, 2005). *Callicarpa formosana* is a small flowering plant which has been reported to be used for treatments in external bleeding, rheumatism and fever (Jones and Kinghorn, 2008). *Scutellaria barbata* is a herb which has been used traditionally in Korea to treat inflammation and used as an anti-tumor agent (Suh et al., 2007). *Clinacanthus nutans* has been reported for treating herpes and virus infection in Thailand (Sangkitporn et al., 1995). *Vernonia amygdalina* was used in the treatment of bacterial infection (Farombi and Owoeye, 2011).

However, the exact mechanism behind the bacterial inhibition of these plant extracts remained to be demonstrated. It remains unclear how these antibacterial plants work or which bacterial pathway or enzymes are being targeted. Additionally, there is still no information available concerning their effects on the structural integrity of bacterial cell membranes. With the increasing rise of multi-drug resistant bacteria strains, the world is currently facing an urgent need to find new antibiotic derivatives (Gardete and Tomasz, 2014). Accumulated evidences are pointing toward the effectiveness of plant-derived compounds in inhibiting multi-drug resistant bacterial strains.

1.2 Objectives

There are a total of five objectives in this project and these objectives are as listed below.

1.2.1 To study medicinal plants for their antioxidation and antibacterial activities (Minimum inhibitory concentration assay)

1.2.2 To perform phytochemical analysis on medicinal plant extracts using a combination of bioautography and GC-MS.

1.2.3 To identify the differentially expressed bacterial proteins when exposed to antibacterial plant extracts

1.2.4 To determine the morphological changes on bacterial cell surface when treated with antibacterial plant extracts

1.2.5 To highlight identified antibacterial protein targets with potential therapeutic applications

CHAPTER 2

LITERATURE REVIEW

2.1 Antioxidants Present In Plants

All over the world, medicinal herbs have long been used by different ethnic groups for thousands of years for therapeutic or health-promoting purposes. *Panax ginseng* has been used for many years to enhance vital energy and to maintain homeostasis of the body, particularly in Asian countries. It is traditionally used as a therapeutic medicine. Nonetheless, the plant is now normally used as a supplement by the healthy or sub-healthy population. The antioxidative effects of *P. ginseng* have been detected in animal studies (Kim et al., 2008; Zhang et al., 2008). Many of these medicinal herbs may exert their beneficial properties, partly by eliminating excess free radicals like reactive oxygen and nitrogen species from our bodies (Hou, 2003; Lipinski, 2011).

Plants are the main sources of antioxidants, comprising of different phenolic compounds such as flavonoids and several classes of non-flavonoids such as phenolic acids, stilbenes and terpenoids. The variation of the structure and position of phenolic compounds lead to the creation of different kinds of antioxidants (Tsai et al., 2008). Majority of antioxidants are comprised of phenolic compounds which are capable of reducing oxidative stress produced by scavenging free radical species (Erkan, 2012). Oxidative damages induced by free

radicals are known to be involved in the onset of many chronic and degenerative diseases such as cancer, diabetes, cardiovascular, neurodegenerative and aging. They are the main contributor to DNA mutations, inactivation of proteins, lipid peroxidation, cell apoptosis or abnormal proliferation (Garcia-Nebot et al., 2014).

Free radicals are generated through normal body metabolism or by ingestion of foreign chemicals and pollutants. Free radicals are atoms with unpaired electrons and because of their reactive nature, these radicals can cause oxidative damage on different cellular compartments, including cell membranes and DNA. Numerous chronic diseases including neurodegenerative and cardiovascular diseases may be caused by intracellular oxidative damage by reactive oxygen species (ROS) (Ju et al., 2012).

Formation of cancers have been linked to accumulated mutations due to oxidative stress by free radicals (Chahar et al., 2011). It has also been suggested that the aging of one's cells is due to oxidative stress cascades, including attack from ROS and deficiency in intracellular anti-oxidative defense (Harman, 2006). Elimination of excess free radicals in our body may prove to be very beneficial to the overall of one's well-being. However, antioxidants are substances which are capable of scavenging these free radicals and protecting the cells from oxidative damage (Gan et al., 2010).

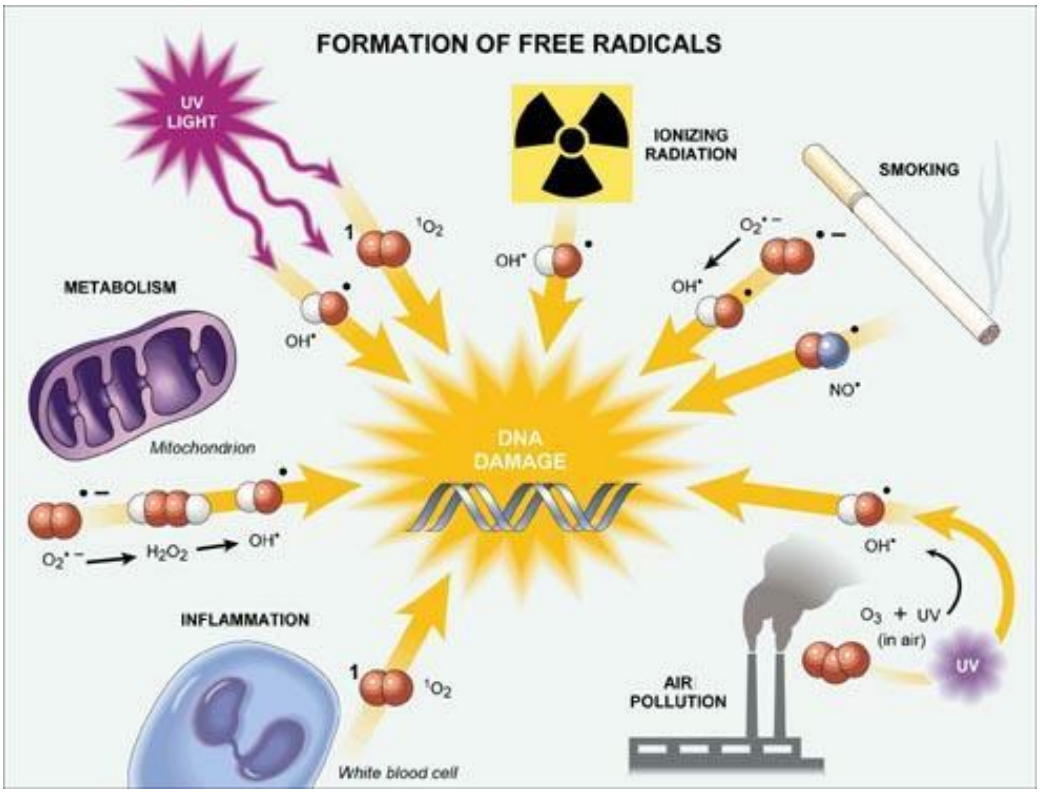


Figure 2.1: Generation of Free-Radicals from Various Scenarios Damage DNA.

Plants are considered reservoir of phytochemical compounds and are considered good sources of antioxidants and radical scavengers (Gescher et al., 1998; Moukette et al., 2015). Medicinal plants are especially rich in various secondary metabolites, including polyphenols and flavonoids, which are capable of eliminating free radicals (Ren et al., 2003). Experiments done *in vitro* and *in vivo* have shown that plants-derived compounds are capable of neutralizing free radicals (Etsuo, 2010; Park et al., 2010; Costa et al., 2013)

2.2 Plant as Antimicrobial Sources

Plants are known to contain antimicrobial substances and these substances have been studied thoroughly to achieve higher levels of food security. For many years, plants have been utilized in traditional medicine for treating different type of diseases (Sakunpak and Panichayupakaranant, 2012). Compound such as quercetin founds in onion, grapes and apple are known to be antioxidant and antibacterial activity (Prasad et al., 2014; Nishimuro et al., 2015; Kobori et al., 2015).

Natural sources have become a valued asset as it is important in drug development in the pharmaceutical industry. The major issue faced by pharmaceutical industry nowadays is to develop an antibiotic which is effective against wider range of bacteria and have minimal side-effect on mankind. Most antibiotics developed have become obsolete and because of the progression of some drug resistant bacteria, new drugs have to be sought out and for which herbal treatment is one possible solution to treat diseases caused by multi-drug resistant bacteria (Ruban and Gajalakshmi, 2012).

There have been several cases of multidrug-resistant bacteria reported worldwide today such as Vancomycin resistant *Enterococcus* (VRE), Methicillin resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant *Tuberculosis* (MDR-TB) (Sharma and Mohan, 2004; Engel, 2010; Stix, 2006). Recently, in

European hospitals and globally, bacteria which are totally or almost resistant to the current available antibiotics have become a threatening situation as treatment options for infected patients are remarkably limited (Freire-Moran et al., 2011).

Unnecessary and over-prescribing of antibiotics lead to the evolution of these pathogenic bacteria to adapt to currently available antimicrobial drugs. If this trend continues, the world may face an urgent need to develop a more powerful antibiotic to combat against these multidrug-resistant bacteria. In recent years, World Health Organization (WHO) supports the use of traditional medicine and has considered it as safe remedies for diseases of both microbial and non-microbial origins (WHO, 2012).

Only recently there was an increase of interest in investigating the healing powers of plants, owing to their diverse applications (Baris et al., 2006). For example, oregano oil was shown to have antimicrobial activities and the substance carvacol was shown to be responsible for the antibacterial activity (Smith-Palmer et al., 1998; Soković et al., 2010). Medicinal plants have become very important materials for research in pharmacology and drug development as the constituents in plant can be used directly as therapeutic agents and also the starting material for the synthesis of synthetic drugs.

The limitless abilities of plants to synthesize aromatic secondary metabolites make them a rich source of bio-resources for drugs discoveries. These

metabolites which include coumarins, phenols, phenolic acids, flavonols, flavones, flavonoids, quinones, and tannins show great antimicrobial activities and serve as a protector against pathogenic microorganisms (Das et al., 2010). Bioactive phytochemicals such as phenolic acids consisted of a single substituted phenolic ring. The presence of various hydroxyl groups in the phenolic compounds make it toxic towards microorganisms (Scalbert, 1991; Urs et al., 1975; Wang et al., 2013).

Flavonoids are also one of plant secondary metabolites. Flavones and flavonols are a class of flavonoids known as anthoxanthins. They have phenolic structures with one carbonyl group and are synthesized by plants when microbial infection occurs (Dixon et al., 1983). Flavonoids are found to be effective against microorganisms as an antimicrobial substance (Bennet and Wallsgrove, 1994; Okoth et al., 2013).

Tannins are polymeric phenolic substances which are soluble in water, alcohol and acetone. Tannins from chestnuts have been shown to be bactericidal towards *Clostridium perfringens* (Ana et al., 2010). Coumarin is another type of phenolic compounds, made of fused benzene and α-pyrone rings and several of these coumarins have been reported with antimicrobial properties (O'Kennedy and Thornes, 1997). The first antibacterial properties of coumarins were discovered by Goth et al. (1945) and it was found to be able to inhibit the growth of several bacterial strains.

Table 2.1: List of Plants with Antimicrobial Activity (Cowan, 1999).

Common name	Scientific name	Compound	Class	Activity
Aloe	*Aloe barbadensis*, *Aloe vera*	Latex	Complex mixture	*Corynebacterium*, *Salmonella*, *Streptococcus*
Apple	*Malus sylvestris*	Phloretin	Flavonoid derivative	General
Basil	*Ocimum basilicum*	Essential oils	Terpenoids	*Salmonella*, bacteria
Bay	*Laurus nobilis*	Essential oils	Terpenoids	Bacteria, fungi
Betel pepper	*Piper betel*	Catechols, eugenol	Essential oils	General
Black pepper	*Piper nigrum*	Piperine	Alkaloid	Fungi, *Lactobacillus*, *Micrococcus*, *E. coli*, *E. faecalis*
Blueberry	*Vaccinium* spp.	Fructose	Monosaccharide	*E. coli*
Buchu	*Barosma setulina*	Essential oil	Terpenoid	General
Buttercup	*Ranunculus bulbosus*	Protoanemonin	Lactone	General
Ceylon cinnamon	*Cinnamomum verum*	Essential oils, others	Terpenoids, tannins	General
Chili peppers, paprika	*Capsicum annuum*	Capsaicin	Terpenoid	Bacteria
Clove	*Syzygium aromaticum*	Eugenol	Terpenoid	General
Fava bean	*Vicia faba*	Fabatin	Thionin	Bacteria
Garlic	*Allium sativum*	Allicin, ajoene	Sulfoxide	General
Henna	*Lawsonia inermis*	Gallic acid	Phenolic	*Staphylococcus aureus*
Olive oil	*Olea europaea*	Hexanal	Aldehyde	General
Onion	*Allium cepa*	Allicin	Sulfoxide	Bacteria, *Candida*
Papaya	*Carica papaya*	Latex	Mix of terpenoids, organic acids, alkaloids	General
Peppermint	*Mentha piperita*	Menthol	Terpenoid	General
Periwinkle	*Vinca minor*	Reserpine	Alkaloid	General
Quinine	*Cinchona* sp.	Quinine	Alkaloid	*Plasmodium* spp.
Turmeric	*Curcuma longa*	Curcumin	Terpenoids	Bacteria, protozoa

Ruban and Gajalakshmi (2012) have investigated *Hibiscus rosa-sinensis* flower extract against human pathogens on its antibacterial activity and the flower extracts have proven to have antimicrobial activity. The inhibitions of bacterial growth were due to the presence of active compounds found in the flower extracts. It has been proposed that these compounds might have acted alone or in combination to inhibit the growth of bacteria. The crude extracts contain various

different kinds of organic compounds such as flavonoids, tannins, alkaloids and triterpenoids. These compounds are all known for its antibacterial effects. Tannins and flavonoids are especially known to be effective antimicrobial agents against a wide array of microorganisms. This may be due to their ability to couple with the extracellular and soluble proteins and with the bacterial cell wall.

The assessment of antimicrobial agent from plant are done through biological evaluation of the extract of the plant in order to ensure efficacy and safety which is then followed by identifying the active principles, the dosage, efficacy, formulation and pharmacokinetic profile of the new drug (Das et al., 2010). Currently, the available screening methods in detecting antimicrobial activities of plant-based products are thin-layer chromatography-bioautographic, diffusion, and microbroth dilution methods. Techniques such as bioautographic and diffusion methods are considered as qualitative techniques because these methods only allow one to know about the presence or absence of substances which has antimicrobial activity. Dilution methods however are known as quantitative techniques due to their method which allow the determination of the minimal inhibitory concentration (Cowan, 1999).

2.3 Antibacterial Study

2.3.1 Minimum Inhibitory Concentration (MIC)

MIC enables us a quantitative measure of antimicrobial activity. This is done by diluting the antimicrobial agents into a series of different concentrations.

The MIC is determined as the lowest concentration of antimicrobial agent which is able to inhibit the growth of microorganism *in vitro*. This data obtained is important in determining the optimal dosage required, in addition, the correct administration route of the antimicrobial agent in therapy.

With the use of microtiter plate or broth microdilution method, MIC can be easily determined for large number of test samples. The use of MIC in diagnostic laboratories is important and useful to confirm resistant of microorganisms to an antimicrobial agent, and it allows the monitoring activities of new antimicrobial agents. In the application of microtiter plate method on medicinal plants, a stock solution of the plant extract is obtained through solvent extraction (Grierson and Afolayan, 1999). The extract concentration will then undergo serial dilution in order to determine the minimum concentration which is able to inhibit a certain amount of bacteria.

2.3.2 Time-Kill Study

A time-kill study is done to determine the killing rate of a bacterial isolate by antimicrobial agent. This study has been widely applied in the development and evaluation of new drug (NCCLS, 1999). A time-kill study gave a more accurate understanding of the antimicrobial agents than MIC (Kurochkina et al., 2012). Most studies have shown that time-kill study technique has better correlation with cure than measured by minimum bactericidal concentration

(MBC) as MBC has shown to have poor correlation of bactericidal activity with cure (Bayer and Lam, 1985).

Time-kill study is also one of the most reliable ways to determine the tolerance of the test subjects (Tuomanen, 1996). It is also a useful method in determining whether two or more antimicrobial agents are in synergy or antagonist against each other (King et al., 1981; Chalkley and Koornhoff, 1985). Time-kill study correlates better with cure in animal models. The time-kill study which is done in different concentrations of a single antimicrobial agent allowed one to compare the rates of decline in the microbial count. Knowing the rate of kill might be more important than knowing the concentration in which 99.9% killing of the final inoculum. Using several concentration of the MIC is recommended in conducting the time-kill study when assessing an antimicrobial agent. At different intervals, mostly at 0, 4, 8, 10 to 12, and 24 hours of incubation, the surviving colonies are counted and then charted onto a graph. The result may be represented in $\log_{10}$ CFU/ml (NCCLS, 1999).

Over time, some of the colony counts might increase after a certain decrease in surviving cells. Selection of resistant mutants, inactivation of the antimicrobial agent, or regrowth of susceptible bacterial cells which have escaped the antimicrobial activity by adhering to the wall of the culture vessel might be the reason behind the regrowth (Layte et al., 1983; Taylor et al., 1983). The importance of such regrowth still remains unclear. There are several factors which

should be taken note of which is, the time whereby the regrowth occurs, the dosage of antibiotic in a clinical settings and the type of bacteria and antibiotic used.

In time-kill study, it is possible to determine whether an antimicrobial agent is bacteriostatic or bactericidal. The term bacteriostatic and bactericidal are pretty straightforward whereby bacteriostatic means that the particular antimicrobial agent only prevents the growth of bacteria by keeping it in the stationary phase while bactericidal means that the agent kills the bacteria (Pankey and Sabbath, 2004). To determine the bactericidal rate, linear regression analysis is used on the resultant curve which is defined as the slope of the regression line with units being the change in the $\log_{10}$ CFU/ml per hour of exposure to the agent.

In a time-kill study, bactericidal activity is designated as 99.9% killing of the final inoculum or a reduction of being equal to $3\log_{10}$ CFU/ml or greater in the viable colony count compared to initial inoculum; whereas bacteriostatic is defined as reduction of less than $3\log_{10}$ CFU/ml (NCCLS, 1999).

Valuable information can be obtained on the potential action of antimicrobial agents *in vitro* by distinguishing whether agents are bacteriostatic or bactericidal. In some instances, bacteriostatic action is more effective than bactericidal action in the treatment of meningitis caused by *Streptococcus pneumonia*. Rapid death of the microorganism by action of bactericidal antibacterial agent may intensify the white blood cell response and prostaglandin

release resulting in cerebral edema and high mortality rate (Pankey and Sabath, 2004).

2.4 Mechanisms of Action of Antibacterial Agents

The creation and discovery of antibiotic is one of the most significant medical achievements known to mankind, as it has greatly crippled and fought off bacterial infections that are frequently severe and lethal. However, due to the incessant and extensive use of antibiotics, antibiotic resistance bacteria cases began to increase, partly due to the strong selective pressure on bacteria. The evolution of antibiotic resistance pathogenic microorganisms such as Methicillin-resistant *Staphylococcus aureus* (MRSA), Penicillin-resistant *Streptococcus pneumoniae* (PRSP) and Vancomycin-resistant *Enterococcus* (VRE) are a major concern to the public health worldwide (Silva et al., 2005).

This poses a serious threat to the treatment of certain bacterial infection and new approaches are needed in the development of the next generation of antibiotics. Researchers nowadays have taken the path of screening and investigating plant materials and their isolated compounds for new antibacterial compounds. However, the examination of bacterial ribonucleic acid (RNA) and protein expression levels on a genome wide scale may prove to be an important tool for drug discovery research (Palzkill, 2001). Potential antibacterial targets were selected from important bacterial pathways such as the bacterial pilus

biosynthesis pathway, protein secretion pathway, cell wall anchoring machinery and protein translational pathway (Bleves et al., 2010; Jones and Hrub, 1998; Stephens and Shapiro, 1997). The major issue faced by pharmaceutical industry nowadays is to develop an antibiotic which is effective against wider range of bacteria and have minimal side-effect on mankind.

The current available antibacterial drugs exert their bacterial inhibiting activities via five major mechanisms which are inhibition of the cell wall synthesis, inhibition of the cell membrane function, inhibition in the protein synthesis pathway, inhibition in the nucleic acid synthesis pathway and the inhibition of essential metabolic pathways (Nester et al., 2004).

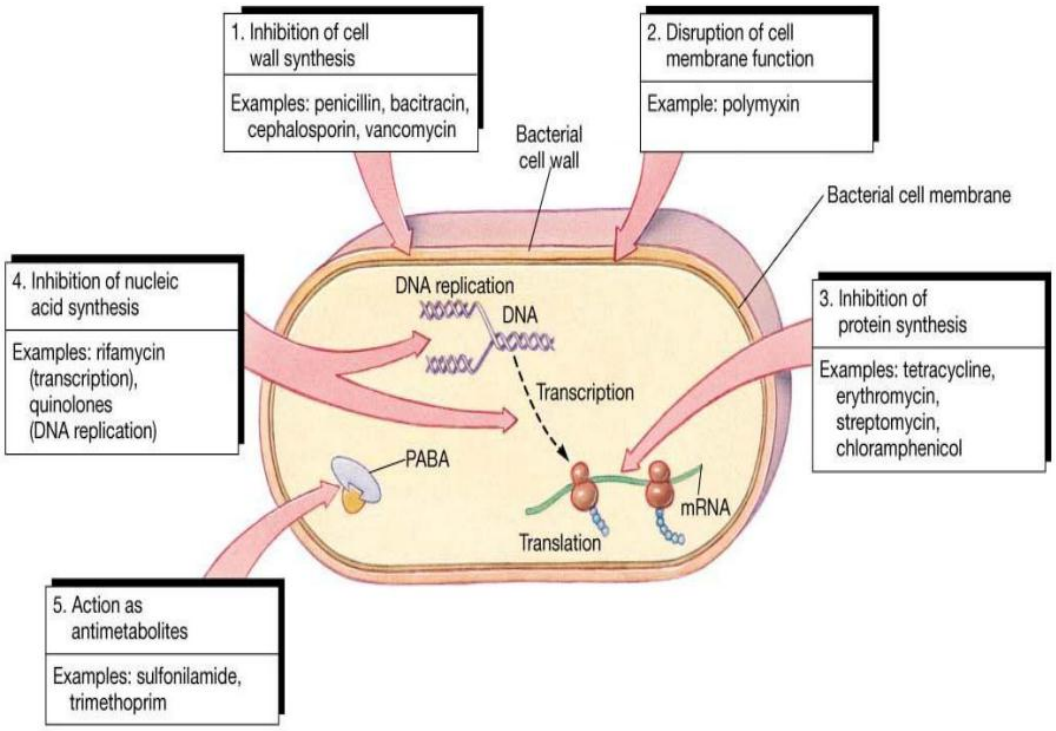


Figure 2.2: Mechanism of Antibacterial Agents.

2.4.1 Inhibition of Cell Wall Synthesis

A number of antimicrobial agents used clinically nowadays mostly target bacterial cell wall synthesis. Example includes beta-lactam based antibiotics. This targeted mechanism is mainly exploited for antimicrobial development. The components involved in the bacterial cell wall synthesis are interesting antimicrobial targets due to the absence of these counterparts in human biology, thus creating intrinsic target selectivity. The bacterial cell wall synthesis includes the synthesis of *N*-acetyl muramic acid (M) linked to *N*-acetyl glucosamines (G), along with an attached pentapeptide (P) side chain collectively referred to as MGP subunits (Palzkill, 2001). D-alanyl-D-alanine, which beta lactam antibiotics are stereochemically related to, is a substrate required for the last step in the synthesis of peptidoglycan. Antibiotic such as penicillin binds and inhibits the carboxypeptidase and transpeptidase enzymes which are needed in the peptidoglycan biosynthesis.

Glycopeptides such as vancomycin are able to inhibit both transglycosylation and transpeptidation during the assembly of peptidoglycan. It binds to the muropeptide subunit when it is transferred out of the cell cytoplasm and inhibits subsequent polymerization. However, this antibiotic is not effective against Gram-negative bacteria due to its inability to penetrate their outer membrane. Thus far, vancomycin has been important for the treatment of infection by resistant *Staphylococcus aureus* strains such as MRSA (Todar, 2008).

2.4.2 Inhibition of Cell Membrane Function

Another mechanism utilized by antimicrobial agents is the inhibition of cell membrane functions. This type of antimicrobial agent disorganizes the structure and inhibits the function of bacterial membranes. Maintaining the integrity of the cytoplasmic and outer membrane is vital to the bacteria survival. Once the membrane stability is shaken, it will rapidly kill the cell. However, this course of action is not selective enough to only target bacterial cells, due to the fact that the bacterial and eukaryotic membranes are nearly identical in their phospholipids.

Polymyxin is the only antibiotic which acts by this mechanism and it is produced by *Bacillus polymyxa* (Storm et al., 1977). This antibiotic is effective against Gram-negative bacteria and it is only limited to topical use. It binds to membrane phospholipids and interferes with the function of bacterial membrane. Polymyxin is normally prescribed to patient with urinary tract infections caused by the bacteria strain, *Pseudomonas*, which are resistant to carbenicillin, gentamicin and tobramycin. This drug is given under close supervision in the hospital due to their potential damage to kidney and other organs (Levin et al., 1999).

2.4.3 Inhibition of Protein Synthesis

Some therapeutically useful antibiotics are able to inhibit some of the step in the complex process of bacterial protein synthesis. Most of these antibiotics target around the events occurring on the ribosome, instead of amino acid attachment on to the tRNA. Many of this class of antibiotics have an affinity or specificity towards 70S as opposed to 80S ribosomes, thus they are considered selectively toxic. Some examples of antibiotics with this mode of action are chloramphenicol, tetracycline, the macrolides and the aminoglycosides (Todar, 2008).

Aminoglycosides such as streptomycin, kanamycin, tobramycin and gentamicin are derived from *Streptomyces* species. Streptomycin has been used substantially as a primary drug in treating tuberculosis. Among them, this type of antibiotic exerts their antimicrobial activities by binding to bacterial ribosomes and stopping the initiation of protein synthesis. The antibiotic streptomycin binds to the 30S subunit of the bacterial ribosomes, specifically to the S12 protein where it has been shown to inhibit the initiation of protein synthesis, through preventing the binding of initiator N-formylmethionine tRNA to the ribosome. It had been shown to prevent the normal dissociation of ribosomes into their subunits thus only the 70S remains, which prevents the formation of polysomes. Thus, these antibiotics act by disrupting the functional formation of ribosomes so that it may no longer able to carry out its normal protein synthesis function (Tenson et al., 2003).

2.4.4 Inhibition of Nucleic Acid Synthesis

Some antibiotics exert their antimicrobial effects by interfering with the synthesis of DNA, resulting in the prevention of growth of cells. However, these antibiotics are not selective and can affect both mammalian cells and bacterial cells alike. Nonetheless, there are two nucleic acid inhibitors created which have selective activities against prokaryotes only and these are quinolones and rifamycins.

Nalidixic acid is a compound belonging to quinolones group. It is a synthetic therapeutic agent that has antibacterial activity mainly on Gram-negative bacteria. It has a bactericidal effect by binding to DNA gyrase, a bacterial enzyme which is essential for the replication and supercoil formation of DNA. DNA gyrase was the first target identified in quinolones screening, during genetic studies with mutant *Escherichia coli* that had mutation mapping in the gyrA (Hooper, 2001).

2.4.5 Inhibition of Metabolic Pathways

Some of these synthetic antimicrobial agents are competitive inhibitors, with structure mimicking essential metabolites or growth factors which are critical in the bacterial metabolic pathway. Thus these types of antimicrobial agents are sometimes known as anti-metabolites due to their natures to specifically binds and

inhibit essential metabolic pathways in pathogenic bacteria. Structurally, competitive inhibitors are nearly identical to a bacterial growth factor or metabolite; however, they are not able to fulfill the metabolic functions in the cell. Some of these antimicrobial agents are either bacteriostatic or bactericidal.

The sulfonamides such as gantrisin and trimethoprim are structurally similar to *p*-aminobenzoic acid (PABA) as shown in Figure 2.3. Due to their similarity, sulfonamides are able to interfere with the utilization of said substrate when it competes with it for its enzyme. Sulfonamides are also known to inhibit tetrahydrofolic acid (THF) which is needed for 1-carbon transfer reaction. Trimethoprim and dihydrofolate (DHF) are similar in structure and it inhibits the second step in THF synthesis which was mediated by DHF reductase. Since animal cells do not construct their own folic acid, thus they are not affected by these antimicrobial agents.

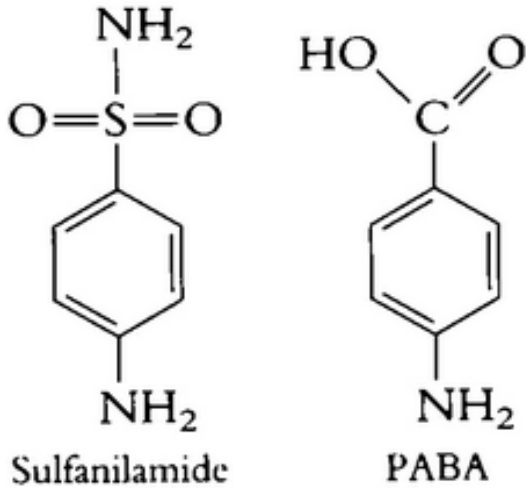


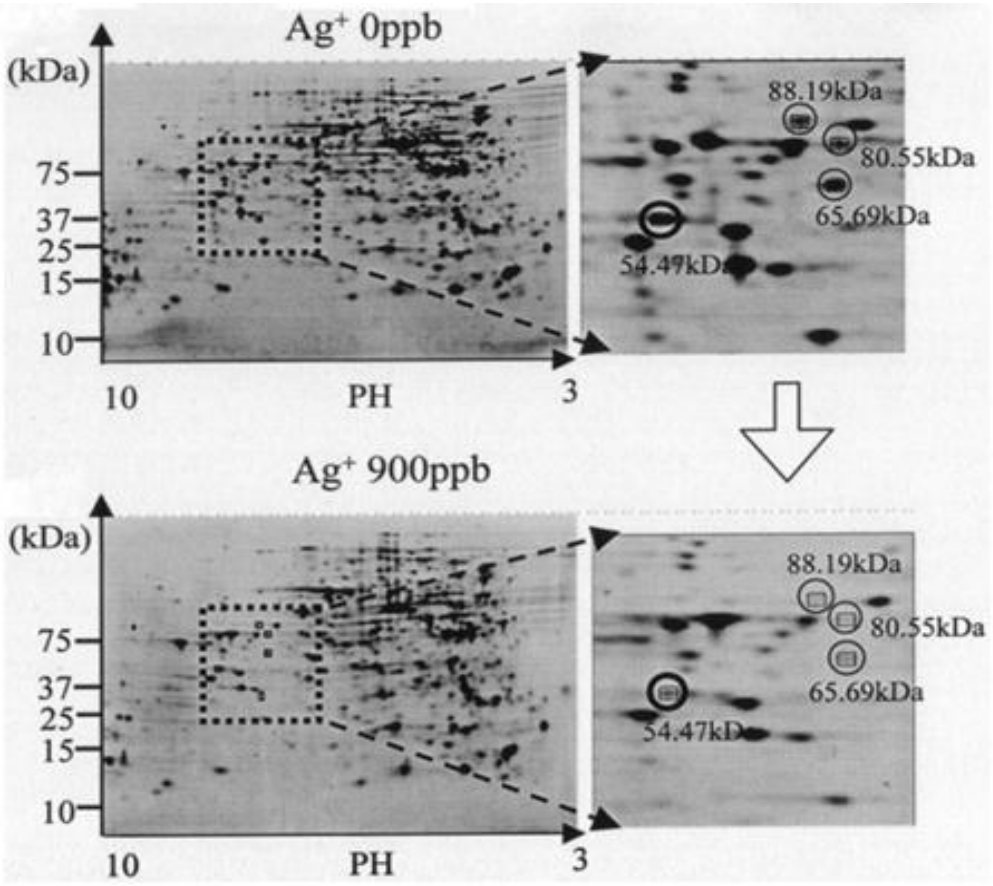
Figure 2.3: Structures of Sulfanilamide and *p*-aminobenzoic acid (PABA).

Through understanding the mode of action taken by antibiotic to target bacterial cells, new drugs can be synthesized to combat bacterial cells efficiently and thoroughly. Thus, researchers nowadays have gone into researching the mechanism behind some antimicrobial agents such as silver. Silver has been known to have antimicrobial activities for centuries but only recently that the understanding behind the mechanism by which it inhibits important bacterial metabolic pathway is known. It was thought that silver atom binds to the thiol group (-SH) in enzymes which subsequently caused the deactivation of the enzymes. In the cell membrane, silver is able to form stable S-Ag bonds with thiol-containing compounds which is involved in the transmembrane energy generation and ion transport (Klueh et al., 2000).

Silver was believed to be able to take part in the catalytic oxidation reactions that form the disulfide bonds. This occurs when silver catalyzed the reaction between oxygen molecule in the cells and hydrogen atoms of thiol groups. These formations of disulfide bonds are probably able to alter the shape of cellular enzymes and influence their function. The changed shapes of protein are able to inactivate key enzymes such as those required in cellular respiration (Davies and Etris, 1997).

Yamanaka et al. (2005) have reported that silver caused differentially expressed proteins in bacteria cells. There were four enzymes which were identified as protein with decreased expression when the cells were treated with

900 ppb of Ag+ solution and these enzymes were 30S ribosomal subunit protein, succinyl coenzyme A synthetase, maltose transporter (MalK) and fructose bisphosphate aldolase as seen in Figure 2.4.



Description	M_r	Accession no. and database	Probability[a]
30S ribosomal subunit protein S2	54.47	26246115; NCBI	69
Succinyl-CoA synthetase	65.69	6980733; NCBI	64
Maltose transporter	88.19	37927753; NCBI	89
Hypothetical protein	80.55	26248233; NCBI	47
Fructose-bisphosphate adolase	36.33	P71295; SwissProt	121

[a] Probabilities were based on the Mowse scoring algorithm.

Figure 2.4: Protein expression is down-regulated when bacteria are exposed to Ag^+ (Yamanaka et al., 2005).

The silver ions were hypothesized to have bound to the 30S ribosomal subunit thus deactivating the ribosome complex required in the translation of proteins. These proteins which have been found to be down regulated upon treatment with silver serve an important function within the cell (Yamanaka et al., 2005). Succinyl-coenzyme A synthetase is an enzyme which were involved in the TCA cycle whereby it catalyzed the conversion of succinyl-CoA to succinate while phosphorylating ADP to produce ATP. Fructose bisphosphate aldolase is an enzyme responsible in the reaction taking place in glycolysis where it is involved in the breaking down of fructose-1, 6-bisphosphate into glyceraldehyde 3-phosphate and dihydroxyacteone phosphate (Slonczewski and Foster, 2009). MalK is a cytoplasmic membrane-associated protein which is involved in the transport of maltose (Bavoil et al., 1980). All of these proteins identified have a major role in energy and ATP production in the cell and thus the decreased expression of any of these proteins can lead to the death of cells (Yamanaka et al., 2005).

Another study was done by Nakayama et al. (2012) by using antibacterial green tea extract (GTE) to see the differentially expressed protein in *S. aureus* and *E. coli*. SDS-PAGE was used to view the proteins from the treated and non-treated bacteria which are shown in Figure 2.5. It was found that once treated with the green tea extract, the protein bands in the GTE-treated lane (Lane 3 and 4) have observed a decreased amount of protein in both bacteria.

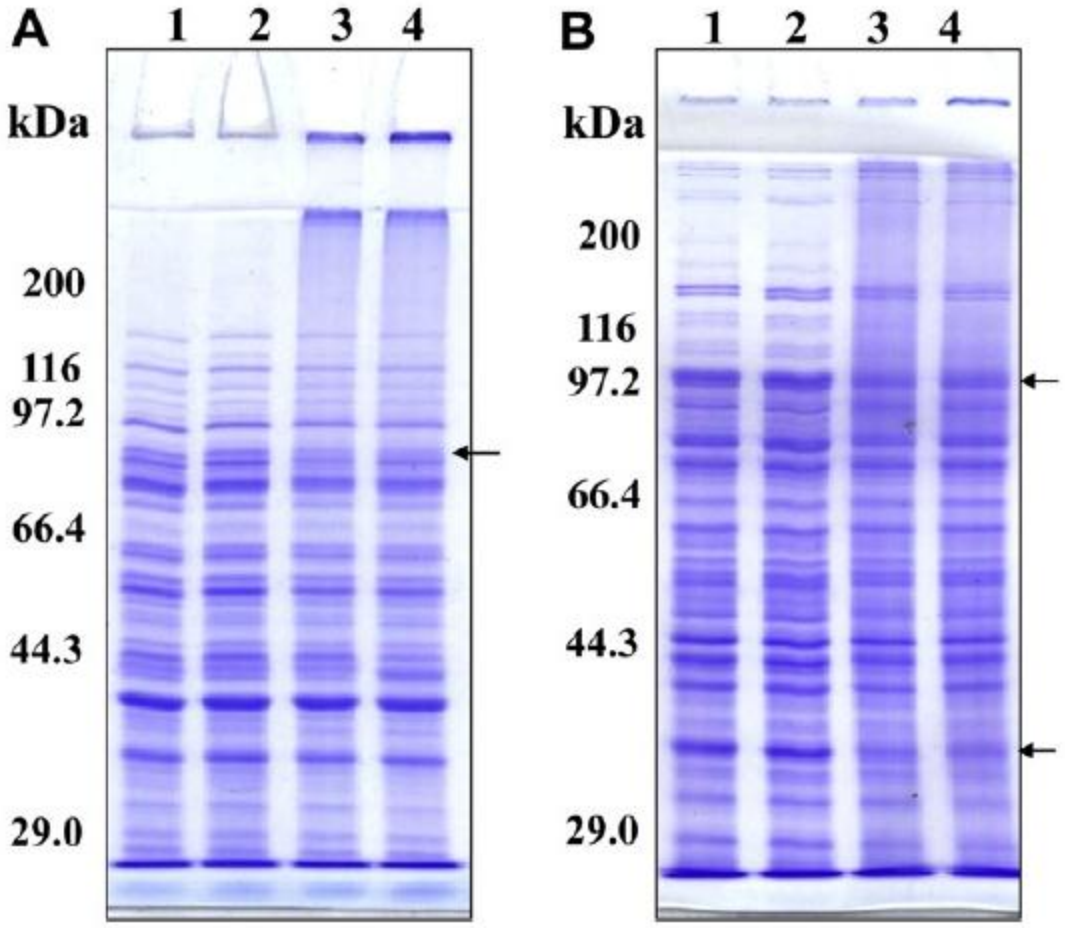


Figure 2.5: SDS-PAGE profiles of cells treated with green tea extract (GTE) and/or NaCl. (A) Untreated *S. aureus* NBRC 13276 (lane 1), treated with NaCl (lane 2), treated with GTE (lane 3), and treated with both NaCl and GTE (lane 4). (B) Untreated *E. coli* O157:H7 (lane 1), treated with NaCl (lane 2), treated with GTE (lane 3), and treated with both NaCl and GTE (lane 4) (Nakayama et al., 2012).

2.5 Bioautography Detection in Thin-Layer Chromatography

Bioautography is an analytical technique which is used to identify organic compounds with inhibition effects on microorganisms. This technique combined the use of thin-layer chromatography (TLC) to separate the compounds, followed

by application in antimicrobial testing. The procedure is identical to the agar diffusion methods (Meyers and Smith, 1964). This technique is used as bioassay-guided fractionation to detect antimicrobial compounds (Nostro et al., 2000; Schmourlo et al., 2004).

The first documented use of this technique was dated back in 1946 when Goodall and Levi (1946) used paper chromatography followed by bioautography to estimate the purity of penicillin. The improved thin layer chromatography-bioautography was introduced by Fisher and Lautner (1961) and Nicolaus et al. (1961). The method was described by them as simple, reproducible and highly sensitive. The most important point in performing bioautography might be the fact that it provides information regarding antimicrobial activities of substances separated from a mixture through planar chromatography. There are three categories of bioautography methods which are agar diffusion or contact bioautography, immersion or also known as agar-overlay bioautography and direct bioautography.

For contact bioautography, antimicrobial compounds diffuse onto an inoculated agar plate from a paper chromatography or thin layer chromatography (Narasimhachari and Ramachandran, 1967). The chromatogram is situated face down onto the inoculated agar for some time to allow diffusion of compounds to take place before the chromatogram is removed and the agar is incubated. Antimicrobial activities can be observed when inhibition spots are present on the

agar surface. The spots represent sites where antimicrobial compounds are diffused from the chromatogram onto the agar surface. However, contact bioautography has its disadvantage. The difficulty in obtaining a complete contact between the agar and the chromatogram may cause some loss in the transfer of active compounds onto the inoculated agar.

In immersion bioautography, the chromatogram will be placed onto a sterile agar plate where molten, seeded agar will cover it. Incubation is done once the agar solidified, and the inhibition zones are visualized with tetrazolium dye, as seen in Figure 2.6 (Harborne, 1998).

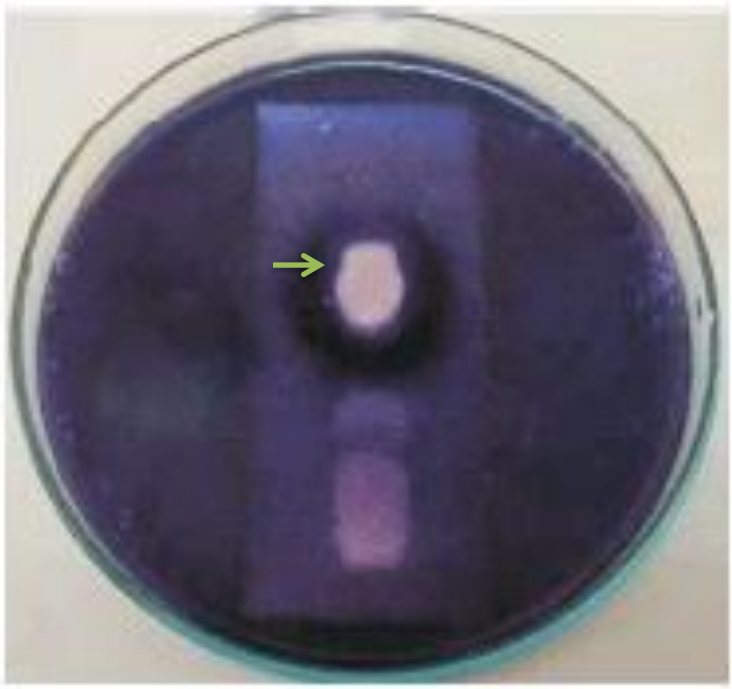


Figure 2.6: Immersion Bioautography Assay against *Staphylococcus aureus*. (Silva et al., 2005).

Immersion bioautography as shown in Figure 2.6 is a mixture of contact and direct bioautography. The antimicrobials compounds are transferred from the TLC plate to the agar layer similar to contact bioautography however the TLC plate stays on the agar plate during incubation and visualization of the agar layer as in direct bioautography. The only disadvantage of this method is the lower sensitivity caused by the dilution of antimicrobial compounds in the agar layer compared with direct bioautography. Contact and immersion bioautography are routinely used back in the 1960s however, direct bioautography has prevailed over them.

In direct bioautography, the developed TLC plate with the separated compounds is sprayed with a suspension of test bacteria. The inoculum used for the test varied with different authors. Meyer and Dilika (1996) has mentioned of using an inoculum of 0.84 absorbance at 560 nm while Schmourlo et al. (2004) has been reported to use a suspension of 10^6 CFU/ml. The bioautogram are then visualized with tetrazolium salts. A clear white zone against a coloured background on the TLC plate shows the antimicrobial activity of the extract (Silva et al., 2005).

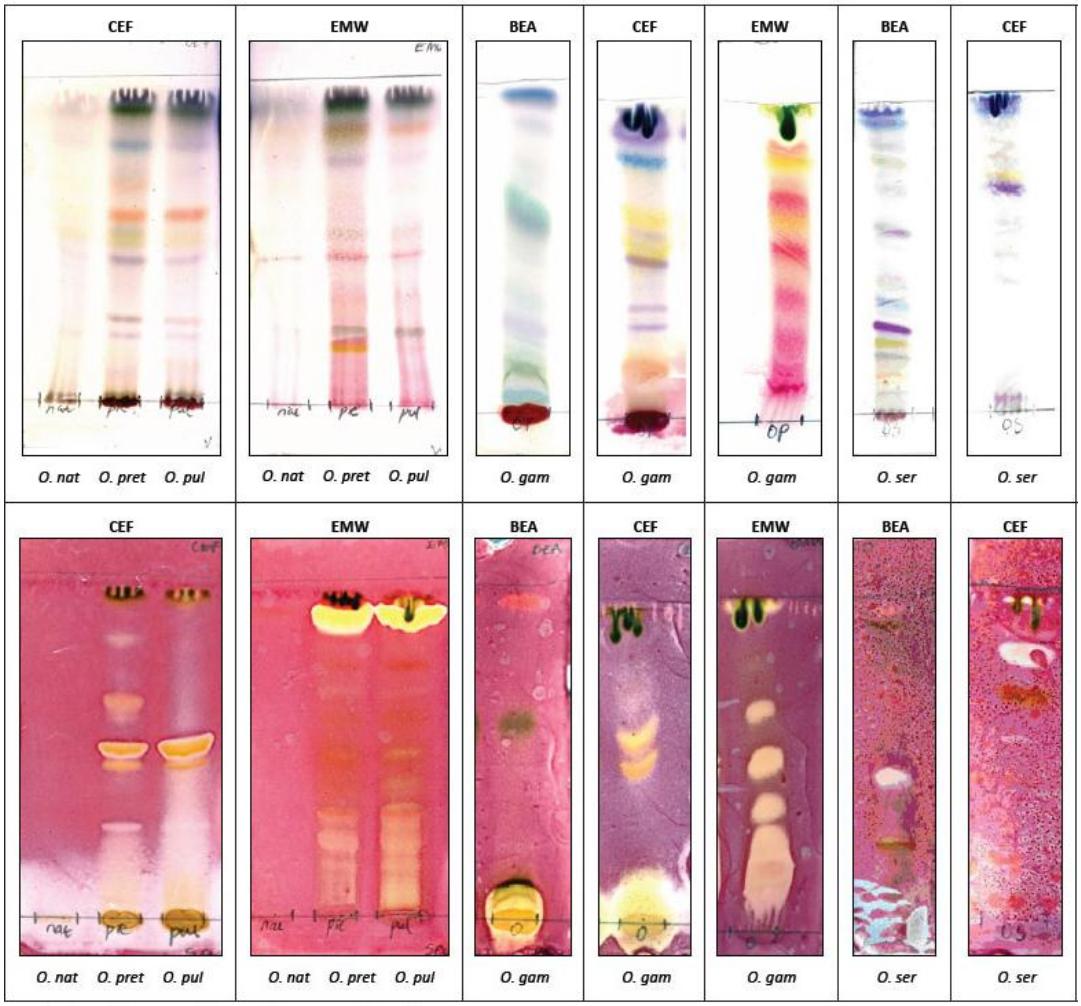


Figure 2.7: Developed Thin-Layer Chromatography Plate and Its Corresponding Bioautography (Makhafola and Eloff, 2012).

TLC-based bioautography is a crucial technique in identifying the localization of antimicrobial compounds present in the crude plant extracts. This technique has allowed easy screening and identification of antimicrobial compounds. As shown in Figure 2.7, the inhibition zones on the bioautogram can be easily compared to the chromatogram, to identify the exact band which shows antimicrobial activity (Makhafola and Eloff, 2012). From the band, the desired

active compounds can be scrapped and analyzed using gas chromatography and NMR.

2.6 Investigation of Morphological Changes of Bacteria Using Scanning Electron Microscope

The presence of bacteria can be found almost anywhere such as in the soil, water and also living on or inside other living organisms. Some of these bacteria are harmful and causes diseases in plants, animals and even humans. However, some bacteria are useful towards humans in the protection of health or assisting in the production of various materials in the industry. These microorganisms may yet have far reaching macroscopic consequences in the life of humans. The agriculture field has taken an interest in these microorganisms as they may have played a role in harming or improving the production of foods, either animal or plant which can easily affect human civilization.

These days, there is an increase in interest in studying bacteria in their natural environment to better understand them. Electron microscopy is one of the techniques which are suitable in showing bacteria in greater detail in their natural state. There are two types of electron microscopy which are scanning electron microscope (SEM) and transmission electron microscope (TEM). SEM is a simple, easy to use electron microscopy and the results are easily interpreted due to the specimens being shown as a three dimensional objects. TEM however

generates a different kind of image than SEM. In TEM, the electron beam passes through the specimen and creates a shadow-like image on a fluorescent screen.

SEM are useful in viewing the surface of microorganisms as it creates good images in the form of three dimensional structures and having a higher magnification power compared to traditional optical microscopes which only focuses on a small area and creating images of structure that are flat. Thus SEM is useful in viewing the integrity and cell surface of a microorganism. SEM has been employed in the study of how antimicrobial agents affect the cell surface of microorganism. In a study conducted by Kim et al. (2011), SEM was used to study the antibacterial activities of silver nanoparticles (Ag-NPs) with respect to Gram-positive *S. aureus*, by observing the morphological changes of treated bacterial cells compared to untreated cells as shown in Figure 2.8. In Figure 2.8 (A), the bacterial cells in control group retained its grape-shaped form whereas when treated with Ag-NPs [Figure 2.8(B)], many fragments were observed on the bacterial cell surface.

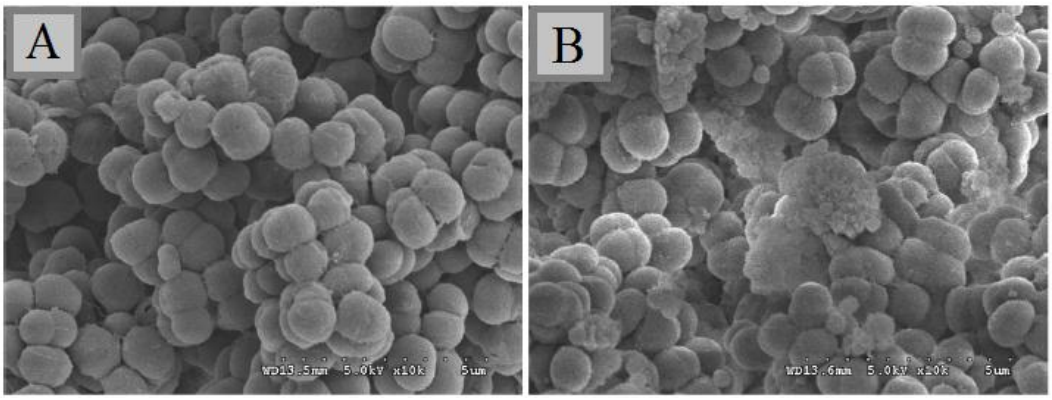


Figure 2.8: Scanning Electron Microscope Images of *S. aureus*. (A) Untreated *S. aureus*, (B) Treated *S. aureus* with Ag-NPs (Kim et al., 2011).

SEM operates through the use of a beam of electrons to project an image of extremely small structures. The electron beam is usually created by a cathode made of tungsten which is then accelerated in an electric field towards the cathode where the specimens to be viewed are located. Deflector plates are positioned in the SEM to scan the beam across the sample to be imaged. To achieve a proper creation, control and detection of the electron beam, the imaging of the sample are to be in a vacuum surrounding (Bacteria World, 2011).

Before the bacteria are subjected to viewing, the bacteria have to be fixed, dehydrated in ethanol, and then subjected to critical-point drying before mounting it onto an SEM stub and coated with a conductive material such as gold or titanium. Preservative such as glutaraldehyde is normally used to preserve biological tissues and it is usually used in preparation for a variety of imaging procedures. Glutaraldehyde is an alkylating agent which provides a much tougher cell that will resist lysis for extended time periods. It is also used as a buffer solution to maintain the pH and osmotic conditions of the specimen (Lohnes and Demirel, 1978).

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemical Reagents

Acetic acid, ammonium sulfate, acetonitrile, acetone, chloroform, ethanol, ethyl acetate, hexane, iron (II) sulphate, sodium carbonate, sodium hydroxide (QRec), methanol (Chemsol), ascorbic acid, sodium nitrite, (Systerm), Mueller Hinton broth, Mueller Hinton agar, Luria bertani broth (Merck), Ampicillin, Ferrozine, Quercetin (Acros), aluminum chloride hexahydrate, Caffeic acid, Catechin hydrate, 1, 1-diphenyl-2-picrylhydrazyl, Gallic acid, glutaraldehyde, phosphate buffered saline, sodium nitroprusside (Sigma Aldrich), ethylenediaminetetraacetic acid, Folin-Ciocalteu, sodium nitroprusside (R&M), bis-acrylamide, Kanamycin, Tris-HCl (Bio Basic Canada), *p*-iodonitrotetrazolium chloride, sodium dodecyl sulfate (Fisher Scientific).

3.2 Sample Collection and Preparation

A total of fifteen plants (Voucher numbers MHR-2012-001 to MHR-2013-015) as shown in Figure 3.1 to Figure 3.15 were collected from different places around the state of Perak mainly in Kampar and Menglembu from April to June of 2012. The plants species were authenticated by Professor Dr. Hean-Chooi Ong at the Institute of Biological Sciences, University of Malaya, Malaysia. Upon

collection, the samples were washed thoroughly with deionized water to remove soils and debris. The samples were then oven dried at 40 °C for 48 hours or until constant weight was achieved. After acquiring the dried samples, the plants were pulverized with a blender and placed in a zip-lock bag, stored in -20 °C until further use.

Figure 3.1: ***Callicarpa formosana***

Figure 3.2: ***Clinacanthus nutans***

Figure 3.3: ***Epiphyllum oxypetalum***

Figure 3.4: ***Gyunura procumbens***

Figure 3.5: ***Hedyotis diffusa***

Figure 3.6: ***Hottunynia cordata***

Figure 3.7: ***Leonurus cardiaca***

Figure 3.8: ***Lonicera japonica***

Figure 3.9: ***Melastoma candidum***

Figure 3.10: ***Opuntia ficus-indica***

Figure 3.11: ***Pereskia bleo***

Figure 3.12: ***Rhoeo discolor***

Figure 3.13: ***Scutellaria barbata***

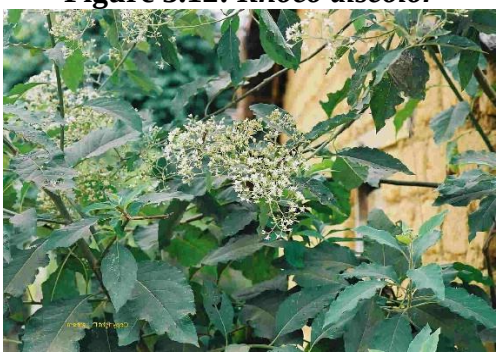


Figure 3.14: ***Vernonia amygdalina***

Figure 3.15: ***Zebrina pendula***

3.2.1 Aqueous Extraction of Plant Extracts

The dried plant samples (1:18 w/v) were then soaked in deionized water and incubated for 1 hour at 90 °C. The extracts were then filtered with filter paper and then subjected to centrifugation at 10000 rpm for 10 min at 4 °C. The supernatants were then collected and kept at -20 °C and used for various tests.

3.2.2 Sequential Extraction of Plant Extracts

After conducting the initial screening of antibacterial activities of the fifteen plants, only three plants which are *M. candidum, C. formosana* and *S. barbata* were shown to have prominent antibacterial activities. Thus, further extractions were done on these plants. Sequential extractions were performed on these plants by extracting it with organic solvents such as hexane, ethyl acetate, acetone, methanol and water by soaking plant samples in the respective solvents and the mixture was shook gently. The extraction using organic solvents were done in sequence according to the increasing polarity of the solvents.

Hexane < Ethyl Acetate < Acetone < Methanol < Water

Increasing Polarity

The extracted solutes were then evaporated using rotatory evaporator and the collected extracts were kept at -20°C until further tests.

3.3 Antioxidant Tests

The plant extracts were used for various antioxidant assays (DPPH radical scavenging assay, ferrous ion chelating assay, nitric oxide scavenging assay and total flavonoid assay). The extracts were also used in antibacterial assay, bioautography, time-kill study, identification of differentially expressed bacterial proteins and investigating the morphological changes of bacteria through scanning electron microscope.

3.3.1 Determination of 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity

DPPH radical scavenging activity of the extracts was assessed using an assay modified from Lim and Quah (2007). A mixture of 0.5 ml of 0.10 mM DPPH in methanol and 0.5 ml of extract was kept in the dark for 30 minutes. The absorbance was then read at 517 nm. A blank was prepared for each sample in which the DPPH solution was replaced with methanol. DPPH radical scavenging activity (%) was calculated as follows:

$$\textbf{DPPH radical scavenging activity (\%)} = (1 - [A_{sample} / A_{control}]) \times 100$$

where $A_{control}$ is the absorbance of control reaction without plant extract and A_{sample} is the absorbance in the presence of a plant extract. Ascorbic acid was used as the positive control.

3.3.2 Determination of Ferrous Ion Chelating (FIC) Assay

Ferrous ion chelating assay was performed according to the method stated from Chew et al. (2009) with slight modification. A volume of 200 µl of plant extract was mixed with 200 µl of 0.10 mM iron (II) sulphate ($FeSO_4$) and leave to stand for 5 minutes. After that, 400 µl of 0.25 mM ferrozine was added into the mixture and left to stand for another 10 minutes before reading it at 562 nm. Ferrozine is light sensitive thus this experiment was done in the absence of light to ensure that the result stays true to the chelating abilities of the plant extracts only and not influenced by other factors. Chelating activity (%) was calculated as follows:

Ferrous Ion Chelating Activity (%) = ($A_{control}$ –A_{sample}/$A_{control}$) X 100%

where $A_{control}$ is the absorbance of control reaction without plant extract and A_{sample} is the absorbance in the presence of a plant extract. Ethylenediaminetetraacetic acid (EDTA) acts as a positive control.

3.3.3 Determination of Nitric Oxide Radical Scavenging Activity

Nitric oxide scavenging activity of the extracts was determined as described by Sreejayan and Rao (1997) with modifications. Briefly, a mixture of 0.8 ml of extract and 0.2 ml of freshly prepared sodium nitroprusside (5 mM, in phosphate buffered saline, pH 7.4) was kept at room temperature for 150 minutes under light source (24 W compact fluorescent light bulbs). Then, 0.6 ml of the

mixture was transferred to a new tube containing 0.6 ml of freshly prepared Griess Reagent (1% sulphanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 5% phosphoric acid).

This mixture was then allowed to stand at room temperature in darkness for 10 minutes. Its absorbance was then read at 546 nm. To correct for background absorbance, each sample measurement was accompanied with a simultaneous reaction in which sodium nitroprusside solution and Griess Reagent were replaced with water. Nitric oxide radical scavenging activity (%) was calculated as follows:

$$\textbf{Nitric oxide scavenging activity (\%)} = (1 - [A_{sample} / A_{control}]) \times 100$$

where $A_{control}$ is the absorbance of control reaction (without plant extract) and A_{sample} is the absorbance in the presence of a plant extract. Ascorbic acid was used as a positive control in this assay.

3.3.4 Total Flavonoid Assay

Total flavonoid contents of the extracts were determined using a colorimetric assay described by Zou et al. (2004). A mixture of 0.2 ml of extract and 0.15 ml of $NaNO_2$ (5% w/v) was first incubated at room temperature for 6 minutes. Next, 0.15 ml of $AlCl_3.6H_2O$ (10% w/v) was added to the mixture, which was then kept at room temperature for 6 minutes. Then, 0.8 ml of NaOH (10% w/v) was added and the absorbance of the mixture was read at 510 nm after standing at room temperature for 15 minutes.

For the blank, the extracts were replaced with water. To correct for background absorbance, each sample measurement was accompanied with a simultaneous reaction in which $AlCl_3.6H_2O$ was replaced with water. A standard curve was prepared with 0 to 500 g/ml quercetin (in 80% ethanol). Total flavonoid content was expressed in mg quercetin equivalents (QE)/g dry matter. A linear graph of the standard curve of quercetin was drawn and the concentrations of the extracts were found through the graph.

3.4 High Performance Liquid Chromatography

Separation of compound was performed with a HPLC system (Shimadzu Corporation). Analysis was performed using published protocol by Jiang et al. (2005) with modification. Samples were separated on a Thermo Scientific ODS Hypersil column (5 μm, 100 mm x 4.6 mm). The mobile phase solvents were as follows: Solvent A: distilled water acidified to pH 2.74 with acetic acid; Solvent B: acetonitrile. After injecting 20 μl of plant extract or phenolic standard, the following elution gradient was applied with a flow rate of 0.8 ml/min: 0–5 min, 5% B; 5–22 min, 9% B; 22–38 min, 11% B; 38–43 min, 18% B; 43–44 min, 23% B; and 44–54 min, 90% B. UV-spectra were monitored at 272 nm for gallic acid, 280 nm for both catechin hydrate and caffeic acid, and 370 nm for quercetin. The column was eluted at room temperature, and all aqueous solvents for HPLC were filtered through 0.45 μm membrane prior to applications (Wong et al., 2014).

3.5 Antibacterial Study

Minimum inhibitory concentration (MIC) and time-kill study were employed in this antibacterial study. First, the plants were screened for their antibacterial activities with aqueous extract using MIC assay. Plants which showed good antibacterial activities in the initial MIC screening were selected and sequential extracted with different organic solvents. Fractionated plant extracts were then tested in MIC assay to determine which solvent extract has the lowest MIC value.

Time-kill study was then carried out with the plant solvent extract with the lowest MIC value in order to evaluate the plant extract's killing rate against the tested bacteria.

3.5.1 Minimum Inhibitory Concentration (MIC) Assay

MIC assay was performed to determine the lowest plant extract concentration required to inhibit growth of the tested bacteria. The assay was performed based on published protocols (Andrews, 2001; Wiegand et al., 2008) with slight modifications. Three Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538, *Staphylococcus aureus* ATCC 25923 and Methicillin resistant *Staphylococcus aureus* ATCC 33591) and three Gram-negative bacteria

(*Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853) were used in the assay.

Plant extract was added into the first row of wells and then serially diluted according to Figure 3.16 and Figure 3.17. Briefly, a bacterial inoculum of 5×10^5 colony-forming unit/ml was prepared in Mueller-Hinton Broth and aliquot into a 96-well sterile microtiter plate.

The plate was then sealed and incubated at 37 °C for 24 hours. Next, 20 µl of 0.4 mg/ml *p*-iodonitrotetrazolium chloride was added to each well, followed by 30 minutes of incubation at 37 °C. Colour changes in each well were monitored visually. The lowest extract concentration that inhibited bacterial growth, indicated by the absence of colour change in the well, was taken as the MIC value. For comparison, the assay was carried out using different concentrations of antibiotics (Ampicillin and Kanamycin).

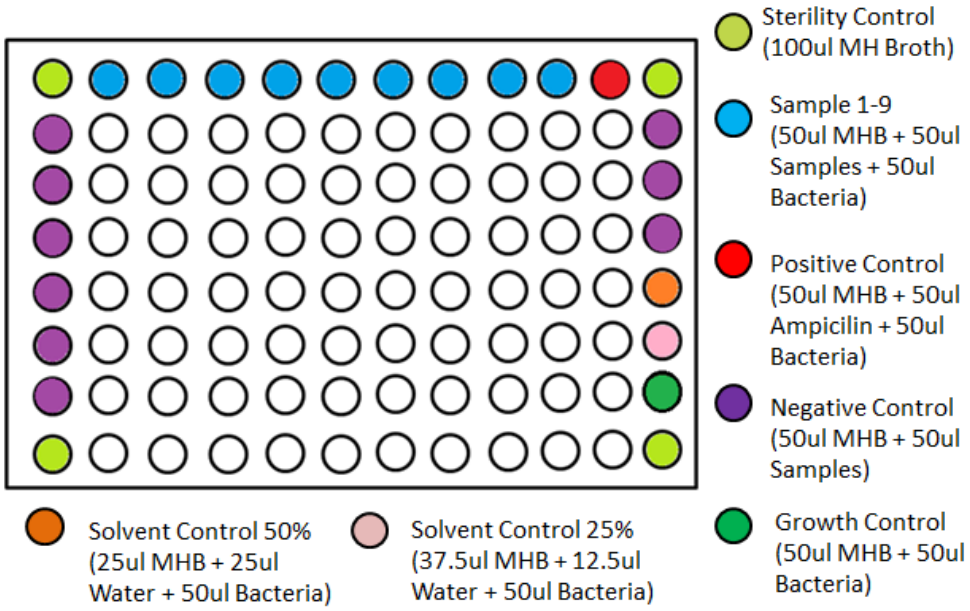


Figure 3.16: Design of the 96-well Plate for MIC Test for Aqueous Extract of Plants.

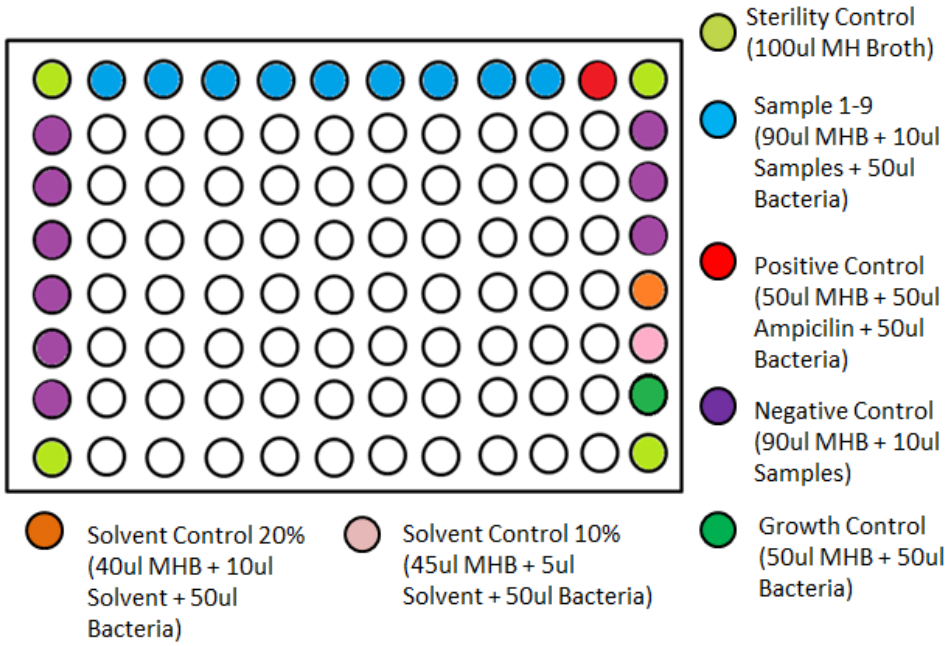


Figure 3.17: Design of the 96-well Plate for MIC Test for Solvent Extract of Plants.

3.5.2 Time-Kill Assay

An overnight bacterial suspension was prepared before transferring into Mueller-Hinton broth (MHB) and incubated for 2 hours at 37 ^{o}C on a 150 rpm shaking incubator to reach logarithmic phase. This culture was then further diluted to reach an inoculum of 1×10^{8} colony-forming units (CFU)/ml. The culture was then transferred to McCartney bottle to achieve an initial bacterial inoculum of 5 x 10^{5} CFU/ml. Respective extracts were prepared and added to the medium at 1×, 2×, 4×, 8× MIC.

Extract free cultures were included as growth control. All culture bottles were incubated for 24 hours at 37 ^{o}C on a 150 rpm shaking incubator. The viable counts were carried out at 0, 2, 4, 6, 8, 10 and 24 hours of incubation. The concern regarding extract carryover was addressed by performing dilution as described previously by Pankuch et al. (1994). 20 µl of the cultures were withdrawn from the culture bottles at their respective time and transferred into 180 µl of MHB for 10-fold serial dilutions. Viable count was determined on Mueller-Hinton agar plates using the drop plate method. The plates were incubated at 37 ^{o}C for 24 hours.

The changes found in $\log_{10}$ colony count at each sampling point from the initial inoculum were calculated. Bactericidal effect was defined as a reduction of 99.9% ($\geq$3 $\log_{10}$) of the total count of CFU/ml with the initial test inoculum

(NCCLS, 1999). Time–kill curves were constructed by plotting the change observed in $\log_{10}$ colony count versus time.

3.6 Determination of Antibacterial Compound

3.6.1 Thin Layer Chromatography Bioautobiography

The thin layer chromatography (TLC) bioautobiography was performed on the methanol fraction against all five bacteria strains (*S. aureus* ATCC 6538, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *E. coli* ATCC 35218 and *P. aeruginosa* ATCC 27853). Exactly 0.2 mg of methanol fraction of the plant extract was spotted at 1.5 cm from the base of preparative chromatography Silica Gel 60 F_{254} TLC plates (Merck).

One solvent system (Methanol: Chloroform; 9:1) was used and run in duplicate. One set of the TLC plate (Set A) was used to extract the spot which has the antibacterial compound, while the other set was used for bioautography (Set B). The TLC set used for bioautography was developed as described above, was placed in a sterile petri dish. Exactly 0.1 ml inoculum of the bacteria culture (1 x 10^8 CFU/ml) was pipetted into 9.9 ml of nutrient agar before distributing over the TLC plates creating a thin layer of seeded agar.

After the agar solidified, the TLC plate was incubated for 24 hours at 37^{o}C. After 24 hours, 0.2 mg/ml of p-iodonitrotetrazolium violet was sprayed on top of the agar. Clear zone on the agar indicates that the bacteria around that zone were inhibited of its growth (Eloff, 2004).

The clear zone was then compared with the other set which was used to extract the spot with antibacterial compound. From Set A TLC, the spot corresponding to the bacterial inhibition zone on Set B TLC was extracted, by scrapping the silica gel with a clean scalpel. The scrapped silica gel was then eluted of its compound with methanol and then centrifuged twice and filtered before subjecting it to gas chromatography analysis.

3.6.2 Gas Chromatography Analysis of TLC Spot

The TLC spot was analyzed by gas chromatography equipped with mass spectrometry (Shimadzu GCMS-QP2010 Plus). The column temperature was set to 110 ^{o}C for 2 minutes, then increased to 200 ^{o}C at the rate of 10 ^{o}C per minute and then increased to 280 ^{o}C at the rate of 5 ^{o}C per minute and which was then held for 9 minutes. The injector temperature was set to 250 ^{o}C (split mode with the ratio adjusted to 20:1, with injection volume 3 μl). The flow rate of the carrier gas, helium was set to 1 ml/min and the ion source was set to 280 ^{o}C. The total running time of the gas chromatography was 36 minutes. The mass spectra were obtained from the range of m/e 40 to 700. The chromatograms of the sample were

identified by comparing its mass spectra with NIST Gas Chromatography library data.

3.7 Identification of Differentially Expressed Bacterial Proteins as Potential Therapeutic Antimicrobial Targets

3.7.1 Microorganism and Culture Condition

A total of six bacteria cultures (*S. aureus* ATCC 6538, *S. aureus* ATCC 25923, Methicillin resistant *S. aureus* ATCC 33591, *E. coli* ATCC 25922, *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853) were used in this study. The bacteria were cultured overnight within a conical flask in an orbital shaker at 37 ^{o}C and shaking at 200 rpm in Luria-bertani broth.

3.7.2 Isolation of Protein for SDS–PAGE Analysis

The cultured bacteria were then treated with the appropriate concentration of the plant extract and sterile water was used as the control instead of the plant extract. The treated cultures were then placed into the orbital shaker at 37 ^{o}C, shaking at 200 rpm. After that, 20 ml of culture was collected at different time interval (5th minute, 30th minute, 60th minute and 90th minute) and centrifuged at 9000 rpm for 10 minutes in 4 ^{o}C to separate the pellet from the supernatant. The pellet was then treated with a bacterial extraction kit and the supernatant

underwent ammonium sulphate precipitation. The precipitated protein was collected by centrifugation at 9000 rpm for 10 minutes in 4 ^{o}C.

3.7.3 SDS-PAGE Analysis

SDS-PAGE gel electrophoresis was carried through using 12% resolving gel (1.5M Tris-HCl pH 8.8, 10% SDS, 40% bis-acrylamide), and 4% stacking gel (0.5 M Tris-HCl pH 6.9, 10% SDS, 40% bis-acrylamide). The protein samples were treated with dithiothreitol (DTT) and then boiled before loading it into the well.

The molecular weight of the dissociated proteins was then estimated through using marker of known molecular weight (Thermo Scientific Spectra Multicolor Broad Range Protein Ladder) which was loaded along with the samples. The SDS-PAGE gel apparatus was connected with constant electric current of 135 mV till the bromophenol blue (BPB) reached the bottom of the plate.

The gels were put into a container and then treated with staining solution consisting of coomassie brilliant blue R-250 dissolved in methanol with acetic acid and distilled water. The gels were left in the staining solution for 30 minutes before destaining it overnight in distilled water until the bands became visible

above the background. Both staining and destaining steps were carried out while shaking in room temperature.

3.8 Investigation of Morphological Changes of Bacteria via Scanning Electron Microscopy

The effect of antibacterial plant extracts toward the morphology of bacteria was investigated by employing the use of scanning electron microscope. The treated bacteria were compared to the control set of bacteria to have a better understanding of how the plant extracts affect the bacteria cell surface.

3.8.1 SEM Sample Preparation

Bacteria cultures (treated and non-treated) were incubated with 2.5% glutaraldehyde overnight. The mixture was spin down and the glutaraldehyde was replaced with phosphate buffer saline (PBS) to wash off any glutaraldehyde residue. The samples were subjected to 3 changes of PBS. After washing with PBS, the samples were hydrated with a series of ascending concentration of ethanol (25% for 5 minutes, 50% for 10 minutes, 75% for 10 minutes and 3 changes of 100% ethanol for 10 minutes each change).

The samples were then dried through freeze drying. Before the samples are to be viewed with SEM, the samples underwent sputter coating with a

conducting material. The samples were lightly dusted onto a double sided adhesive conductive carbon tape which was mounted on a copper stage. The amounts of samples that adhere onto the carbon tape are to be as little as possible in order to allow a thorough coating to avoid electrical discharge when viewing it with SEM. The specimens were coated with platinum with a sputter coater (JEOL JFC-1600 Auto Fine Coater) as shown in Figure 3.18.

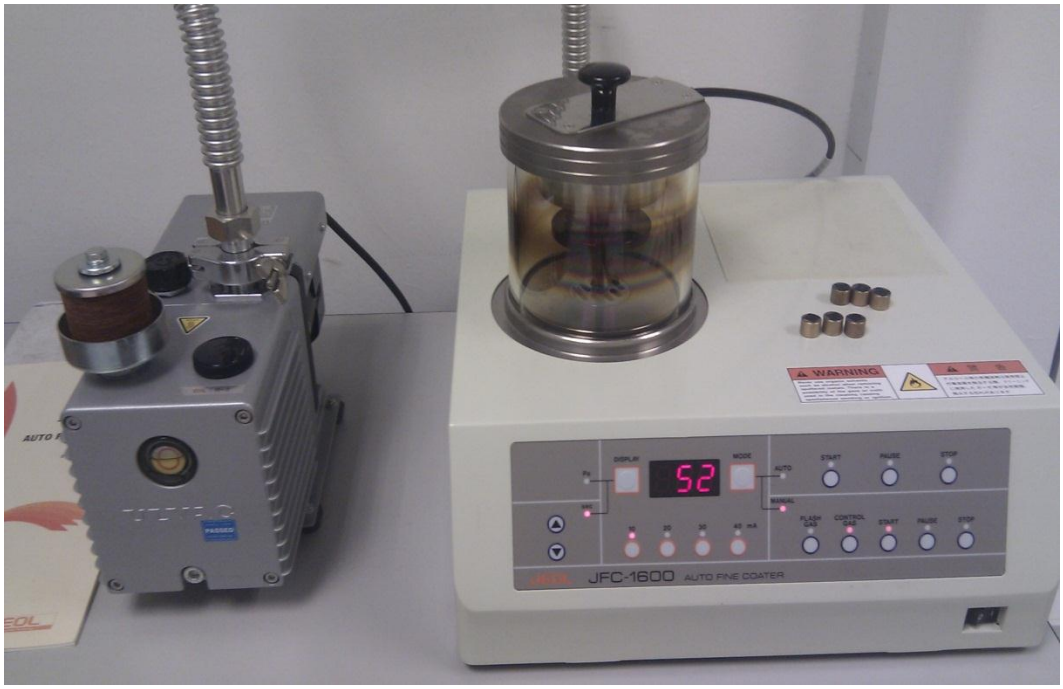


Figure 3.18: JEOL JFC-1600 Auto Fine Coater.

3.8.2 Capturing Images with Scanning Electron Microscope

The specimens were examined on a Scanning Electron Microscope (JEOL JSM-6701F Field Emission Scanning Electron Microscope) as shown in Figure

3.19. Magnification and working distance were adjusted for the position and type of specimen.

Figure 3.19: Field-Emission Scanning Electron Microscopy.

CHAPTER 4

RESULTS

4.1 Antioxidant Study of Plant Extracts

4.1.1 Determination of 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity of Plant Extracts

The DPPH radical scavenging activities of plant extracts with varying concentration of 1, 2, 4, 8 and 16 mg/ml were determined. The result was summarized in Table 4.1 and a graph of the result is depicted in Figure 4.1. The ascorbic acid acts as a positive control in this test. An example of the calculation is depicted in Appendix A.

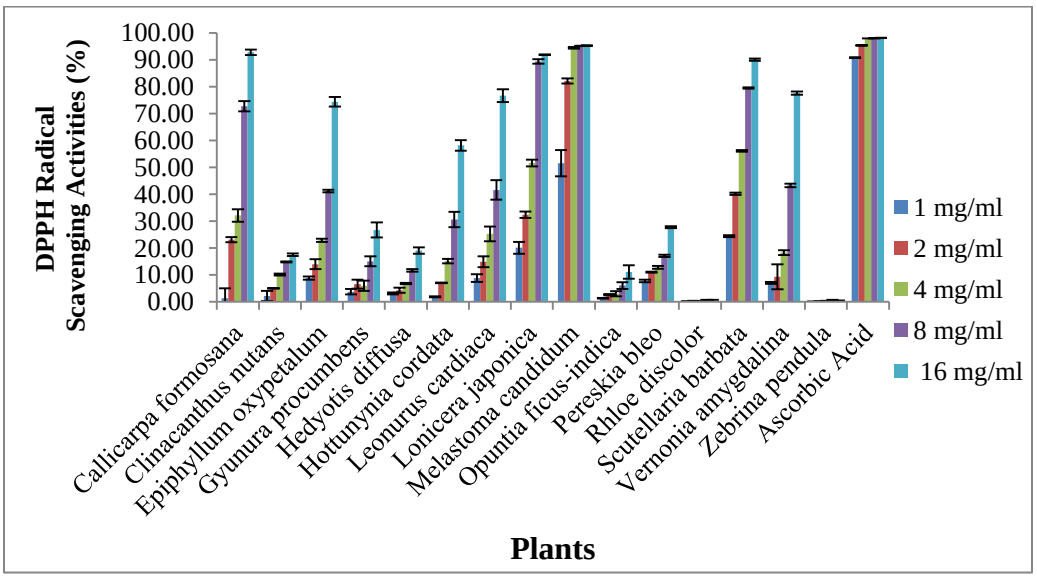


Figure 4.1: DPPH Radical Scavenging Activity of Different Plants with Varying Concentration.

Table 4.1: DPPH Radical Scavenging Activities of Different Concentrations of the Plant Extracts.

	% of DPPH Radical Scavenging Activities of Extracts				
Plants	**1 mg/ml**	**2 mg/ml**	**4 mg/ml**	**8 mg/ml**	**16 mg/ml**
C. formosana	1.27±3.72	23.06±0.95	32.09±2.36	72.76±1.91	92.84±1.02
C. nutans	2.09±1.91	5.00±0.16	10.15±0.34	14.85±0.20	17.54±0.46
E. oxypetalum	8.80±0.55	13.94±1.85	22.82±0.58	41.16±0.45	74.36±1.79
G. procumbens	3.73±0.97	6.56±1.68	5.98±1.92	15.02±1.87	26.72±2.78
H. diffusa	3.13±0.34	4.25±0.94	6.79±0.26	11.64±0.49	19.03±1.15
H. cordata	1.81±0.16	7.07±0.11	15.12±0.84	30.57±2.85	58.18±1.98
L. cardiaca	8.80±1.39	14.85±2.04	25.23±2.74	41.58±3.63	76.68±2.39
L. japonica	20.09±2.18	32.39±1.21	51.64±1.24	89.44±0.82	91.89±0.11
M. candidum	51.56±4.86	82.18±0.94	94.44±0.28	95.16±0.05	95.30±0.09
O. ficus-indica	1.33±0.11	2.66±0.23	2.99±0.92	6.06±1.25	11.04±2.48
P. bleo	7.73±0.48	11.01±0.20	12.81±0.55	17.13±0.41	27.75±0.36
R. discolor	0.00±0.27	0.00±0.40	0.00±0.27	0.00±0.66	0.00±0.71
S. barbata	24.38±0.34	40.17±0.38	56.08±0.31	79.51±0.26	90.07±0.41
V. amygdalina	7.01±0.33	9.33±4.63	18.28±0.93	43.28±0.63	77.61±0.60
Z. pendula	0.00±0.14	0.00±0.24	0.00±0.38	0.00±0.69	0.00±0.51
Ascorbic Acid	90.78±0.13	95.33±0.08	97.98±0.03	98.01±0.03	98.16±0.03

Data are reported as mean±SE values (n=3)

The results in Table 4.1 and Figure 4.1 have shown that the DPPH scavenging activities are concentration dependent whereby the higher the concentration of plant extracts, the higher the scavenging activities. At concentration of 16 mg/ml, *C. formosana, L. japonica, M. candidum* and *S.*

barbata have shown to have the highest scavenging activities which were 92.84%, 91.89%, 95.30% and 90.07% respectively whereas *R. discolor* and *Z. pendula* does not show to have any DPPH radical scavenging activities at all.

4.1.2 Ferrous Ion Chelating (FIC) Assay

In FIC assay, ferrozine was used due to its reactivity with ferrous ion to form stable red coloured complex species. Reduction of this coloured complex species by plant extracts determines the plant chelating activity. Ethylenediaminetetraacetic acid (EDTA) acts as a positive control as EDTA is a powerful metal chelating agent. The ferrous ion chelating activity of the fifteen plants were summarized in Table 4.2 and a graph comparison of the result was shown in Figure 4.2.

From the study done, it is shown that the chelating activities are concentration-dependent. The higher the concentration of plant extracts, the higher the chelating activity. This study has shown that all plants have the potential to be good ferrous ion chelating agents as most of the plants have high chelating activities while only *O. ficus-indica* has shown to have the lowest chelating activity. An example of the calculation is in Appendix B.

Table 4.2: Ferrous Ion Chelating Activities of Different Concentrations of the Plant Extracts.

	% of Ferrous Ion Chelating Activities of Extracts			
Plants	**1 mg/ml**	**2 mg/ml**	**5 mg/ml**	**10 mg/ml**
C. formosana	22.05±2.15	44.95±4.93	69.49±1.87	73.08±2.19
C. nutans	81.10±0.40	83.08±0.40	88.07±0.60	90.96±0.60
E. oxypetalum	21.40±0.94	33.71±1.07	56.53±1.70	81.82±0.66
G. procumbens	77.25±1.20	78.95±1.63	81.79±0.90	82.15±1.68
H. diffusa	76.59±0.54	80.34±0.38	83.11±0.15	84.81±0.21
H. cordata	49.24±3.18	76.59±2.58	87.19±2.45	91.48±1.32
L. cardiaca	30.49±5.92	49.88±1.22	70.44±2.98	80.52±5.79
L. japonica	19.23±2.89	32.44±3.02	51.25±4.08	64.96±1.56
M. candidum	58.41±0.18	60.35±0.27	67.48±2.41	74.31±0.93
O. ficus-indica	15.43±2.26	20.76±1.31	30.00±4.90	46.61±2.50
P. bleo	58.46±1.61	75.59±1.07	85.36±0.23	86.32±0.18
R. discolor	20.49±2.17	38.67±0.73	66.69±3.26	89.13±2.29
S. barbata	65.40±1.51	71.20±0.62	86.20±0.93	88.50±4.65
V. amygdalina	71.68±1.14	84.27±0.68	85.64±0.69	86.38±0.62
Z. pendula	10.16±0.61	24.35±2.67	58.47±3.11	86.74±0.59
EDTA	90.21±0.18	98.17±1.83	100.00±0.00	100.00±0.00

Data are reported as mean±SE values (n=3)

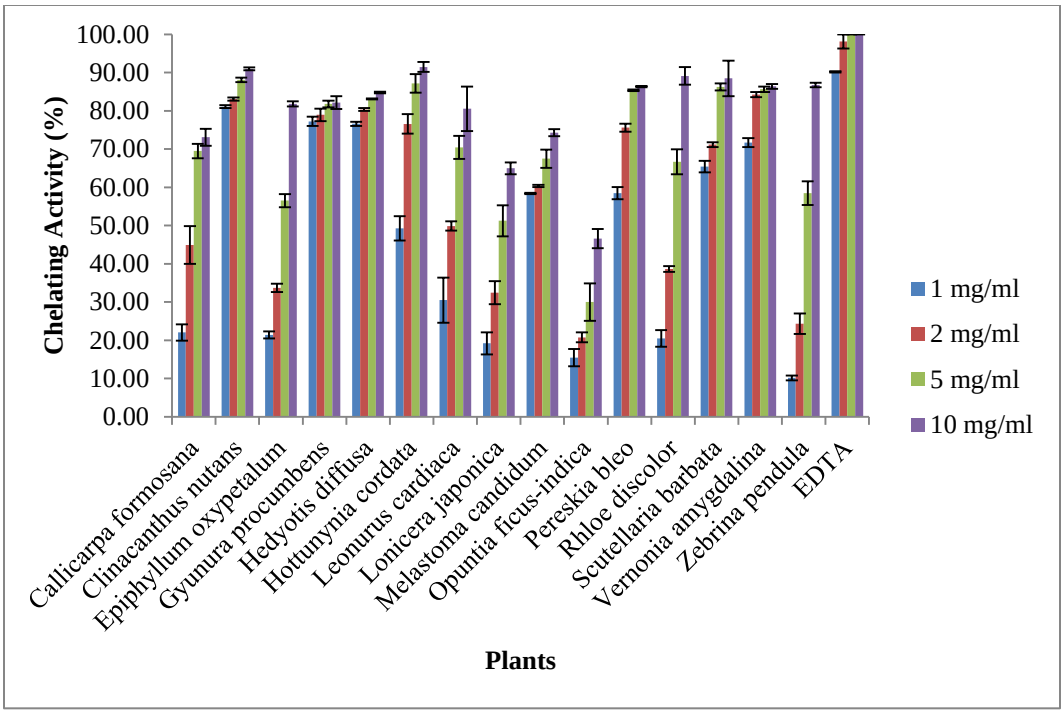


Figure 4.2: Ferrous Ion Chelating Activities of Different Plants with Varying Concentrations.

4.1.3 Determination of Nitric Oxide Scavenging Activity of Plant Extracts

The nitric oxide scavenging activities of plant extracts were carried out in this study. Ascorbic acid acts as a positive control. The result of the study is summarized in Table 4.3 and a graph comparison of the result is depicted in Figure 4.3. From the result shown, the nitric oxide scavenging activities of the plant extracts are concentration-dependent as the higher the concentration, the higher the scavenging activities. Most of the plants have shown to have high nitric oxide scavenging activities except *O. ficus-indica, R. discolor and Z. pendula* where their nitric oxide scavenging activities are the lowest among all of the plants. An example of the calculation is in Appendix C.

Table 4.3: Nitric Oxide Scavenging Activities of Different Concentrations of the Plant Extracts.

	% of Nitric Oxide Scavenging Activity of Extracts		
Plants	**1 mg/ml**	**5 mg/ml**	**10 mg/ml**
C. formosana	67.55±1.64	74.15±4.93	85.47±1.87
C. nutans	46.61±2.18	78.02±0.58	96.95±1.50
E. oxypetalum	58.15±1.47	63.74±0.68	74.80±0.93
G. procumbens	38.16±2.19	70.01±2.03	85.37±1.33
H. diffusa	46.67±3.07	75.96±4.74	79.67±0.77
H. cordata	48.13±2.08	79.26±1.53	94.41±0.54
L. cardiaca	54.33±2.05	71.10±0.81	83.03±1.88
L. japonica	64.04±3.20	69.09±1.25	72.36±0.71
M. candidum	72.42±1.76	76.68±3.06	78.76±0.27
O. ficus-indica	8.92±1.89	34.72±0.91	34.04±0.51
P. bleo	29.38±1.35	66.73±2.33	84.96±1.03
R. discolor	12.59±0.75	41.56±1.37	40.35±1.87
S. barbata	32.42±2.37	66.81±2.45	77.29±2.82
V. amygdalina	60.77±0.37	70.74±0.31	80.59±0.71
Z. pendula	4.93±0.40	7.39±2.96	18.19±0.73
Ascorbic acid	90.67±1.41	97.45±1.95	97.76±1.64

Data are reported as mean±SE values (n=3)

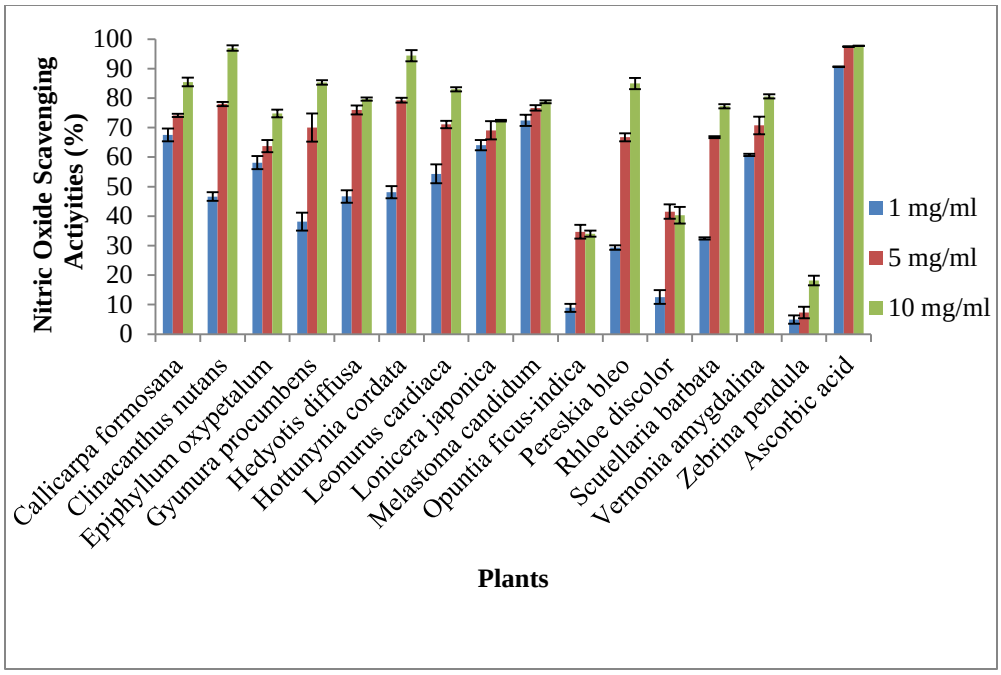


Figure 4.3: Nitric Oxide Scavenging Activities of Different Plants with Varying Concentrations.

4.1.4 Determination of Total Flavonoid Contents of Plant Extracts

The total flavonoid contents (TFC) of fifteen plants extracts were determined and the results are displayed in Table 4.4. Quercetin was used as the standard unit to determine TFC of the plant extracts. *M. candidum* has shown to have the highest content of flavonoid compounds with 103.47 mg QE/g dry matter followed by *S. barbata*, and *C. formosana* with 91.55 mg QE/ g dry matter and 87.70 mg QE/g dry matter respectively. An example of the calculation is in Appendix D.

Table 4.4: Total Flavonoid Contents of Plant Extracts.

Plant Extracts	Total Flavonoid Content (mg QE/g dry matter)
C. formosana	87.70±0.99
C. nutans	2.08±0.05
E. oxypetalum	6.94±0.04
G. procumbens	6.90±0.32
H. diffusa	6.30±0.10
H. cordata	6.82±0.18
L. cardiaca	9.86±0.15
L. japonica	74.68±0.22
M. candidum	103.47±1.42
O. ficus-indica	1.77±0.02
P. bleo	1.83±0.07
R. discolor	1.43±0.08
S. barbata	91.55±1.19
V. amygdalina	4.82±0.01
Z. pendula	0.92±0.09

Data are reported as mean±SE values (n=3)

4.2 High Performance Liquid Chromatography (HPLC)

Plant extracts which show high antioxidant activities were subjected to HPLC analysis and the results are summarized in Table 4.5. The chromatograms and calculations of the phenolic constituents are in Appendix E and F (Wong and Yong, 2013).

Table 4.5: Summary of Concentration of Phenolic Constituents.

Plants	Concentration of phenolic constituents (μg per mg of dried sample)			
	Gallic acid	**Quercetin**	**Caffeic Acid**	**Catechin**
C. formosana	0.04 ± 0.04	0.43 ± 0.01	n.d	0.66 ± 0.03
C. nutans	n.d	0.25 ± 0.01	n.d	n.d
H. diffusa	0.06 ± 0.00	0.73 ± 0.02	n.d	0.08 ± 0.00
M. candidum	0.61 ± 0.03	0.40 ± 0.01	n.d	n.d
P. bleo	n.d	0.52 ± 0.02	n.d	n.d
V. amygdalina	n.d	0.57 ± 0.03	0.08 ± 0.01	1.31 ± 0.08

n.d, not determined; Data are reported as mean±SE values (n=3)

4.3 Minimum Inhibitory Concentration (MIC)

4.3.1 Minimum Inhibitory Concentration (MIC) with Aqueous Plant Extracts

Aqueous extracts of fifteen plants were screened for its antibacterial activities towards six different bacteria (*Staphylococcus aureus* ATCC 6538 and

ATCC 25923, Methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 33591, *Escherichia coli* ATCC 35218 and ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853). Table 4.6 shows the MIC of the plant extracts against its respective bacteria (Wong and Yong, 2013).

Table 4.6: Minimum Inhibitory Concentration of Various Plant Aqueous Extracts against Gram-Positive Bacteria.

	Minimum Inhibitory Concentration (mg/ml)		
	***S. aureus* ATCC 6538**	***S. aureus* ATCC 25923**	**MRSA ATCC 33591**
C. formosana	16.67±4.2	12.5±0.00	25±0.00
C. nutans	>50	>50	>50
E. oxypetalum	>50	>50	>50
G. procumbens	>50	>50	>50
H. diffusa	50±0.00	50±0.00	>50
H. cordata	50±0.00	50±0.00	>50
L. cardiaca	>50	>50	>50
L. japonica	>50	>50	>50
M. candidum	2.3±0.8	6.3±0.00	1.56±0.00
O. ficus-indica	>50	>50	>50
P. bleo	41.7±8.3	50±0.00	>50
R. discolor	>50	>50	>50
S. barbata	2.5±0.00	2.5±0.00	5±0.00
V. amygdalina	>50	50±0.00	>50
Z. pendula	>50	>50	>50

MRSA, Methicillin-resistant *Staphylococcus aureus*. Data are reported as mean±SE values (n=3)

Table 4.7: Minimum Inhibitory Concentration of Various Plant Aqueous Extracts against Gram-Negative Bacteria.

	Minimum Inhibitory Concentration (mg/ml)		
	***E. coli* ATCC 35218**	***E. coli* ATCC 25922**	***P. aeruginosa* ATCC 27853**
C. formosana	>50	>50	>50
C. nutans	>50	>50	>50
E. oxypetalum	>50	>50	>50
G. procumbens	>50	>50	>50
H. diffusa	>50	>50	>50
H. cordata	>50	>50	>50
L. cardiaca	>50	>50	>50
L. japonica	33.3±8.3	>50	>50
M. candidum	8.3±2.1	12.5±0.00	8.3 ± 2.1
O. ficus-indica	>50	>50	>50
P. bleo	>50	>50	>50
R. discolor	33.3±8.3	>50	>50
S. barbata	>50	>50	>50
V. amygdalina	>50	>50	>50
Z. pendula	>50	>50	>50

Data are reported as mean±SE values (n=3).

4.3.2 Minimum Inhibitory Concentration (MIC) with Different Organic Solvents

After the initial screening of antibacterial activities with aqueous plant extracts, *C. formosana, M. candidum* and *S. barbata* were chosen for further studies of MIC with organic solvent extract (Wong and Yong, 2013).

Table 4.8: Minimum Inhibitory Concentration of *Melastoma candidum* Against Various Bacteria.

	Minimum Inhibitory Concentration (mg/ml)				
	Hexane	**Ethyl Acetate**	**Acetone**	**Methanol**	**Cold Water**
***S. aureus* ATCC 6538**	5±0	n/a	4.17±0.83	0.625±0	7.18±1.79
***S. aureus* ATCC 25923**	n/a	n/a	n/a	1.25±0	10.77±0
MRSA ATCC 33591	4.17±0.48	5±0	3.44±0	0.31±0	4.3±0.5
***E. coli* ATCC 35218**	n/a	n/a	n/a	2.5±0	n/a
***E. coli* ATCC 25922**	n/a	n/a	n/a	5±0	n/a
***P. aeruginosa* ATCC 27853**	n/a	n/a	n/a	5±0	43.1±0

MRSA, Methicillin-resistant *Staphylococcus aureus;* n/a. no activity; Data are reported as mean±SE values (n=3)

Table 4.9: Minimum Inhibitory Concentration of *Callicarpa formosana* Against Various Bacteria.

	Minimum Inhibitory Concentration (mg/ml)				
	Hexane	**Ethyl Acetate**	**Acetone**	**Methanol**	**Cold Water**
***S. aureus* ATCC 6538**	10±0	1.67±0.24	1.67±0.24	2.5±0	5±0
***S. aureus* ATCC 25923**	10±0	1.67±0.24	1.25±0	1.25±0	33.33±4.81
MRSA ATCC 33591	10±0	0.63±0	1.25±0	0.83±0.12	12.5±0
***E. coli* ATCC 35218**	n/a	10±0	n/a	10±0	n/a
***E. coli* ATCC 25922**	n/a	10±0	n/a	n/a	n/a
***P. aeruginosa* ATCC 27853**	n/a	5±0	n/a	10±0	n/a

MRSA, Methicillin-resistant *Staphylococcus aureus;* n/a. no activity; Data are reported as mean±SE values (n=3).

Table 4.10: Minimum Inhibitory Concentration of *Scutellaria barbata* Against Various Bacteria.

	Minimum Inhibitory Concentration (mg/ml)				
	Hexane	**Ethyl Acetate**	**Acetone**	**Methanol**	**Cold Water**
***S. aureus* ATCC 6538**	n/a	2.14±0.00	1.82±0.00	2.15±0	10.00±0
***S. aureus* ATCC 25923**	n/a	2.14±0	1.82±0	2.15±0	10.00±0
MRSA ATCC 33591	n/a	8.55±0	5.56±0	4.30±0	n/a
***E. coli* ATCC 35218**	n/a	8.55±0	n/a	n/a	n/a
***E. coli* ATCC 25922**	n/a	8.55±0	n/a	n/a	n/a
***P. aeruginosa* ATCC 27853**	n/a	n/a	n/a	n/a	n/a

MRSA, Methicillin-resistant *Staphylococcus aureus*; n/a. no activity; Data are reported as mean±SE values (n=3).

Table 4.11: Minimum Inhibitory Concentration of Ampicillin and Kanamycin Against Various Bacteria.

	Ampicillin (mg/ml)	**Kanamycin (mg/ml)**
***S. aureus* ATCC 6538**	0.02±0	0.02±0
***S. aureus* ATCC 25923**	0.02±0	0.02±0
MRSA ATCC 33591	1.22±0	0.63±0
***E. coli* ATCC 35218**	1.00±0.2	2.5±0
***E. coli* ATCC 25922**	0.02±0	0.039±0
***P. aeruginosa* ATCC 27853**	0.6±0	2.5±0

MRSA, Methicillin-resistant *Staphylococcus aureus;* Data are reported as mean±SE values (n=3).

Ampicillin and kanamycin are two of the antibiotics used in this assay and these antibiotics act as the positive controls. From the initial screening of aqueous extract of the fifteen plants, only three plants (*M. candidum, C. formosana* and *S. barbata*) have shown to have good antibacterial activities. These three plants then underwent sequential extraction with hexane, ethyl acetate, acetone, methanol and water. From the second MIC assay (Table 4.8 to 4.10), *M. candidum* demonstrated the highest antibacterial activity compared to the other two plants (Wong and Yong, 2013). The more polar solvents which are methanol and acetone have shown to be able to extract most of the antibacterial compounds as these two solvent extracts show good antibacterial activities compared to ethyl acetate and hexane.

4.4 Evaluation of Antimicrobial Compounds

4.4.1 Direct Bioautography

Direct bioautography was used to determine the antibacterial compound separated on the thin layer chromatography. Methanol extracts from the plants have been shown to be most effective of all solvent extracts in term of antibacterial activities and thus it was chosen in this study. The extracts were placed on the thin layer chromatography plate and were left to run its course.

Duplicate of this thin-layer chromatography (TLC) was done where one of it underwent direct bioautography (Set B) while the other one was scrapped of its antibacterial compound (Set A) at the corresponding spots, by comparing both plates. After drying the excess mobile phase solvent, the plate (Set B) was placed onto the agar plate and fresh agar with bacteria culture was poured onto the plate. The culture was left for 24 hours in an incubator. After 24 hours, *p*-iodonitrotetrazolium chloride was sprayed onto the agar surface and it was used to identify the inhibition zone as shown in Figure 4.4 and Figure 4.5.

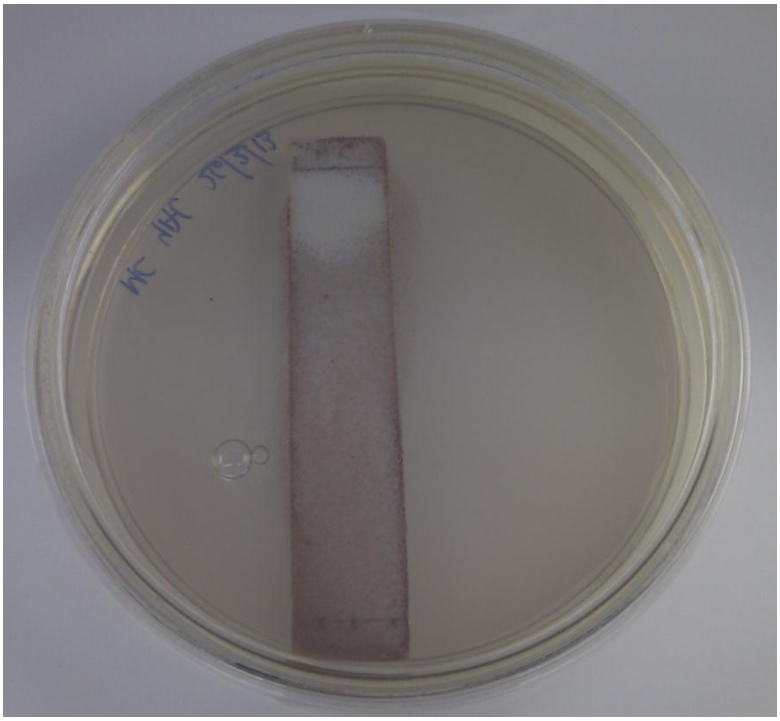


Figure 4.4: Direct Autobiography of *M. candidum* Methanol Extract

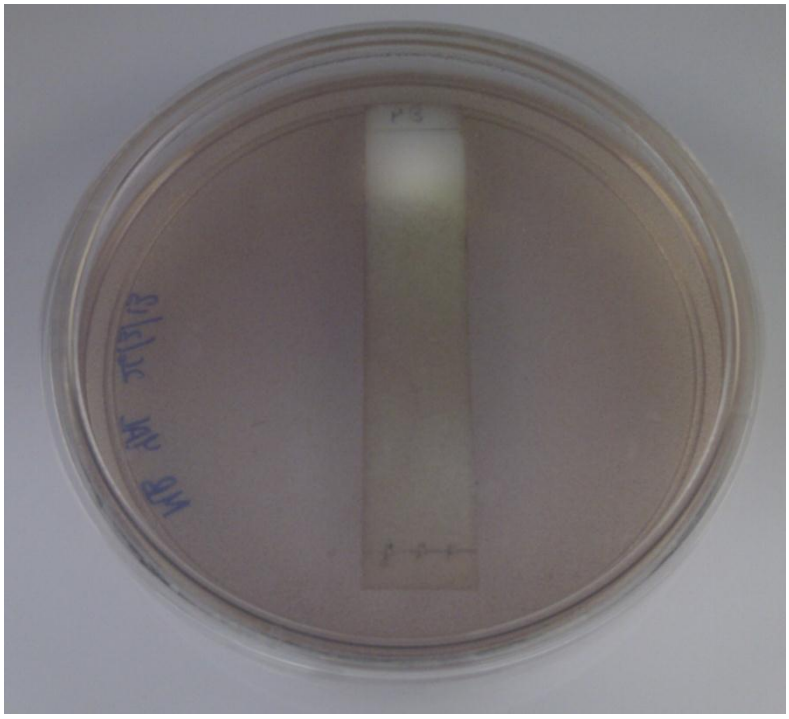


Figure 4.5: Direct Autobiography of *C. formosana* Methanol Extract

4.4.2 Determination of Compounds through Gas Chromatography-Mass Spectrometry (GC-MS)

From the inhibition zone seen in the direct bioautography TLC plate (Set B), a clean scrapper was used to scrap the corresponding spots (which represented the inhibition zone) on TLC plate (Set A). The scrapped samples were then extracted using methanol and filtered before subjecting to analysis with GC-MS.

4.4.2.1 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of Active Fraction of *M. candidum* Methanol Extract

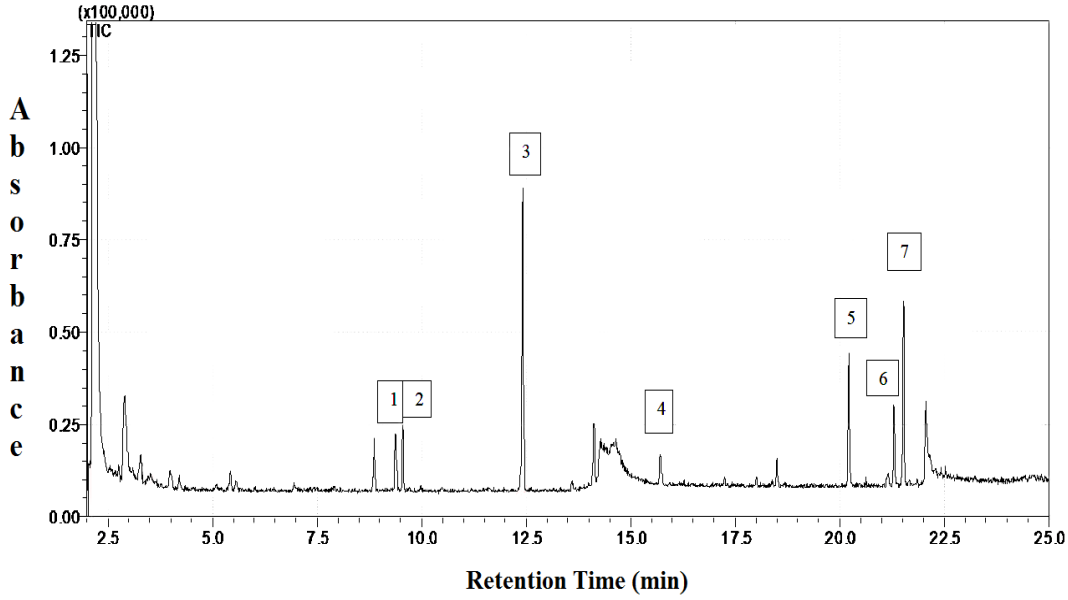


Figure 4.6: Gas Chromatography Analysis of the Active Fraction of *M. candidum* Methanol Extract (Wong et al., 2014).

Table 4.12: Major Chemical Compounds Identified from the Active Fraction of *M. candidum* Methanol Extract by GC-MS (Wong et al., 2014).

Peak Number	Retention time (min)	Name of the compounds	Molecular Formula	Molecular weight	Quality (%)
1	9.408	Butanoic acid, butyl ester	$C_8H_6O_2$	134	80
2	9.542	5-methyl-2-heptanamine	$C_8H_{19}N$	129	80
3	12.442	2,4-bis(1,1-dimethylethyl)-phenol	$C_{14}H_{22}O$	206	90
4	15.725	2-amino-1-phenyl-1-propanone	$C_9H_{11}NO$	149	79
5	20.233	1-butyl 2-(2-ethylhexyl) phthalate	$C_{20}H_{30}O_4$	334	85
6	21.317	Hexadecanoic acid, 15-methyl, methyl ester	$C_{18}H_{36}O_2$	284	82
7	21.542	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy, methyl ester	$C_{18}H_{28}O_3$	292	79

Figure 4.6 shows the chromatogram of the GC analysis done on the active fraction of *M. candidum* (methanol extract), taken from the thin layer chromatography. Several peaks were found and the chemical compounds were identified and summarized in Table 4.12 (Wong et al., 2014).

4.4.2.2 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of the Active Fraction of *C. formosana* Methanol Extract

Figure 4.7 shows the chromatogram of the GC analysis done on the active fraction of *C. formosana* (methanol extract), taken from the thin layer chromatography. Several peaks were found and the chemical compounds were identified and summarized in Table 4.13 (Yong et al., 2015).

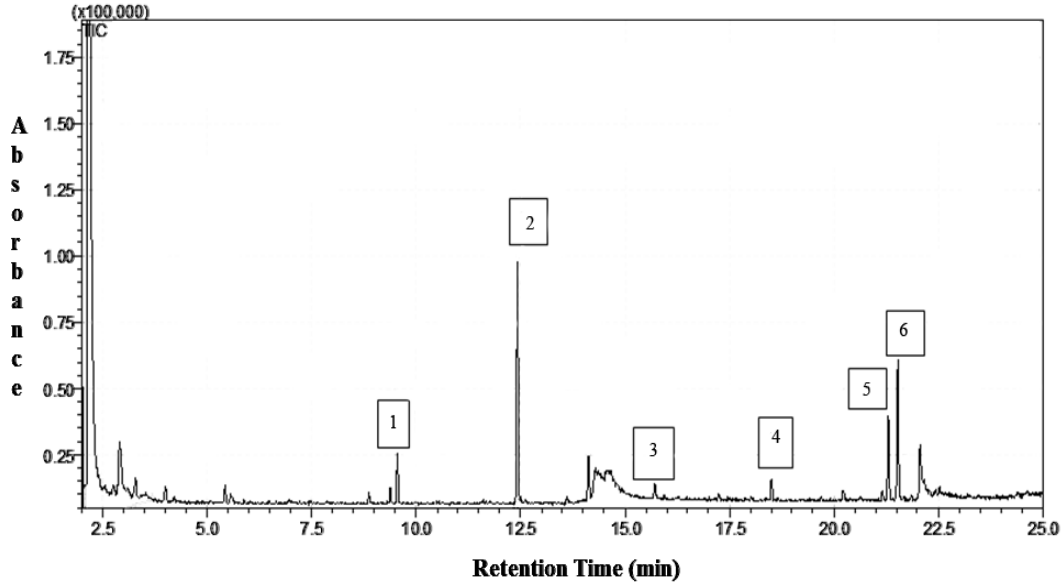


Figure 4.7: Gas Chromatography Analysis of the Active Fraction of *C. formosana* Methanol Extract (Yong et al., 2015).

Table 4.13: Major Chemical Compounds Identified from the Active Fraction of *C. formosana* Methanol Extract by GC-MS (Yong et al., 2015).

Peak Number	Retention time (min)	Name of the compounds	Molecular Formula	Molecular weight	Quality (%)
1	9.57	5-methyl-2-heptanamine	$C_8H_{19}N$	129	81
2	12.42	2,4-bis-(1,1-dimethylethyl)-phenol	$C_{14}H_{22}O$	206	91
3	15.72	2-amino-1-phenyl-1-propanone	$C_9H_{11}NO$	149	80
4	18.50	Aminononadecane	$C_{19}H_{41}N$	283	83
5	21.30	Hexadecanoic acid, 15-methyl, methyl ester	$C_{18}H_{36}O_2$	284	86
6	21.53	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy, methyl ester	$C_{18}H_{28}O_3$	292	79

4.5 Identification of Differentially Expressed Bacterial Proteins as Potential Therapeutic Antimicrobial Targets

Different bacteria were treated with *M. candidum, C. formosana* and *S. barbata* extracts and the bacterial proteins were resolved on denaturing SDS gel to see if they were differentially expressed after treatments. In this study, 17 differentially expressed proteins were successfully identified (Figure 4.8 to Figure 4.13). The differentially expressed proteins were sequenced, identified and compiled in Table 4.12. The protein sequences are listed in Appendix G.

Table 4.14: Identification of Differentially Expressed Proteins from *S. aureus* ATCC 6538.

No.	No. of Peptide Sequence Identified	Protein Residue	No. of Amino Acid Sequence Identified	Sequence Covered (%)	Annotation	Plant
A	7	322	62	19.25	Alpha Hemolysin	*Melastoma candidum*
B	1	681	11	1.61	Triacylglycerol lipase	*Callicarpa formosana*
C	4	619	43	6.95	N-acetylmuramoyl-L-alanine amidase	
D	6	322	71	22.05	Alpha Hemolysin	

Table 4.15: Identification of Differentially Expressed Proteins from *P. aeruginosa* ATCC 27853.

No.	No. of Peptide Sequence Identified	Protein Residue	No. of Amino Acid Sequence Identified	Sequence Covered (%)	Annotation	Plant
E	8	559	126	22.54	30S ribosomal protein s1	*Melastoma candidum*
F	5	548	71	12.96	60 kDa Chaperonine	
G	3	282	49	17.38	Elongation factor Ts	

Table 4.16: Identification of Differentially Expressed Proteins of *E. coli* ATCC 35218.

No.	No. of Peptide Sequence Identified	Protein Residue	No. of Amino Acid Sequence Identified	Sequence Covered (%)	Annotation	Plant
H	14	728	167	22.94	Formate C-acetyltransferase	
I	10	476	113	23.74	Glutamate Decarboxylase	*Melastoma*
J	13	471	132	28.03	Tryptophanase	*candidum*
K	9	394	107	27.16	Elongation Factor Tu 2	
L	5	486	73	15.02	Flagellin	*Scutellaria barbata*

Table 4.17: Identification of Differentially Expressed Proteins of *E. coli* ATCC 25922.

No.	No. of Peptide Sequence Identified	Protein Residue	No. of Amino Acid Sequence Identified	Sequence Covered (%)	Annotation	Plant
M	10	704	124	17.61	Elongation Factor G	*Melastoma candidum*
N	17	728	149	20.46	Formate C-acetyltransferase	
O	9	394	116	29.44	Elongation factor Tu 2	
P	7	350	75	21.43	Outer membrane protein A	
Q	1	99	14	14.14	Stringent starvation protein A	

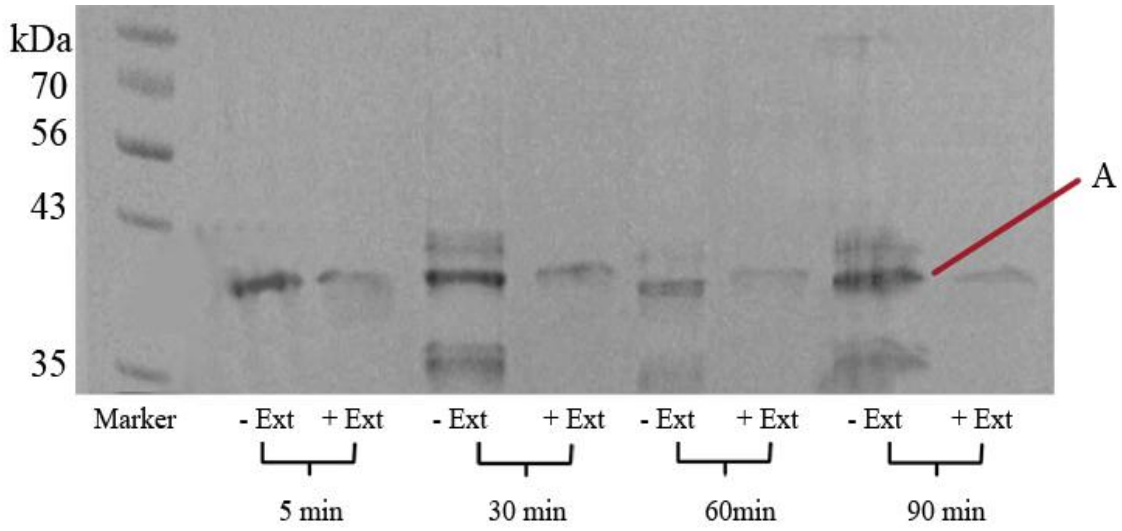


Figure 4.8: Protein expression profile of *S. aureus* ATCC 6538 (supernatant) following exposure to *Melastoma candidum* Extract. (-) and (+) indicated the absence or presence of plant extracts in bacterial culture medium, respectively (Wong and Yong, 2014).

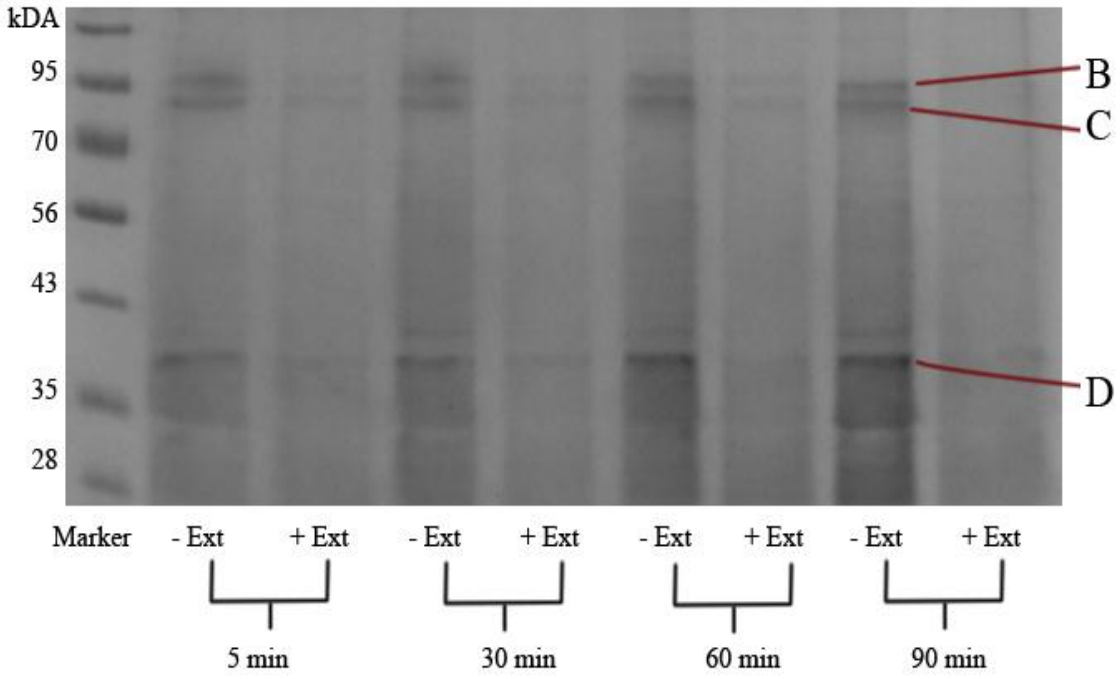


Figure 4.9: Protein expression profile of *S. aureus* ATCC 6538 (supernatant) following exposure to *C. formosana* extract. (-) and (+) indicated the absence or presence of plant extracts in bacterial culture medium, respectively (Yong and Wong, 2014).

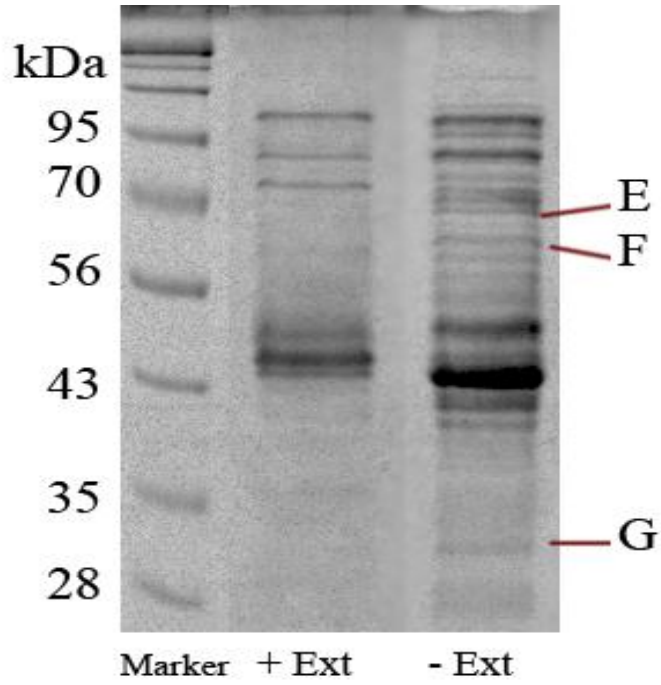


Figure 4.10: Protein expression profile of *P. aeruginosa* ATCC 27853 (pellet) following exposure to *M. candidum* extract. (-) and (+) indicated the absence or presence of plant extracts in bacterial culture medium, respectively.

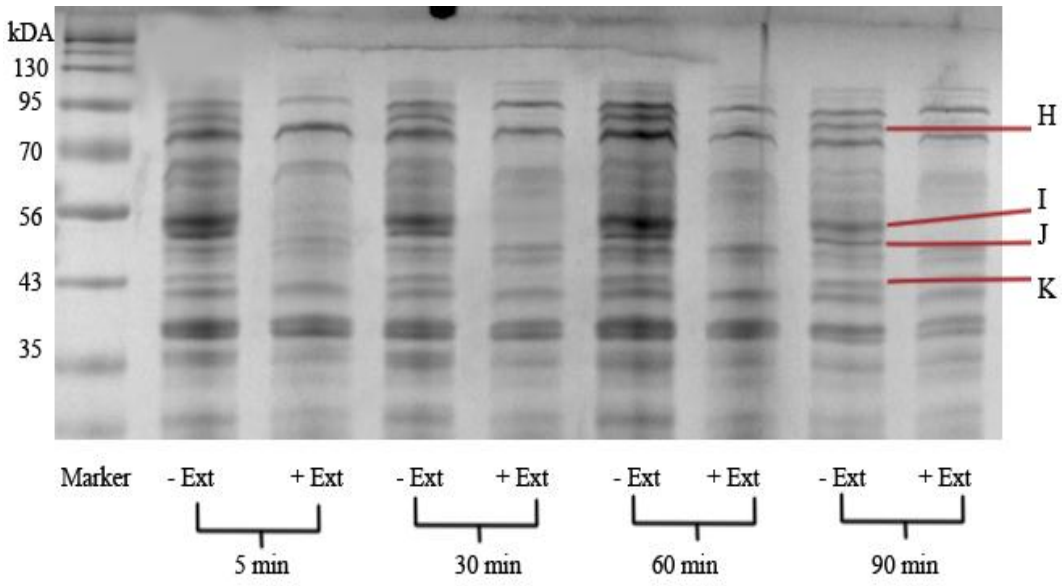


Figure 4.11: Protein expression profile of *E. coli* ATCC 35218 (pellet) following exposure to *Melastoma candidum* Extract. (-) and (+) indicated the absence or presence of plant extracts in bacterial culture medium, respectively (Wong and Yong, 2014).

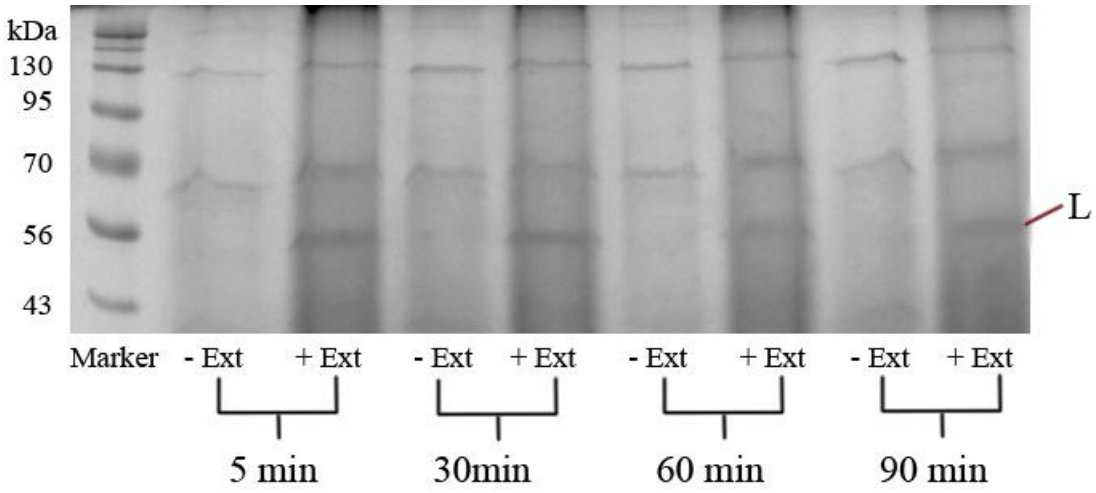


Figure 4.12: Protein expression profile of *E. coli* ATCC 35218 s) following exposure to *S. barbata* extract. (-) and (+) indicated the absence or presence of plant extracts in bacterial culture medium, respectively (Yong and Wong, 2014).

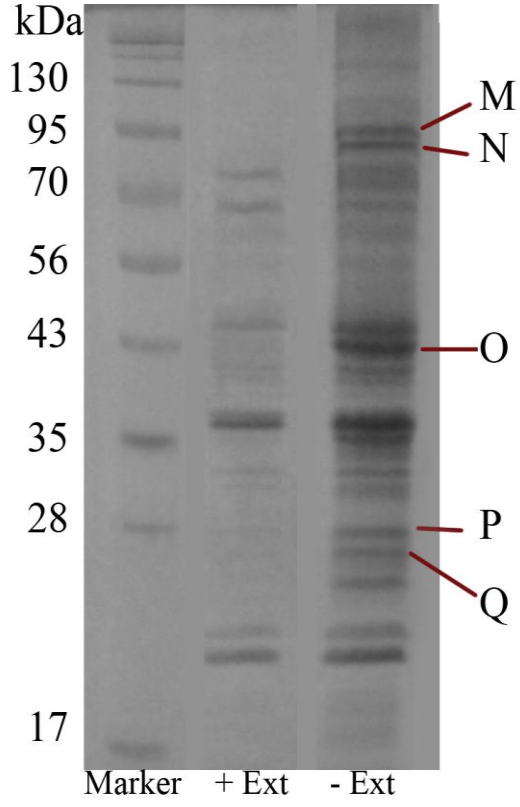


Figure 4.13: Protein expression profile of *E. coli* ATCC 25922 (pellet) following exposure to *M. candidum* extract. (-) and (+) indicated the absence or presence of plant extracts in bacterial culture medium, respectively (Yong and Wong, 2014).

4.6 Time-Kill Assay

In the time-kill assay, the results are presented in terms of mean changes in cells (log_{10} CFU/ml) of viable colonies. Bactericidal activity is defined as reduction being equal to $3log_{10}$ CFU/ml or greater in the viable colony count, compared to initial inoculum. The results are represented in Table 4.18 and Table 4.19 for *M. candidum* extract against Gram-positive bacteria and Gram-negative bacteria respectively.

At 1× MIC of *M. candidum*, all of the bacteria demonstrated regrowth. At 2× MIC, *S, aureus* ATCC 6538 showed sign of bacteriostatic activity after 8 hours of treatment. *S. aureus* ATCC 25923 demonstrated bacteriostatic activity after 10 hours of treatment. For MRSA ATCC 33591, bacteriostatic effect is noted after 24 hours of treatment. No bactericidal activity was noted. For *E. coli* ATCC 35218 and *E. coli* ATCC 25922, no bacteriostatic or bactericidal effect was noted. For *P. aeruginosa* ATCC 27853, after 24 hours of treatment, bactericidal effect was noted.

In 4× MIC, *S. aureus* ATCC 6538 has demonstrated bacteriostatic activity after 2 hours of treatment. After 24 hours, bactericidal activity was noted. For *S. aureus* ATCC 25923, bacteriostatic activity is only observed after 10 hours of treatment with the extract and at 24^{th} hour, bactericidal effect was noted. For MRSA ATCC 33591, bacteriostatic effect was noted after 10 hours of treatment. In *E. coli* ATCC 35218, bacteriostatic activity was observed at the 2^{nd} hour and

regrowth of bacteria was observed at the 4th hour. *E. coli* ATCC 25922 does not show any sign of bacteriostatic and bactericidal activity. For *P. aeruginosa* ATCC 27853, bactericidal activity was noted after 24 hours of treatment.

At 8× MIC, *S. aureus* ATCC 6538 has demonstrated bacteriostatic activity after 2 hours of treatment and then at the 8th hour, bactericidal activity was noted. For *S. aureus* ATCC 25923, bacteriostatic activity is only observed after 4 hours of treatment with the extract and at 24 hours, bactericidal effect was noted. For MRSA ATCC 33591, bacteriostatic effect was noted after 10 hours of treatment and bactericidal activity was seen after 24 hours. In *E. coli* ATCC 35218, bactericidal activity was observed after 24 hours of treatment. Treated *E. coli* ATCC 25922 shows sign of bactericidal activity at the 4th hour of treatment. For *P. aeruginosa* ATCC 27853, bacteriostatic activity was noted at the 6th hour while bactericidal activity was noted after 10 hours of the treatment.

Table 4.18: Mean Changes in Term of log_{10} CFU/ml from Initial Bacterial Concentrations Following Incubation of 2, 4, 6, 8, 10 and 24 h During Time–kill Assays at 1×, 2×, 4× and 8× MIC of *M. candidum* Against Gram-Positive Bacteria.

Hour / Bacteria	2h	4h	6h	8h	10h	24h
***S. aureus* ATCC 6538**						
1× MIC	1.3	1.3	1.2	1.0	1.6	2.0
2× MIC	1.3	1.1	1.1	-1.0[a]	-0.7[a]	-1.0[a]
4× MIC	-0.8[a]	-0.9[a]	-1.2[a]	-1.6[a]	-2.0[a]	-5.7[b]
8× MIC	-0.1[a]	-0.9[a]	-0.9[a]	-5.7[b]	-5.7[b]	-5.7[b]
Growth Control	1.5	1.5	2.8	3.1	5.7	5.7
***S. aureus* ATCC 25923**						
1× MIC	1.3	1.3	1.6	1.3	1.3	1.6
2× MIC	1.1	0.5	0.3	0.0	-0.2[a]	-5.7[b]
4× MIC	0.5	0.7	0.4	0.0	-0.9[a]	-5.7[b]
8× MIC	0.8	0.0	-0.8[a]	-0.4[a]	-1.0[a]	-5.7[b]
Growth Control	2.5	3.7	4.5	5.6	5.8	5.0
MRSA ATCC 33591						
1× MIC	0.4	0.7	0.6	0.5	1.1	2.0
2× MIC	0.4	0.5	0.2	0.5	0.1	-0.5[a]
4× MIC	0.3	0.2	0.3	0.1	-1.0[a]	-1.0[a]
8× MIC	0.2	0.4	0.0	0.0	-0.7[a]	-5.7[b]
Growth Control	2.4	2.6	4.3	4.6	5.6	5.7

MRSA, Methicillin resistant *Staphylococcus aureus*; CFU, colony-forming units;
a <3 log_{10} reduction in CFU, implies bacteriostatic effect.
b ≥3 log_{10} reduction in CFU, implies bactericidal effect.

Table 4.19: Mean Changes in Term of log_{10} CFU/ml from Initial Bacterial Concentrations Following Incubation of 2, 4, 6, 8, 10 and 24 h During Time–kill Assays at 1×, 2×, 4× and 8× MIC of *M. candidum* Against Gram-Negative Bacteria.

Hour / Bacteria	2h	4h	6h	8h	10h	24h
***E. coli* ATCC 35218**						
1× MIC	0.9	1.4	2.0	2.0	2.0	2.0
2× MIC	1.2	1.2	1.9	2.0	2.0	2.0
4× MIC	-0.9 [a]	1.1	1.3	1.7	2.4	2.9
8× MIC	1.1	0.6	0.7	0.6	0.6	-5.7 [b]
Growth Control	1.5	1.9	3.1	5.6	5.7	5.7
***E. coli* ATCC 25922**						
1× MIC	1.2	1.1	0.8	0.7	1.4	2.0
2× MIC	1.2	1.1	1.2	0.6	1.1	1.5
4× MIC	1.4	1.1	1.0	0.9	0.7	0.1
8× MIC	0.3	-5.7 [b]	-5.7 [b]	-5.7 [b]	-5.7 [b]	-5.7 [b]
Growth Control	4.9	4.9	5.6	5.7	5.8	6.0
***P. aeruginosa* ATCC 27853**						
1× MIC	2.0	1.4	1.1	0.8	0.6	-5.7 [b]
2× MIC	1.8	1.4	0.9	0.5	0.3	-5.7 [b]
4× MIC	2.0	1.8	1.0	0.6	0.3	-5.7 [b]
8× MIC	0.9	0.3	-0.7 [a]	-1.0 [a]	-5.7 [b]	-5.7 [b]
Growth Control	4.5	4.7	5.5	4.7	4.7	4.7

MRSA, Methicillin resistant *Staphylococcus aureus*; CFU, colony-forming units;
a <3 log_{10} reduction in CFU, implies bacteriostatic effect.
b ≥3 log_{10} reduction in CFU, implies bactericidal effect.

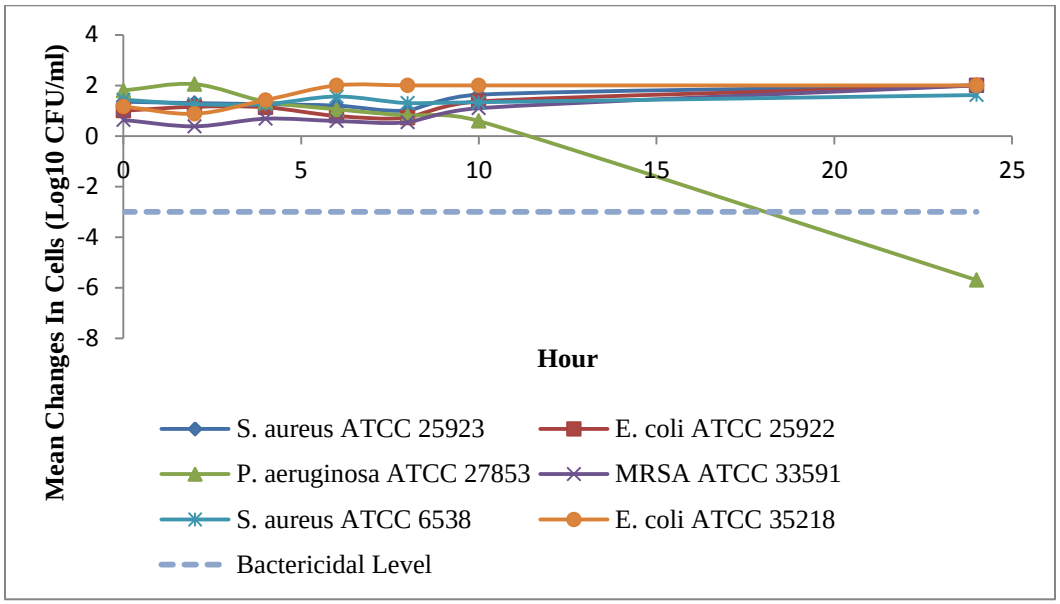


Figure 4.14: Mean Changes in Cells (Log_{10} CFU/ml) When Treated with 1× MIC of *Melastoma candidum*.

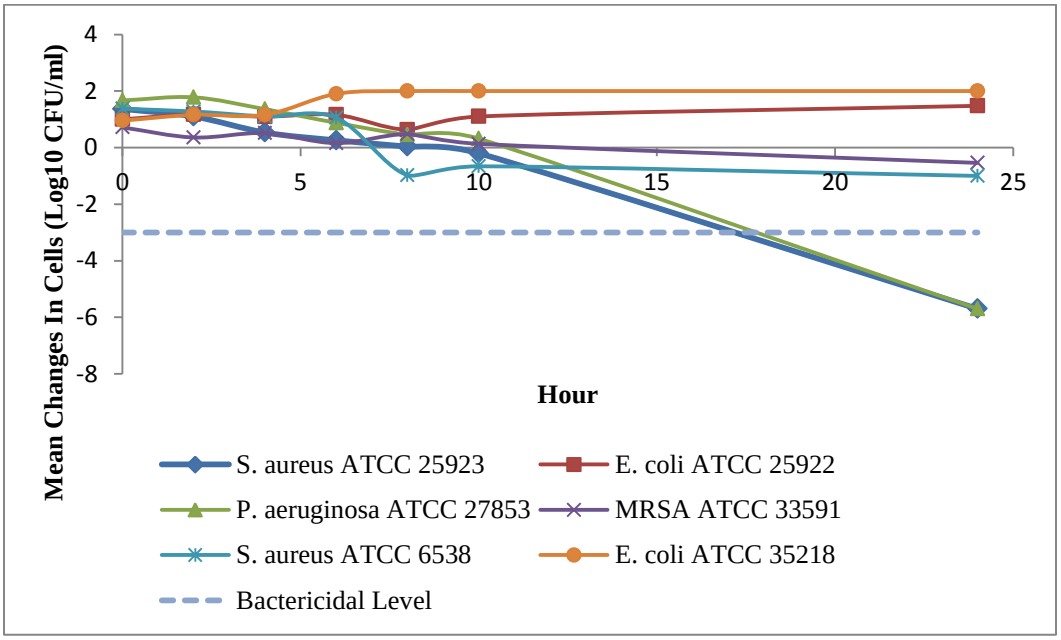


Figure 4.15: Mean Changes in Cells (Log_{10} CFU/ml) When Treated with 2× MIC of *Melastoma candidum*.

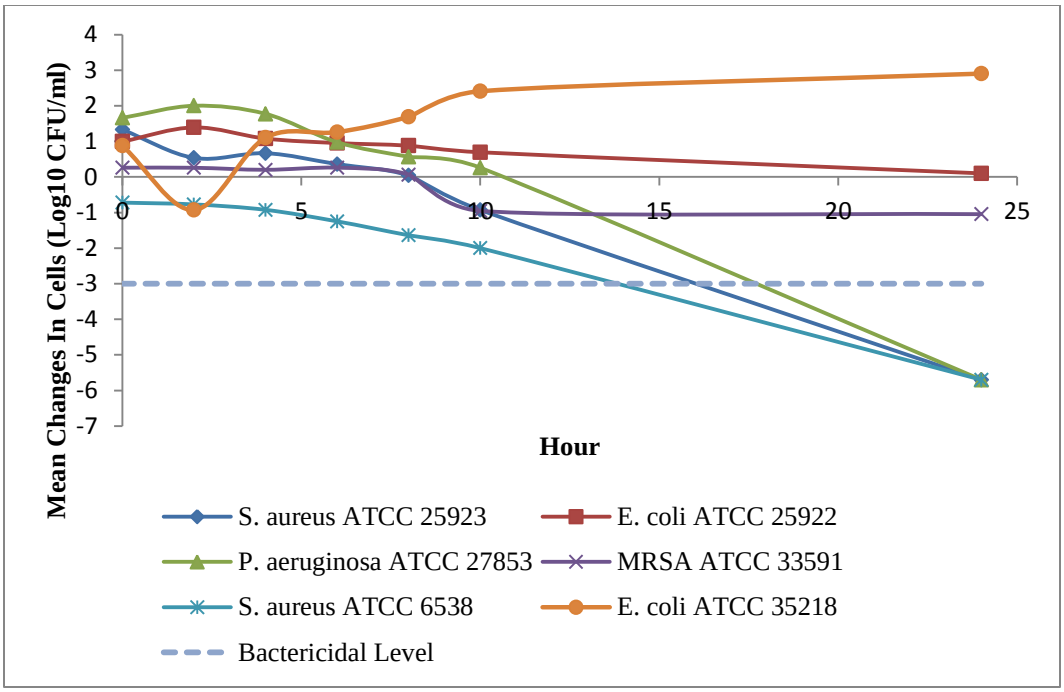


Figure 4.16: Mean Changes in Cells (Log_{10} CFU/ml) When Treated with 4× MIC of *Melastoma candidum*.

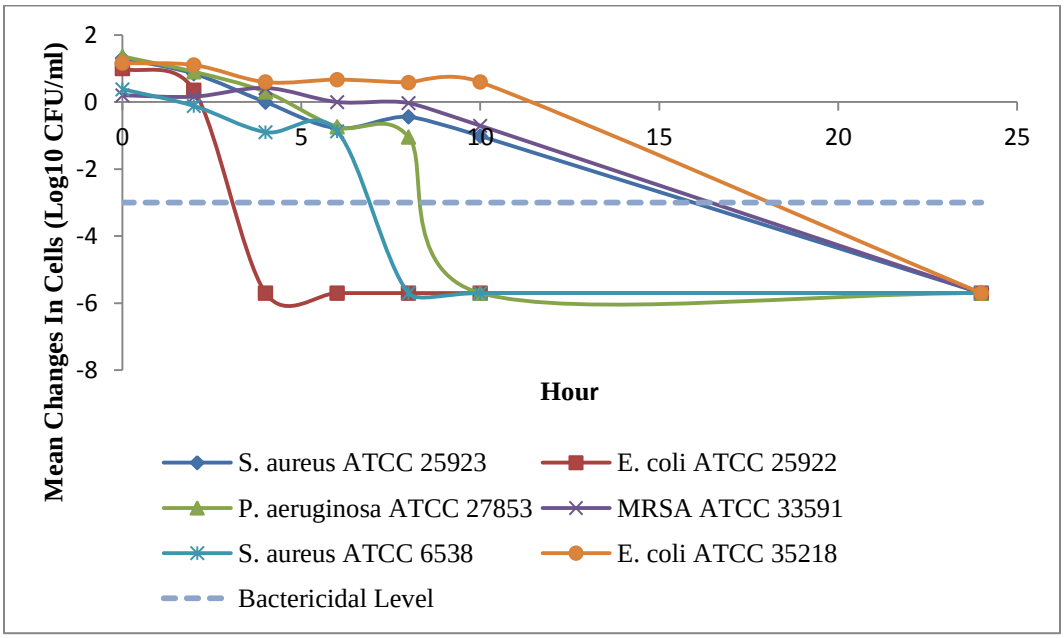


Figure 4.17: Mean Changes in Cells (Log_{10} CFU/ml) When Treated with 8× MIC of *Melastoma candidum*.

4.7 Investigation of Morphological Changes via Scanning Electron Microscopy

Using scanning electron microscope, the morphological changes on bacteria (*S. aureus* ATCC 6538, *S. aureus* ATCC 25923, MRSA ATCC 33591, *E. coli* ATCC 35218, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853) were determined, through treatment of the antibacterial plant extracts as shown in Figure 4.19 to Figure 4.34. The extracts have shown to be able to affect the bacteria morphologically where the treated cells structure deviate from the untreated bacteria cells (Control).

In *S. aureus* ATCC 6538, the control cells were grape-shaped and their cell surfaces are intact and there was no damage to be seen. However in the treated cells with *M. candidum* and *S. barbata* extracts, the cells did not retain its grape-shape and there are many fragments on the cell surfaces, making it uneven indicating the damage of cell surfaces (Yong et al., 2015).

In *S. aureus* ATCC 25923, the control cells were grape-shaped and their cell surfaces are intact. However bacterial cells treated with *M. candidum* and *C. formosana* extracts appeared to be damaged and in the case of bacterial cell treated with *C. formosana*, it can be seen that some of the bacteria cells appear to have indentation.

In MRSA ATCC 33591, the untreated bacterial cells retained its grape-shape and its surfaces appeared to be smooth while in the treated bacterial cells, the cells appeared to be heavily damaged and it does not retain its grape-shaped surface. Fragments of the bacterial cells could be seen, indicating the damaged bacterial cells.

In *E. coli* ATCC 35218, the control cells shown to be in perfect rod shape and no damage on the cell surfaces were detected. However, in the treated cells, irregular fragments appeared and the cells did not retain much of its rod shape. The cells' surface was uneven and the cells are damaged extensively (Yong et al., 2015).

In *E. coli* ATCC 25922, the untreated bacterial cells are shown to be in perfect rod shape and there were no damages found on its surface. Though in the treated bacterial cells, it can be seen that some of the bacterial cells does not grow into its full length and the surface of the cells are fragmented. Some of the bacterial cells seem to be bending onto itself as well.

In *P. aeruginosa* ATCC 27853, the controlled cells retained its rod shape while the *M. candidum* treated cells' shape looked to be irregular.

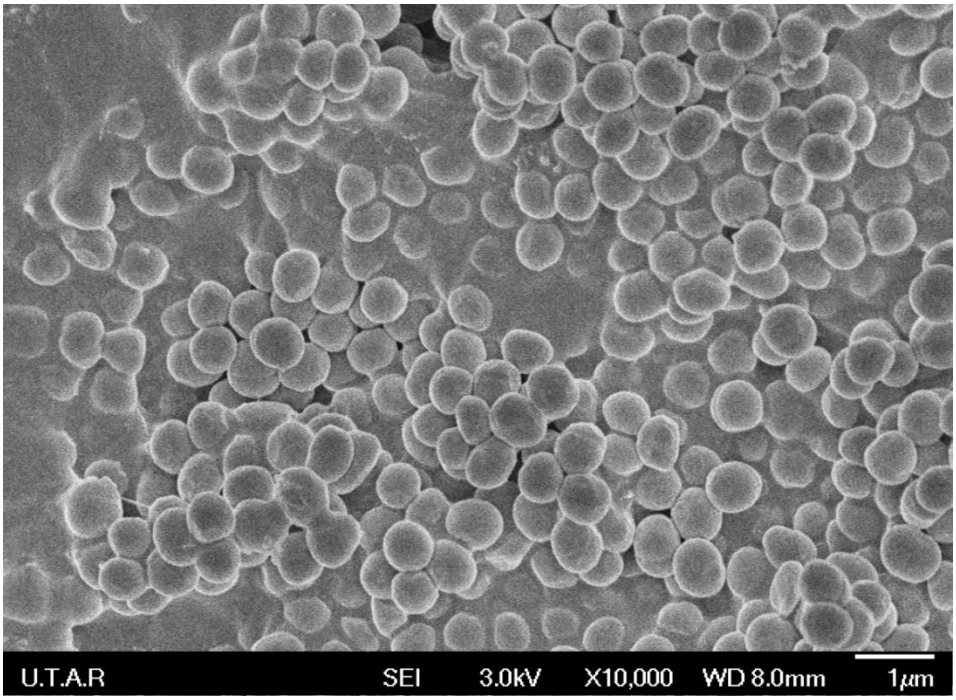


Figure 4.18: Morphology of untreated *S. aureus* ATCC 6538 (Control) (Yong et al., 2015).

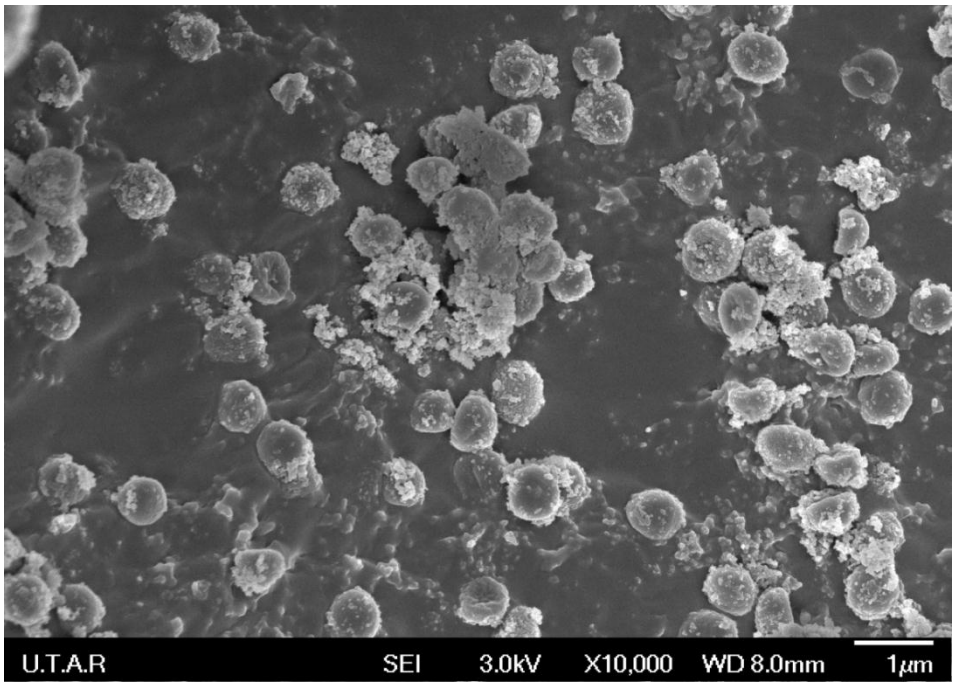


Figure 4.19: Morphology of *S. aureus* ATCC 6538 treated With *Melastoma candidum* Extract (Yong et al., 2015).

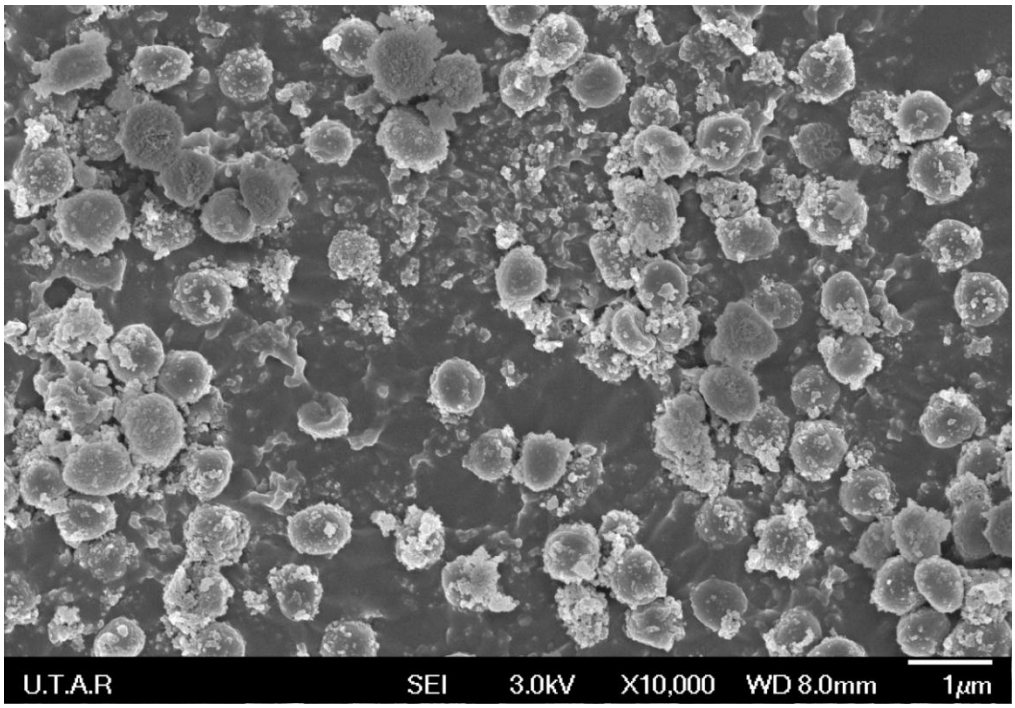


Figure 4.20: Morphology of *S. aureus* ATCC 6538 treated With *Scutellaria barbata* Extract.

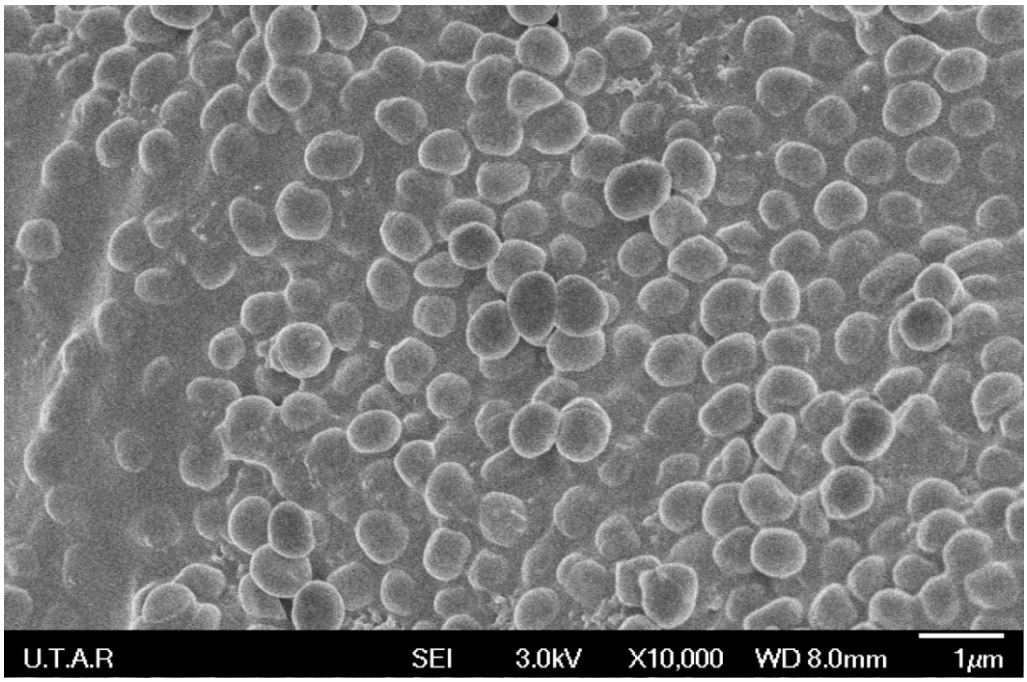


Figure 4.21: Morphology of untreated *S. aureus* ATCC 25923 (Control).

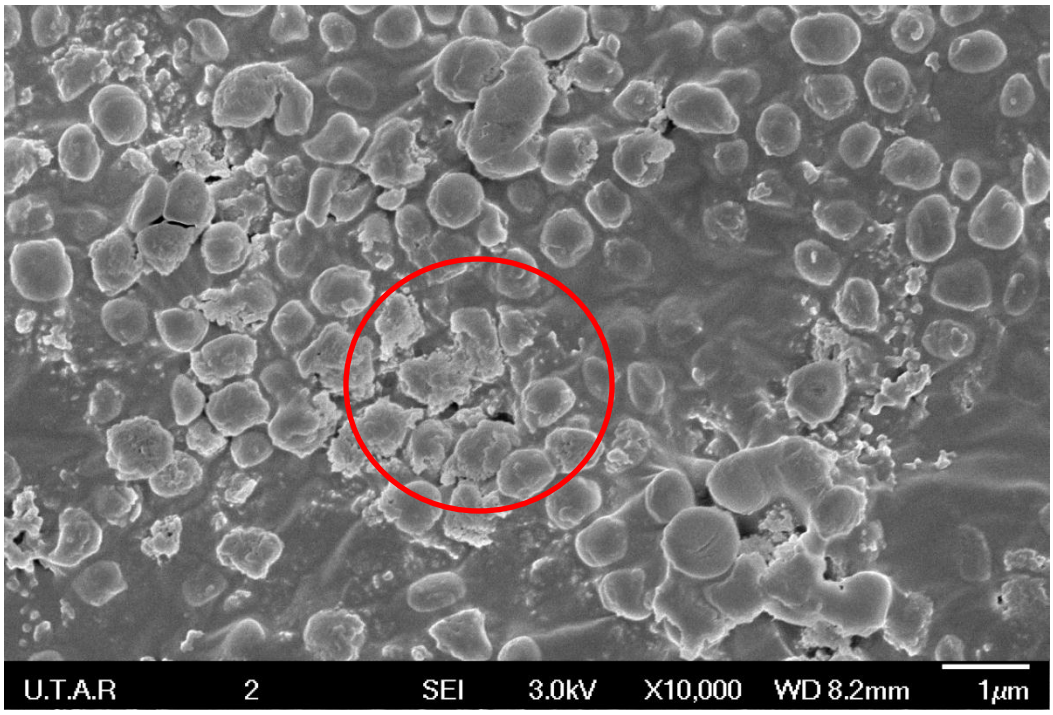


Figure 4.22: Morphology of *S. aureus* ATCC 25923 treated With *Melastoma candidum* Extract.

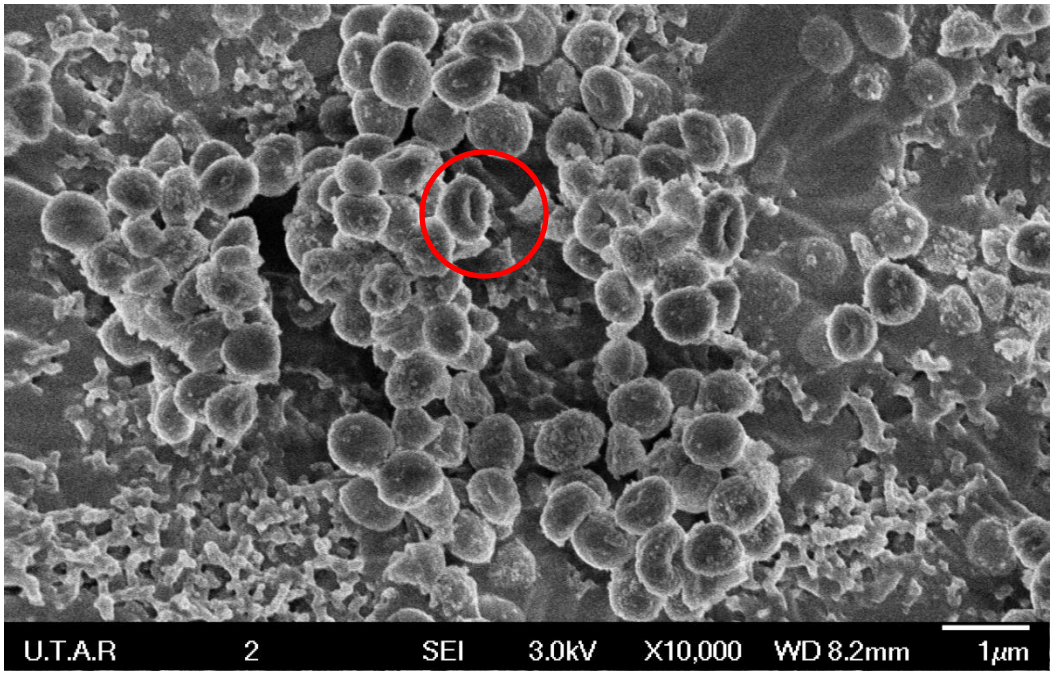


Figure 4.23: Morphology of *S. aureus* ATCC 25923 treated With *Callicarpa formosana* Extract.

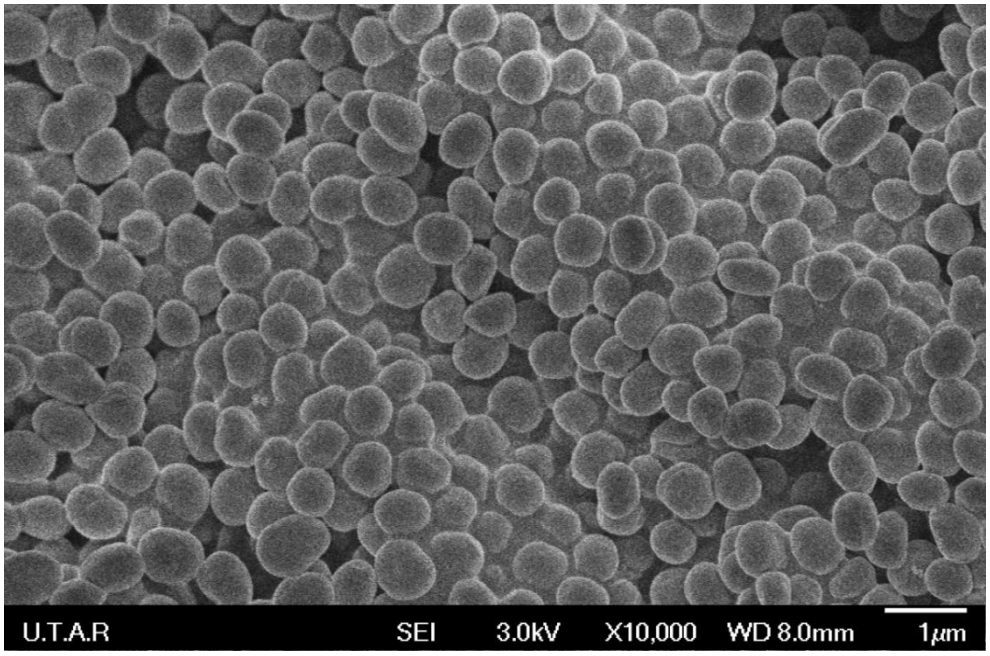


Figure 4.24: Morphology of untreated Methicillin-Resistant *S. aureus* ATCC 33591 (Control).

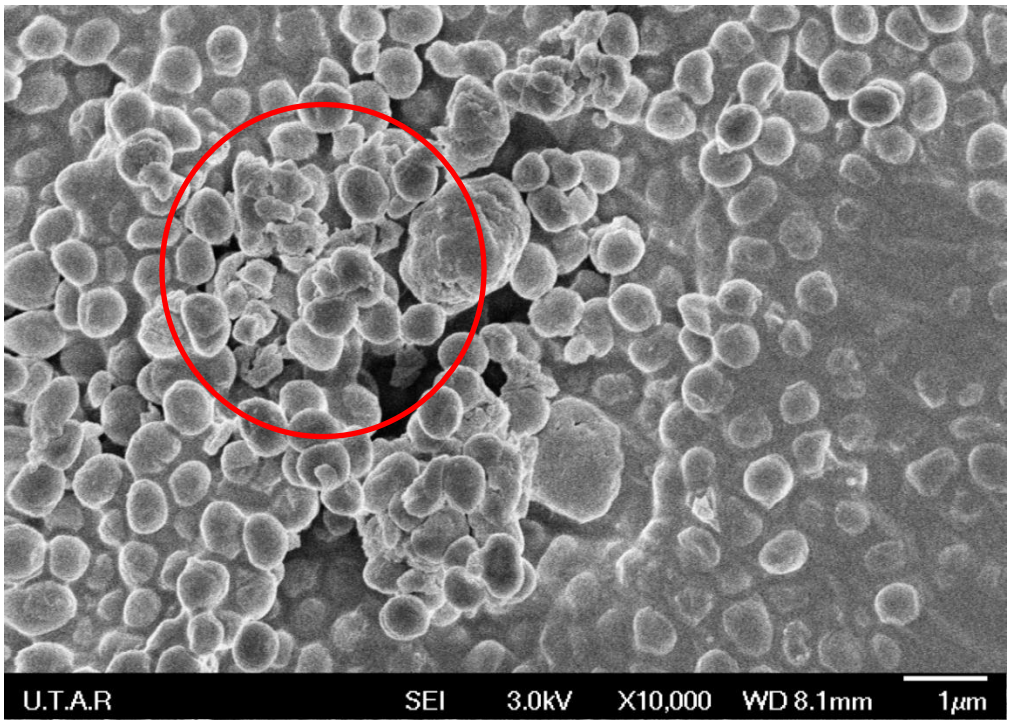


Figure 4.25: Morphology of Methicillin-Resistant *S. aureus* ATCC 33591 treated With *M. candidum* Extract.

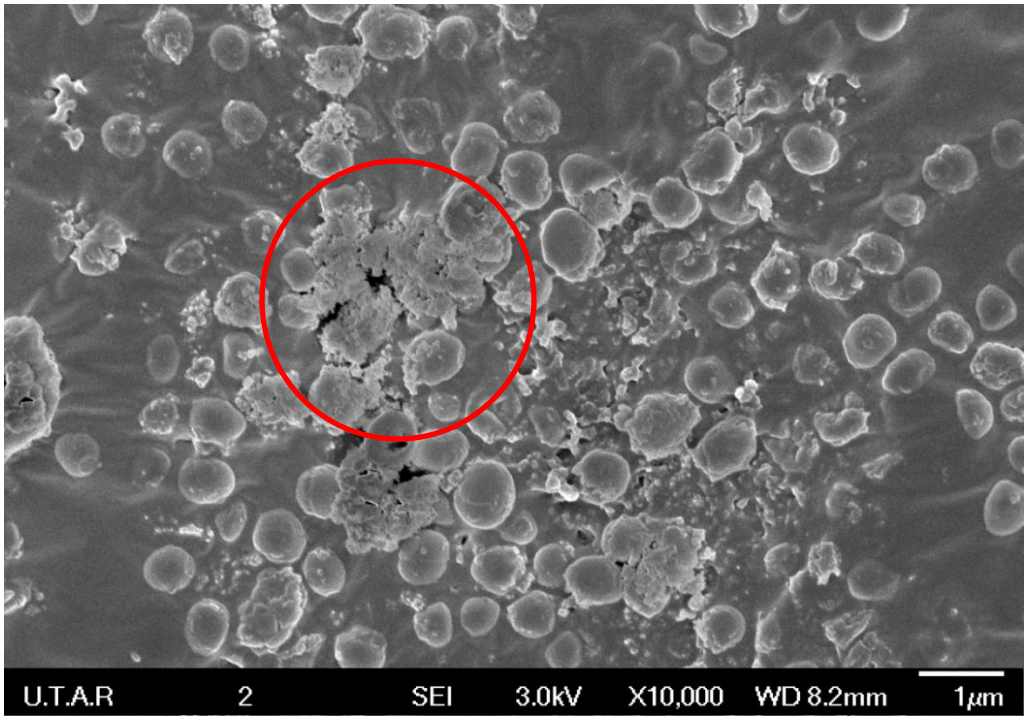


Figure 4.26: Morphology of Methicillin-Resistant *S. aureus* ATCC 33591 treated with *Callicarpa formosana* Extract.

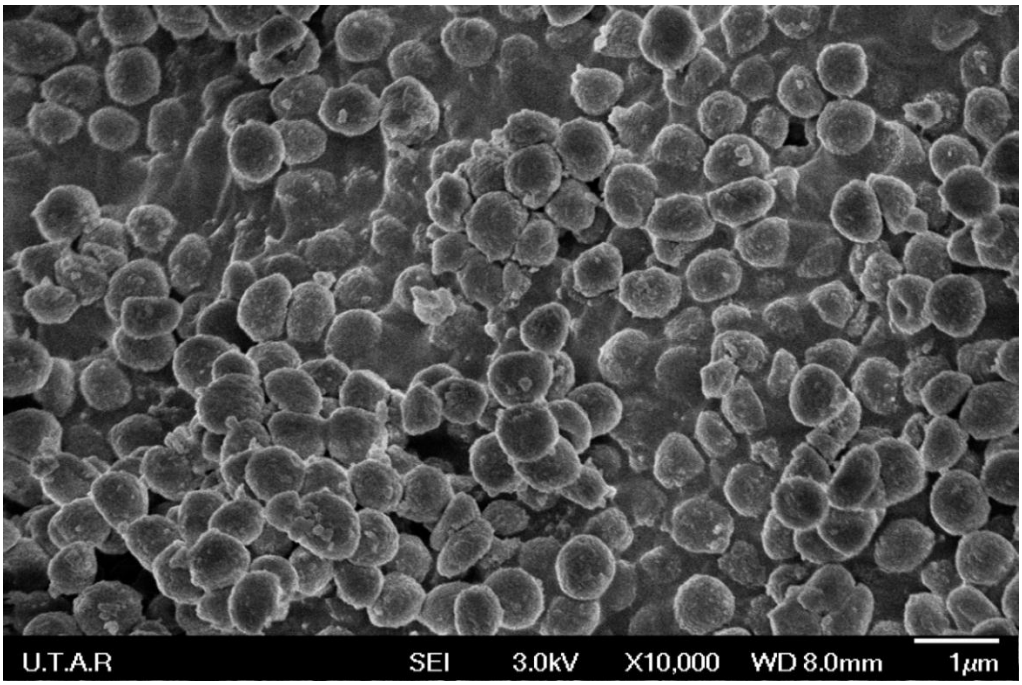


Figure 4.27: Morphology of Methicillin-Resistant *S. aureus* ATCC 33591 treated with *Scutellaria barbata* Extract.

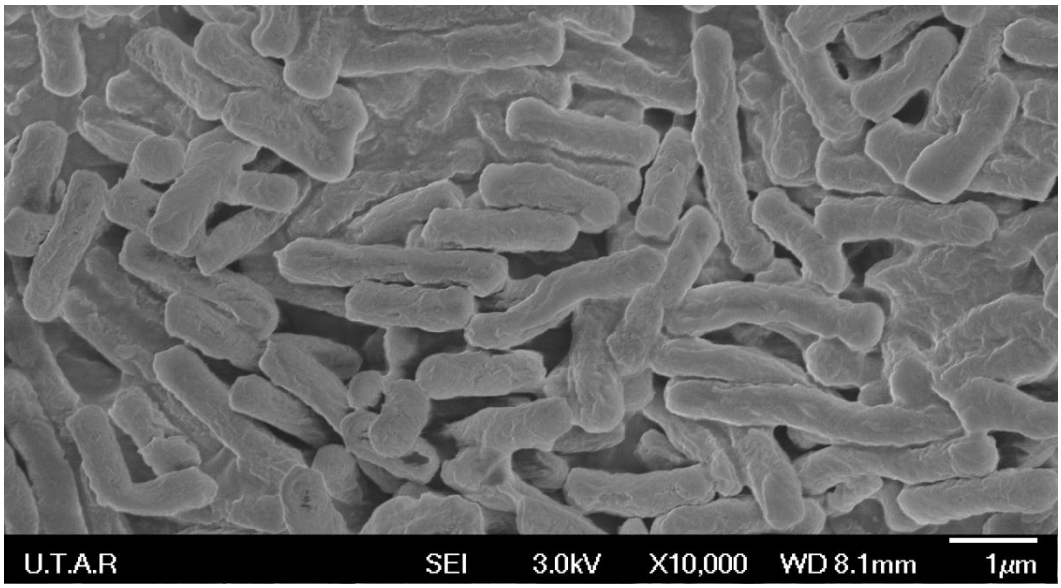


Figure 4.28: Morphology of untreated *E. coli* ATCC 35218 (Control) (Yong and Wong, 2014).

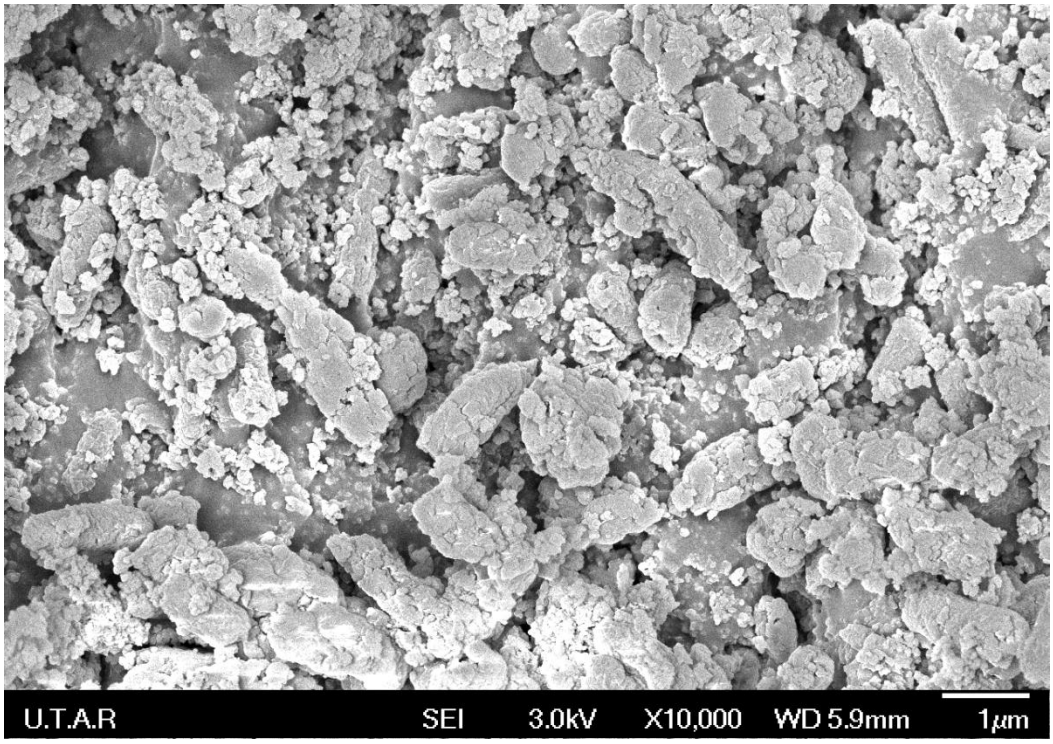


Figure 4.29: Morphology of *E. coli* ATCC 35218 treated With *M. candidum* Extract (Yong and Wong, 2014).

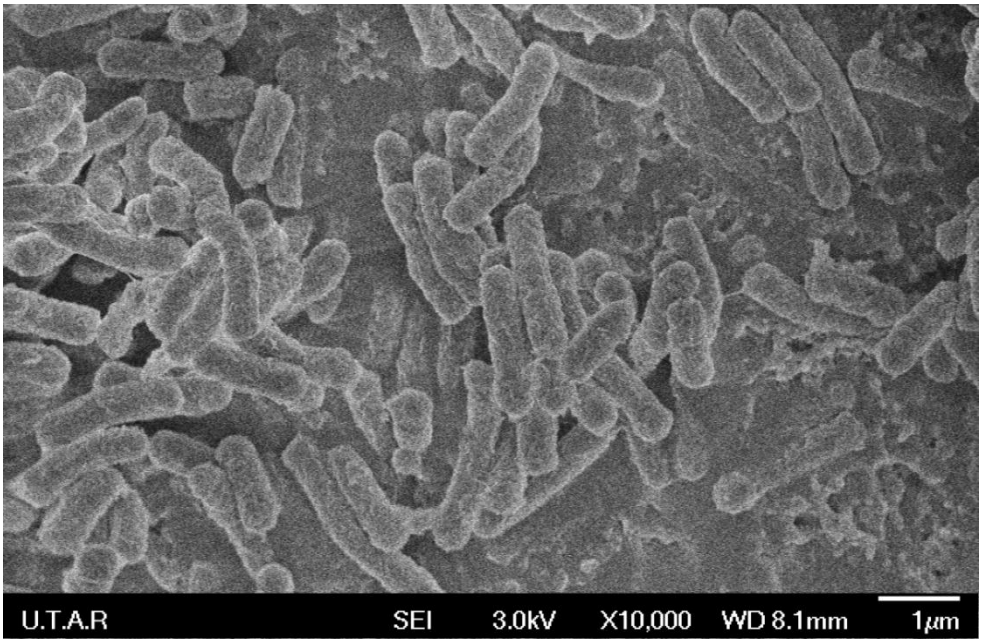


Figure 4.30: Morphology of untreated *E. coli* ATCC 25922 (Control).

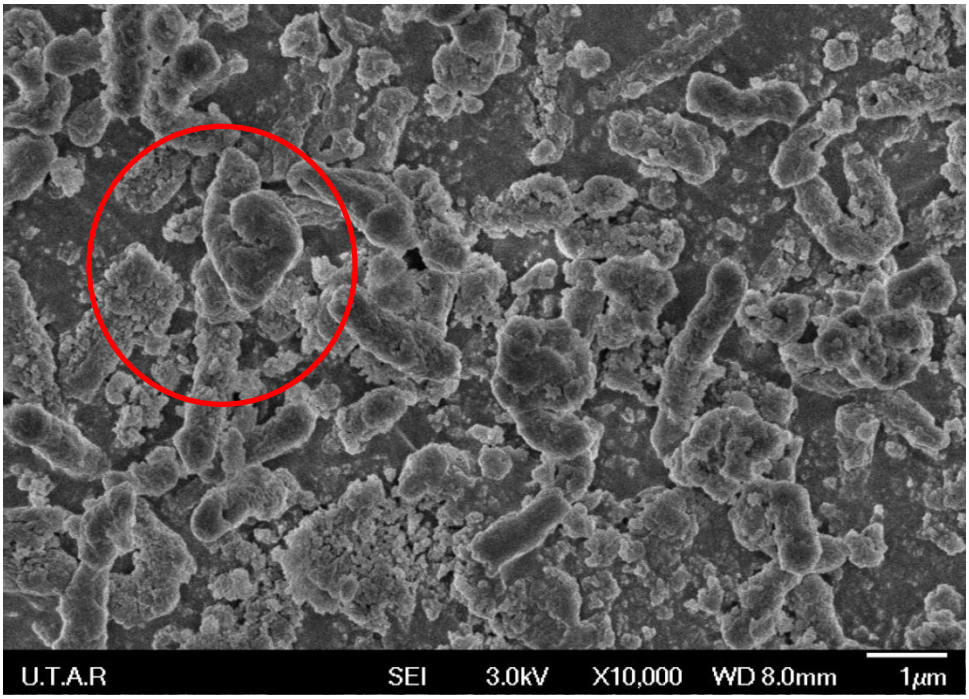


Figure 4.31: Morphology of *E. coli* ATCC 25922 treated With *M. candidum* Extract.

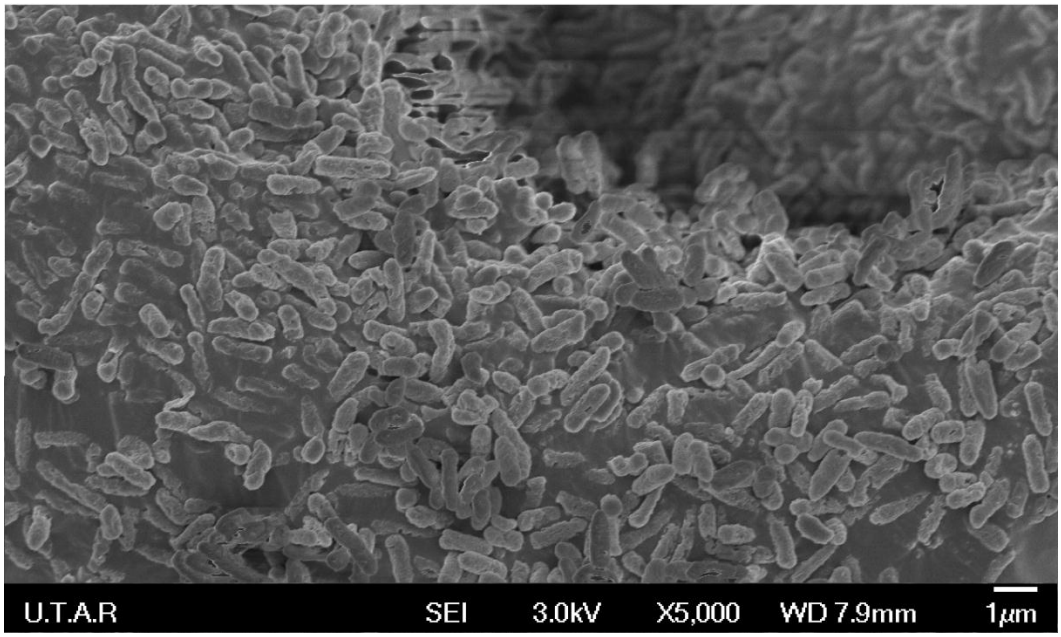


Figure 4.32: Morphology of untreated *P. aeruginosa* ATCC 27853 (Control).

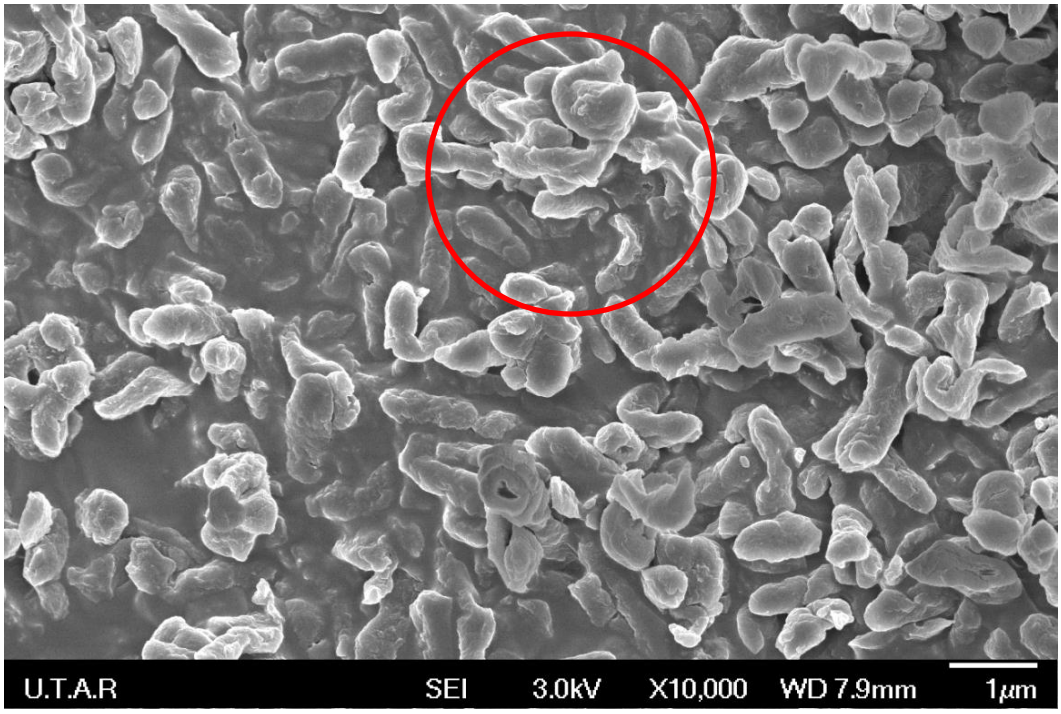


Figure 4.33: Morphology of *P. aeruginosa* ATCC 27853 treated With *M. candidum* Extract.

CHAPTER 5

DISCUSSION

5.1 Antioxidant Study of Plant Extracts

5.1.1 Determination of 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity of Plant Extracts

Free radicals have been known to cause several human diseases (Harman, 1958; Halliwell and Gutteridge, 1997). 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) is known as a stable free radical with an unpaired valence electron located at one atom of nitrogen bridge (Eklund et al., 2005). DPPH radical has a deep violet colour in solution and become a pale yellow once it was neutralized by antioxidants. The strong absorption of the violet colour at 520 nm made it possible for the quantification of the DPPH radical scavenging activity. The change in the optical absorption at 520 nm can be measured using UV-spectroscopy.

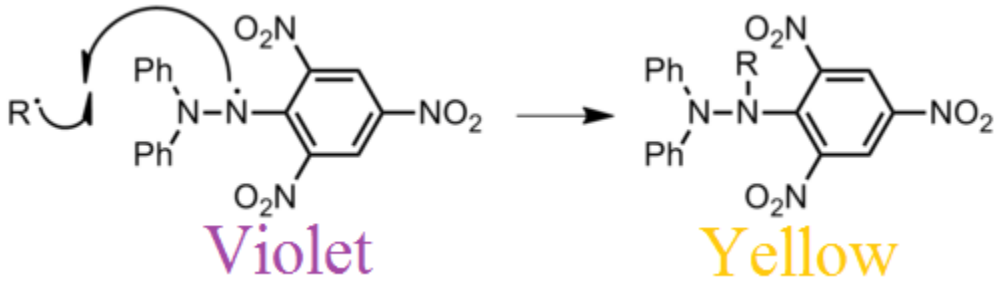


Figure 5.1: DPPH Radical Reaction.

Thus scavenging of DPPH radicals is the basis of a common antioxidant assay. *Callicarpa formosana, Lonicera japonica, Melastoma candidum* and *Scutellaria barbata* have shown to have good DPPH radical scavenging activities. This could be implicated that there are high content of antioxidants in these plants. Antioxidants are able to protect and prevent damages caused by free radicals (Devasagayam et al., 2004). At concentration of 16 mg/ml, these four plant extracts were shown to have activities similar to that of ascorbic acid which is a good antioxidant.

5.1.2 Ferrous Ion Chelating (FIC) Assay

In FIC assay, ferrozine was used to determine the chelating effect of plant extracts. Ferrozine is among the compounds used for quantitating iron colorimetrically. It is a very effective chelator for ferrous iron and has been used in the determination of iron in biological samples (Fish, 1988). Ferrozine reacts with ferrous ion to form stable red coloured complex species which absorbs strongly at 550 nm. The antioxidant activities are able to be determined through the measurement of colour reduction.

From the result as shown in Table 4.2, most of these plants have shown to be very good ferrous ion chelator compared to control. The ability of plant to chelate metal ions is of great importance. There have been reports stating that transition metals such as iron have a major role in the formation of oxygen free

radicals in living organisms. The formation of these radicals can cause DNA damage, lipid peroxidation and protein modification (Welch et al., 2002). Beside this, a variety of disease and disorder may occur such as Alzheimer's disease (Finefrock et al., 2003). Chelating these metal ions can be a preventive mechanism against these free radical reactions (Lopes et al., 1999; Sugihara et al., 2001). Furthermore, this chelation can prevent metal redox cycling, formation of lipid intermediates and formation of insoluble metal complexes (Hider et al., 2001; Moridani et al., 2003; Rice-Evans et al., 1996).

EDTA, a known metal chelator has been known to be used medically to treats acute to chronic lead and other heavy metal poisoning. However, there are also side effects that came with EDTA treatment such as allergic reaction and even organ damages (Ruth DeBusk et al., 2002). Thus, the use of plant as a natural source of metal chelator may prove to be beneficial. For instance, a study done by Zhang et al. (2006) has shown that polyphenols are able to reduce liver damage generated by iron overload in mice.

5.1.3 Determination of Nitric Oxide Scavenging Activity of Plant Extracts

Reactive nitrogen species (RNS or Nitric oxide, NO•) has a vital role in the generation of free radicals. NO• is a reactive radical which has a role in diverse physiological processes such as blood pressure regulation, defense mechanism, neurotransmission and immune regulation (Rao et al., 2011).

However, the overproduction of this reactive nitrogen species is known as nitrosative stress and it has been implicated to be an important contributor to the development of neurodegeneration (Chung, 2007).

Nitric oxide in our body can acts as free radical, electrophile and an oxidizing agent. Despite its stability as a free radical, nitric oxide may react with oxygen to form nitrogen dioxide which can initiate auto-oxidation of fatty acid in lipid membranes of a biological system, thus resulting in membrane damage because of its ability to cleave hydrogen from unsaturated fatty acids (Steinert et al., 2010).

In this assay, fifteen plants were tested of their antioxidant activities by measuring the NO radical scavenging activities. Sodium nitroprusside will give rise to nitric oxide at a physiological pH. The nitric oxide reacts with oxygen to produce nitrite ions which can be determined with Griess reagent. The scavenger of this nitric oxide will compete with oxygen, leading to the reduced production of nitrite ions. Thus the scavenging activity of the extracts on nitric oxide can be measured (Tsikas, 2007). Most of the plants except *Opuntia ficus-indica, Rhloe discolor* and *Zebrina pendula* have shown to have good nitric oxide scavenging activity. It is known that plants contain abundance of antioxidants and it is important in preventing damages done due to nitric oxide free radical.

5.1.4 Determination of Total Flavonoid Contents of Plant Extracts

Total flavonoid assay was carried out to determine the flavonoid contents in the plants. Flavonoids are polyphenolic compounds which are present in most of the plants. Flavonoids are classified into flavanols, flavanones, flavones, iso-flavones, catechins, anthocyanins, proanthocyanidins which possess potent antioxidant activity. Recently, flavonoids have aroused the interest of researcher due to their potential and beneficial effects on human health.

From the result gotten, *C. formosana, M. candidum* and *S. barbata* have shown to have the highest content of total flavonoids. Flavonoids have been used for centuries in the treatment of human diseases (Choudary, 2010). Isolated flavonoids were shown to have various bioactivities such as antibacterial activity (Alarcón et al., 2008). A study done by Ali and Dixit (2012) has shown that flavonoid has antibacterial activity against bacteria which causes urinary tract infection.

5.2 High Performance Liquid Chromatography (HPLC)

Phytochemical analyses were done using HPLC to determine the presence of specific phytochemical in the plant extracts. Concentration plots of four standard phenolic compounds (gallic acid, quercetin, caffeic acid and catechin) were prepared (Appendix F) and used to quantify phenolic constituents of these medicinal plants extracts. The concentration of each constituent was calculated

and summarized in Table 4.5. Quercetin was detected in all five extracts in the following descending order: *V. amygdalina* > *P. bleo* > *C. formosana* >*M. candidum* > *C. nutans*. Gallic acid was detected in *M. candidum* which was 15-fold higher than which was observed in *C. formosana*. Catechin was only observed in *C. formosana* and *V. amygdalina*. Caffeic acid was detected in all plants except *V. amygdalina*.

Quercetin, a flavonoid-type flavonol derivative was detected at a moderate level in *M. candidum* and *C. formosana*. It was previously reported that quercetin and other flavonol derivative to be able to inhibit growth of *S. aureus* (Liu et al., 2009; Rodr´ıguez Vaquero et al., 2007). Thus it is possible that quercetin found in *M. candidum* has a significant role in the inhibitory activity against *S. aureus*.

HPLC analysis has also indicated the presence of catechin, a flavonoid type flavan-3-ol derivative in *C. formosana*. Catechin derivative which has been found to be abundant in green tea has been previously reported as the bioactive compounds responsible for bacterial inhibitory activities (Daglia, 2012). Catechin may be the compound contributing in a similar manner in inhibitory activity observed in *C. formosana*.

Gallic acid, a non-flavonoid hydroxybenzoic acid derivative was detected in both *C. formosana* and *M. candidum*. Chanwitheesuk et al. (2007) had identified gallic acid as the compound responsible for the inhibitory action of

Caesalpinia mimosoides against both Gram-negative and Gram-positive bacteria. Previous studies have highlighted the importance of synergistic effects among the variety of plant-derived bioactive compounds (Eloff, 1998; Rates, 2001). It is highly likely that the detected quercetin, catechin and gallic acid could interact in a synergistic manner to contribute to the bacterial inhibitory activities found in *C. formosana* and *M. candidum.*

5.3 Antibacterial Assay of Plant Extracts

The indiscriminate use of antimicrobial drugs in treating infectious diseases causes the occurrence of multiple drug resistance bacteria (Davis, 1999; Service, 1995). To add to this problem, antibiotics are also known to have adverse effects on the host such as allergic reactions, hypersensitivity and immune-suppression (Ahmad et al., 1998). This situation has led the scientist to search for new antibacterial substances and effective therapeutic agents given the distressing frequency of antibiotic resistance in bacteria (Monroe and Polk, 2000; Bhavnani and Ballow, 2000). Due to the side effects and the resistance build against antibiotics by pathogenic microorganisms, many researches were focused in isolating the biologically active compounds of medicinal herbs (Essawi and Srour, 2000). Antibacterial agent of plant origin has immense therapeutic potential and alleviating many of the side effects which comes with synthetic antibiotic.

There are several ways in investigating antibacterial effects of plant extract, and one of the ways is determining the minimum inhibitory concentration (MIC). Agar diffusion and dilution tests are some of the ways in determining MIC of the plant extracts, however the former methods has its limitation as it is unable to quantify the data. Dilution test such as the utilization of microplate and *p*-iodonitrotetrazolium chloride as an indicator of bacterial metabolism offers a quick and sensitive method as only viable cells are detected. It is a flexible and popularly used technique which works well with fast-growing bacteria such as *S. aureus*, *E. coli* and *P. aeruginosa* (Eloff, 1998). This method works well with human and plant fungal pathogens and also microaerophilic acidogenic oral bacteria (Eloff et al., 2005; Masoko et al., 2007).

Fifteen plants were initially screened for their antibacterial activities. These plants undergo aqueous extraction to extract its compounds. These extracts were then subjected to microplate MIC tests in order to distinguish which plants have the most antibacterial activity. *M. candidum, C. formosana* and *S. barbata* were shown to have good antibacterial activities with *M. candidum* showing antibacterial activity towards Gram-positive and Gram-negative bacteria while *C. formosana* and *S. barbata* only shows good antibacterial activity towards Gram-positive bacteria only.

These three plants then underwent sequential extraction with hexane, ethyl acetate, acetone, methanol and water. Each of these extracts were then subjected

to MIC test again and from the test done, it was shown that plant extracted with the solvent methanol have good antibacterial activity due to it having the lowest MIC value. From the observation, it can be rationalize that the antibacterial compounds are polar in nature thus dissolving and diffusing in methanol which is a polar solvent. Among the three plants, *M. candidum* is a great potential source of antibacterial agents because its extracts are able to inhibit both Gram-positive and Gram-negative bacteria.

M. candidum methanol extract then underwent time-kill assay to study its bacteriostatic and bactericidal effect and the result is depicted in Table 4.18 and 4.19. Time-kill study was performed due to its ability in determining the kinetics of bacterial killing *in vitro* and to distinguish whether the killing of bacteria is concentration dependent whereby the rate and extent of killing increases as the concentration of the antibacterial concentration increases (Pankey and Sabbath, 2004).

M. candidum seemed to have great potential in bacteriostatic and bactericidal effect. Incubating the bacteria with *M. candidum* extract at 4× MIC showed a rapid reduction in the viable cell count at 24 hours for *S. aureus* ATCC 25923, *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 27853. The reduction was greater than that of the rate of kill seen in bacteria treated with only 1× MIC except for *P. aeruginosa* ATCC 27853 where the extract was already bactericidal at that concentration. The reduced bacteria cell counts during interval between 10

and 24 hours of incubation period showed that the extract was greatly bactericidal, seeing that there was no regrowth after incubating for 24 hours. The inhibition of the bacteria growth and efficacy of the *M. candidum* extract were observed to be dose dependent. Different bacteria reacted differently to the concentration of the antibacterial compounds. Some bacteria are more resistant to the extracts than the other. Antibacterial activity in plant might be significantly influenced by microbial infection. The phenolic compounds that are synthesized in the plants are a response towards the infection (Schinor, 2007).

Distinguishing whether an antibacterial agent is bacteriostatic and bactericidal towards a particular bacterium is important when administering an antibacterial agent into human. Some infection are best treated with antibacterial agent that exhibits bacteriostatic action as it is considered more gentle and would not trigger a response from dying bacteria which might be toxic to human cells (Pankey and Sabath, 2004).

5.4 Evaluation of Antimicrobial Compounds

Bioautography was performed in order to determine the area of antibacterial compound. *p*-iodonitrotetrazolium chloride was employed to detect the inhibition area and the scrapped area was extracted of its compound and subjected to gas chromatography-mass spectrometry analysis.

In our GC-MS analysis, the compounds were identified through comparing it to the NIST library. A total of seven compounds (Table 4.12) were identified in *M. candidum* methanol fraction and a total of six compounds (Table 4.13) were identified in *C. formosana* respectively. The compounds detected from the analysis were butanoic acid, 2-heptanamine, 2, 4-bis-(1, 1-dimethylethyl)-phenol, 1-propanone, 1-butyl 2-(2-ethylhexyl) phthalate, hexadecanoic acid, benzenepropanoic acid, cathinone, and aminononadecane. Most of the compounds found in the analysis were known to be antimicrobial compounds.

From the analysis done, there are several compounds [2-heptamine, 2, 4-bis-(1, 1-dimethylethyl)-phenol, 1-propanone, hexadecanoic acid, benzenepropanoic acid] which were found to be present in both plants. Butanoic acid and 1-butyl 2-(2-ethylhexyl) phthalate were found only in *M. candidum* fraction while aminononadecane was found in *C. formosana*.

One of the compounds which shows the most promising antibacterial compound was 2, 4-bis-(1, 1-dimethylethyl)-phenol which was found in both methanol fractions of both plants. A study done by Abdullah et al. (2011) on the antibacterial activity of Malaysia mango kernel has found that 2, 4-bis-(1, 1-dimethylethyl)-phenol was identified as the major compound responsible for the antibacterial activity. 2, 4-bis-(1, 1-dimethylethyl)-phenol is a phenolic compound, one of a class of plant secondary metabolites known to act as

precursors to structural polymers or serve as signal molecules (Nicholson and Hammerschmidt, 1992; Dakora, 1996; Mauch-Mani and Métraux, 1998).

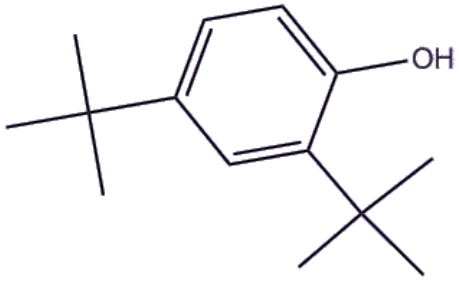


Figure 5.2: Structure of 2, 4-bis-(1, 1-dimethylethyl)-phenol.

These structurally diverse groups of plant compounds are known to play a role in plant defense against pathogens and also activators of plant defense gene. The oxidation of phenols leads to the formation of quinones and free radicals that are able to inactivate enzymes and other proteins which are important to the pathogens (Patil and Dimond, 1967; Appel, 1992). Moreover, oxidized phenols have shown to have enhanced antimicrobial activity and thus these compounds may be responsible in blocking pathogen development (Urs and Dunleavy, 1975).

Another potential antibacterial compound was 1-butyl 2-(2-ethylhexyl) phthalate which was only found in methanol fraction of *M. candidum.* Phthalates were reported to have antimicrobial and other pharmacological activities. The presence of phthalate compound has previously been reported in the aerial parts of *B. retusa* (Sanseera, 2012) and *C. serrata* (McNeil et al., 2012). Both of these plant species demonstrated inhibitory activities against *E. coli*, *S. aureus* and *P. aeruginosa*. Srinivasan et al. (2009) has reported that the high percentage of

phthalate in *L. indica* flower was responsible for the antibacterial activity against Gram-negative bacteria *E. coli* and *S. typhimurium.*

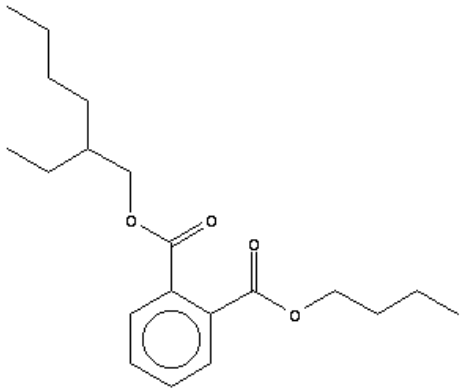


Figure 5.3: Structure of 1-Butyl 2-(2-ethylhexyl) Phthalate.

Hexadecanoic acid also known as palmitic acid was found in both of the plants and this compound has been reported with antibacterial activities (Velmurugan et al., 2012; Jain et al., 2012; Dhankhar et al., 2012; Pavithraa et al., 2009). Furthermore, it was well known that this compound is a very strong antiseptic thus having a wider range of antimicrobial activity against certain pathogenic microbes (Uznanski and Bryszewska, 2010). Meanwhile, a closely related derivative (hexadecanoic acid, ethyl ester), fractionated using ethanol from Brazilian *M. subsericea*, was reported with antibacterial activities in a separate study (Fernandes et al., 2013).

Benzenepropanoic acid is an aromatic acid which has been reported in propolis. The propolis extracts have shown to have antibacterial activity against bacteria (Koru et al., 2007; Uzel et al., 2005; Duarte et al., 2006). Literature

search shows that 5-methyl-2-heptanamine has previously been reported in *Juglans regia* tree bark extract. The extract has shown to have antibacterial effect against Gram-positive and Gram-negative bacteria (Ara et al., 2013). The bromo derivative of butanoic acid, butylester has also been tested with antibacterial activities (Koz'minykh et al., 2004). Interestingly, synthetic derivatives of 2-amino-1-phenyl-1-propanone have previously been reported with inhibitory activities against *E. coli* and *S. aureus* (Chitra et al., 2011; Gul et al., 2005).

From the analysis and literature search, it can be safely assumed that most of the compounds found in the inhibition zone of the methanol extract of *M. candidum* and *C. formosana* are antibacterial agents and may have worked synergistically in order to exert its antibacterial effect towards the bacteria.

5.5 Identification of Differentially Expressed Bacterial Proteins as Potential Therapeutic Antimicrobial Targets

For the analysis of proteomic, seventeen differentially expressed bacterial proteins from different bacteria (*S. aureus* ATCC 6538, *E. coli* ATCC 35218, *E. coli* ATCC 25922 *and P. aeruginosa* ATCC 27853) were identified and summarized in Table 4.14 to Table 4.17 upon treatment with its respective plant extracts. Figure 4.9 to Figure 4.14 show the differentially expressed proteins of the different bacteria studied in SDS-PAGE gels. Fewer bands were observed in the treated bacteria cells compared to the untreated bacteria cells and also the protein bands' intensities for treated bacteria were lower than the untreated cells.

These differentially expressed proteins were identified and these proteins more or less played very important roles in the bacteria.

There are mainly five mechanisms of actions which antibacterial agents took which are inhibition of the cell wall synthesis, inhibition of the cell membrane function, inhibition in the protein synthesis pathway, inhibition of nucleic acid synthesis and the inhibition of metabolic pathways (Nester et al., 2004). From the result gotten from this study, the determination of the antibacterial target was possible. Formate C-acetyltransferase, tryptophanase, glutamate decarboxylase, triacylglycerol lipase and stringent starvation protein A are proteins that are responsible in the metabolism pathway of bacteria and are found to be down regulated in their expression once treated with plant extracts. The plant extracts have also shown to be able to disrupt the protein synthesis as elongation factor Ts, elongation factor Tu 2, elongation factor G, 60 kDa chaperonine and 30S ribosomal protein S1 which are important in the process of synthesizing protein were found to have decreased.

The plant extracts have also been shown to be able to disrupt the cell wall synthesis. Once treated with the extract, there was a decrease in N-acetylmuramoyl-L-alanine amidase as compared to the normal non-treated control bacteria. The cell membrane of the bacteria is also disrupted as there was shown to be a decrease in outer membrane protein A in the bacterial cell pellet. Flagellin on the bacteria, *E. coli* has also been found in the supernatant. This suggests that

the extract may have been managed to disrupt the flagellin from the bacteria cells. Some bacteria such as *S. aureus* are able to secrete alpha-hemolysin, a substance which enables the bacteria to invade foreign cell by creating pore on it. However, once treated with the *M. candidum* extracts, the alpha-hemolysin found in the supernatant of *S. aureus* ATCC 6538 has shown to have decreased.

α-Hemolysin is a pore-forming exotoxin secreted by many pathogens such as *S. aureus* which targets the host immune cells (Sibbald, 2006). This exotoxin was found to have decreased once the bacteria was treated with *M. candidum* and *C. formosana* as shown in Figure 4.9 and Figure 4.10. α-hemolysin is a small β-barrel toxin and is secreted as a water soluble monomer, able to bind and oligomerize into a heptameric structure on the cell membrane of the host (Song et al., 1996). Many studies have shown that this exotoxin causes tissue barrier disruption and lead to the subsequent lethal infection.

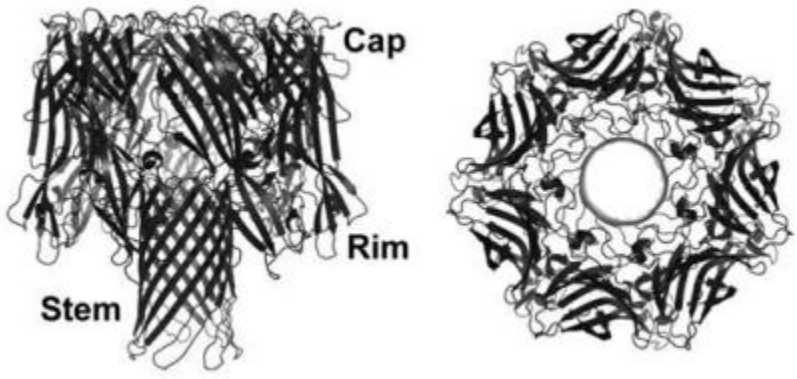


Figure 5.4: Structure of α-hemolysin. The Regions of the Toxin that Separate the Entry of the Pore (Cap), the Membrane-Interfacing Region (Rim), and the Membrane Perforating Stem (Song et al., 1996).

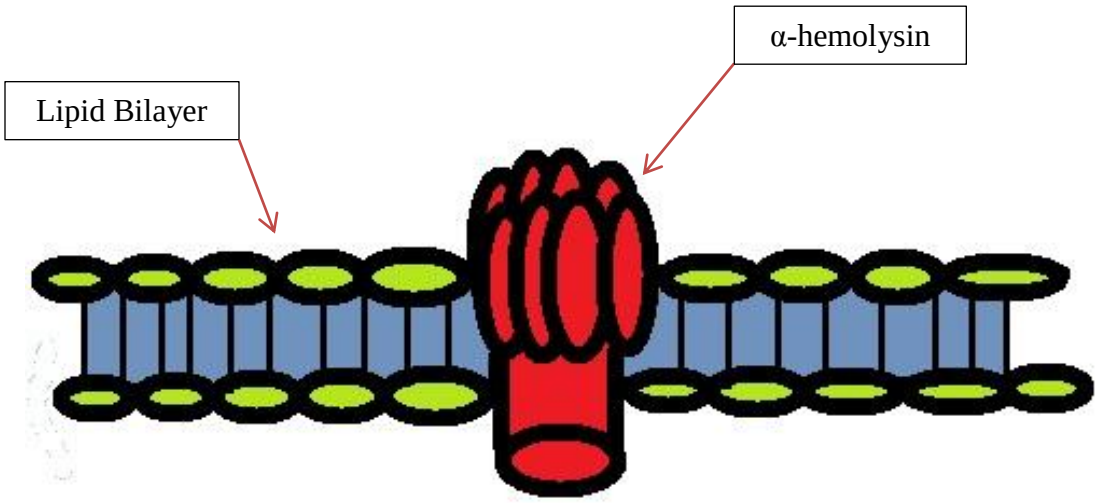


Figure 5.5: α-Hemolysin Mechanism of Action.

This toxin secreted by *S. aureus* is lethal to animals especially to rodents and rabbits (Bernheimer, 1965). However, *S. aureus* strain which is deficient in α-hemolysin has significantly reduced virulence in animal infection models. By targeting this toxin, the severity and virulence of an infection may lessen. α-hemolysin is also known to increase severity in an already known infection. Kennedy et al. (2010) has demonstrated that α-hemolysin contributes to the severity of the infection in the skin of the mouse model as shown in Figure 5.6. Mice with skin infection were injected with α-hemolysin producing *S. aureus*. Mice with pre-immunization have shown formation of dermonecrotic skin lesions while mice which have been immunized show no signs of formation of dermonecrotic skin lesions.

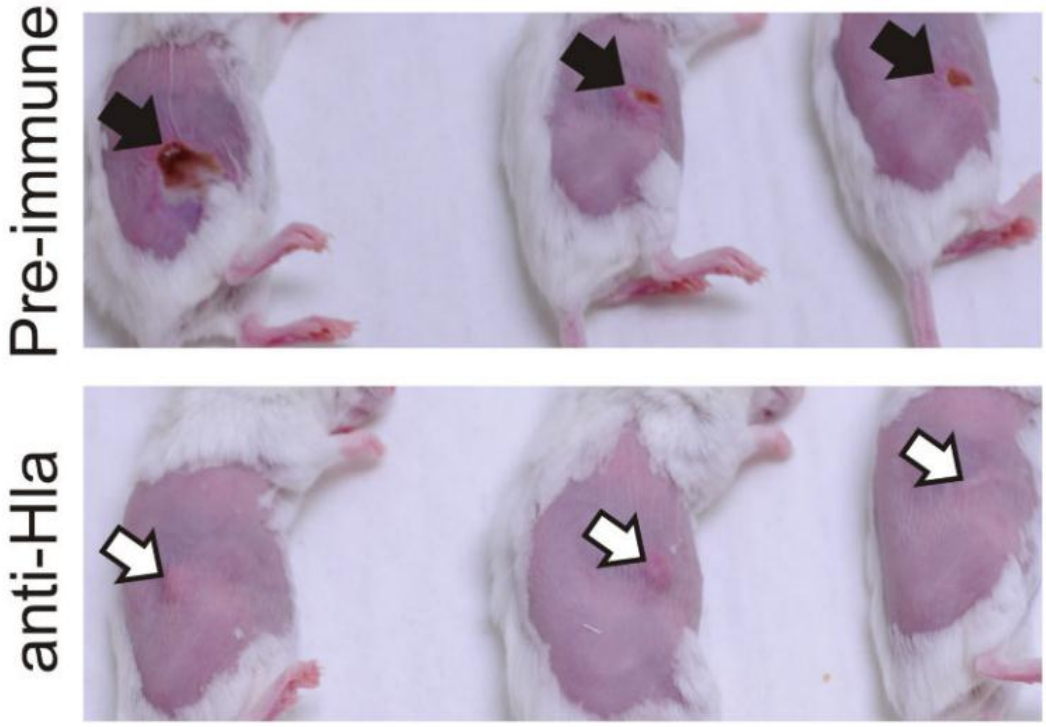


Figure 5.6: Mice pre-immunized and mice which have been immunized against α-hemolysin (Hla). Black arrows indicate dermonecrosis and white arrows indicate abscess formation without dermonecrosis (Kennedy et al., 2010).

Three proteins (α-hemolysin, triacylglycerol lipase and N-acetylmuramoyl-L-alanine amidase) were shown to have been differentially expressed in *S. aureus* ATCC 6538 once treated with *C. formosana*. The protein α-hemolysin has been discussed previously of its function and it's significant as an antibacterial target. Triacylglycerol lipase which was found to have been reduced compared to the untreated control is an enzyme which is responsible for the hydrolysis of triglycerides into free fatty acids and glycerol (Kim et al., 1997).

N-acetylmuramoyl-L-alanine amidase is an enzyme that participated in the biosynthesis of peptidoglycan. Peptidoglycan cell wall in bacteria provides a

strong protective outer layer. It is a very dynamic structure and it must expand during cell growth and it must be cleaved during cell division (Perkins, 1980). This enzyme was shown to have decreased once treated with *C. formosana* in *S. aureus* ATCC 6538 compared to the control. N-acetylmuramoyl-L-alanine amidase is an example of autolysin. Autolysins exist in bacteria with peptidoglycan as peptidoglycan matrix are very rigid thus this enzyme is required to break it down in small section in order for the growth and division of cells to occur. Autolysin also plays a role in the formation of biofilm as suggested by Chen et al. (2013). Their data suggest that *S. aureus* biofilms are formed under conditions of controlled secretion and proteolysis of autolysin, a determinant for the release of DNA biofilm matrix. Thus decreasing this enzyme might be able to inhibit the growth of bacteria since it was required in the cell division and growth of bacteria.

When treated with *M. candidum* extract, three differentially expressed proteins were found in *P. aeruginosa* ATCC 27853, namely 30S ribosomal protein S1, 60 kDa chaperonin and elongation factor Ts as shown in Figure 4.11 and Table 4.11. These three proteins were found to have reduced once treated with the extract as compared to the control.

30S ribosomal protein S1 has been thought to participate in the translation initiation complex formation in protein biosynthesis by assisting the position of 30S in the translation initiation region. This protein has been shown to be reduced

once treated with *M. candidum* extract. A study done by Delvillani et al. (2011) has suggested that the depletion of S1 ribosomal protein impair bacterial growth due to the effect it has on translation. This shows that S1 ribosomal protein is fairly important for the growth of bacteria and its depletion due to the extract might make it as a potential antibacterial target.

60 kDa chaperonin are known to prevent misfolding of polypeptide, stimulate proper folding of newly synthesized polypeptides and promote refolding of denatured protein generated under stress conditions (Kurochkina et al., 2012). When the bacteria are treated with the plant extract, 60 kDa chaperonin has been shown to have decreased. 60 kDa plays an important part in post translational and a decrease in this protein may jeopardize protein function in bacteria thus affecting the bacteria growth and activity.

The role of elongation factor-thermo stable (EF-Ts) in the bacteria cell is to serves as a guanine nucleotide exchange factor for EF-Tu by catalyzing the release of guanosine diphosphate from EF-Tu. This will allow EF-Tu to bind to a new guanosine triphosphate molecule thus catalyzing another aminoacyl tRNA addition (Burner et al., 2013). Treated bacteria cells have shown to have a decrease in this protein and this will cause disruption in the protein biosynthesis.

Four differentially expressed proteins were found in *E. coli* ATCC 35218 when treated with *M. candidum* (Formate C-acetyltransferase, glutamate

decarboxylase, tryptophanase and elongation factor Tu 2) as shown in Figure 4.10. Four of these proteins have been found to be reduced in its expression once treated with the extract compared to the control.

Formate C-acetyltransferase or its common name pyruvate formate-lyase is one of the central enzymes in the anaerobic metabolism in *E. coli* and it is responsible for the conversion of pyruvate and CoA to formate and acetyl-CoA by a non-oxidative route. Acetyl-CoA is an important component in the citric acid cycle (Knappe et al., 1974).

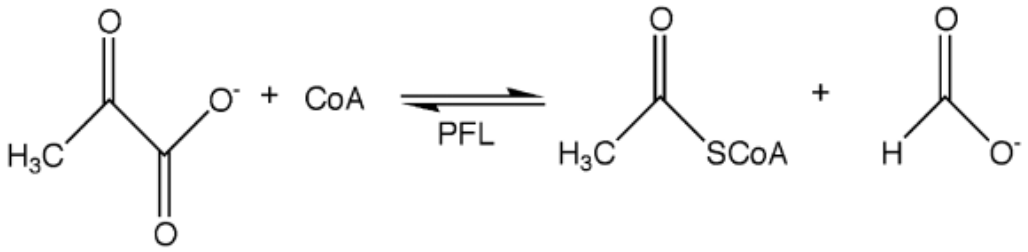


Figure 5.7: Enzyme Reaction of Formate C-acetyltransferase.

Formate C-acetyltransferase is important when *E. coli* is under anaerobic condition such as in the gut of other living organisms. In our study, this enzyme has been found to be reduced once treated with plant extract in *E. coli* ATCC 35218 and *E. coli* ATCC 25922.

Another metabolic pathway enzyme which has been found decreased in its expression when treated with plant extract is glutamate decarboxylase. Glutamate decarboxylase (GAD) converts glutamate into ɣ-aminobutyric acid (GABA) in a

pyridoxal 50-phosphate (PLP)-dependent manner. This reaction takes up protons which constitutionally increases the pH of the medium (Gale and Epps, 1994).

Figure 5.8: Reaction of Glutamate decarboxylase.

Enteric bacteria utilize the GAD reaction to balance out the influx of proton originated from the acidic environment in the human stomach. As can be implied from its role, GADs of enteric bacteria such as *E. coli* are active during acidic conditions only. As the pH rises, the enzyme critically loses its activity and it shows no activity at or above pH 6. This will ensure that the bacteria are able to survive under low pH in the human stomach by neutralizing the acid around it.

Tryptophanase is an enzyme that catalyzes the chemical reaction as shown below in Figure 5.9.

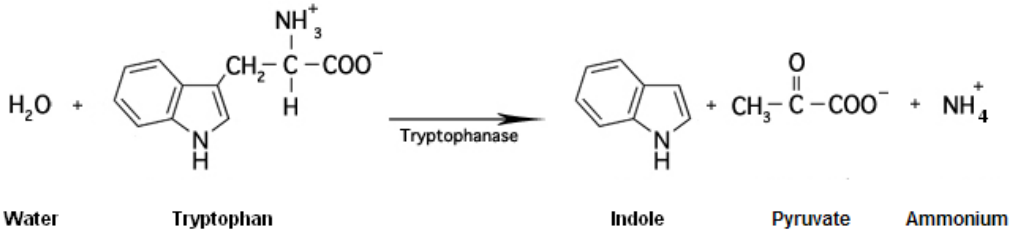


Figure 5.9: Enzyme Reaction of Tryptophanase.

The enzyme tryptophanase catalyzes the degradation of tryptophan to indole, ammonia, and pyruvic acid. This ability allows tryptophan to be used as a source of carbon, nitrogen, and energy. The enzyme is found exclusively in bacteria and primarily in those bacteria which are normal inhabitants of the intestinal tracts of animals. Tryptophanase is necessary to the bacterium per se in the enteric environment (Botsford and Demoss, 1972).

Elongation factor thermo unstable (EF-Tu 2) was found to be reduced in *E. coli* ATCC 35218 and *E. coli* ATCC 25922 when treated with plant extract. Elongation factors are a part of the mechanism which synthesizes new protein through translation at the ribosome. EF-Tu assists in the moving of aminoacyl-tRNA onto a free site on the ribosome by binding onto a charged *t*RNA molecule forming a complex. EF-Tu increases the accuracy in protein translation in three ways. EF-Tu delays GTP hydrolysis when the *t*RNA in the ribosome's A site is not a match to the *m*RNA codon thus decreasing incorrect translation. It also delays the aminoacyl-*t*RNA to enter fully into A site once it detaches itself from it. This will give an opportunity to ensure incorrect amino acids from adding onto polypeptide chain. The third mechanism has yet to have a full understand of its function where EF-Tu check the association of aminoacyl-*t*RNA and reject it when the amino acid is not correctly bound onto its respective tRNA. As EF-Tu 2 is essential in the elongation of the protein, it is very interesting to note that the bacteria treated with *M. candidum* have shown sign of decrease in this protein.

Protein translation process is an essential to the bacteria survival and it is highly conserved across different species of bacteria (Myasnikov et al., 2009).

E. coli ATCC 35218 treated with *S. barbata* has shown that there are more flagellin in the supernatant as compared to the non-treated control as shown in Figure 4.13. Flagellin is a protein which forms the major component of the flagella filaments. Flagellin is a globular protein responsible for the mobility of the bacteria and its structure. Flagella contribute to pathogenicity. The production of flagella in pathogenic bacteria promotes colonization and invasion of mucosa. Flagella are required for motility, invasion, adhesion and secretion of virulence factors in mucosa (Ramos et al., 2004). The increase of flagellin in the supernatant of the treated cell might suggest that it was degraded from the bacteria body due to the plant extract.

Five differentially expressed proteins were found in *E. coli* ATCC 25922 treated with *M. candidum* extract. These proteins are elongation factor G, formate c-acetyltransferase, elongation factor Tu 2, outer membrane protein A and stringent starvation protein A. Two of these proteins (Formate c-acetyltransferase and elongation factor Tu 2) have been explained previously.

Elongation factor G (EF-G) is nearly similar in its role as EF-Tu 2. Its role in the protein biosynthesis is to catalyze the translocation of the tRNA and mRNA along the ribosome after each round of polypeptide elongation. The translation

process as shown in Figure 5.10 occurs by binding the GTPase EF-G that causes the deacylated tRNA at the P site of the ribosome to move to the E site. In the A site of ribosome, the peptidyl-tRNA moves to the P site upon GTP hydrolysis. The decrease in this protein might jeopardize the protein translation process as elongation factor plays an important role in the elongation of the protein (Steitz, 2008).

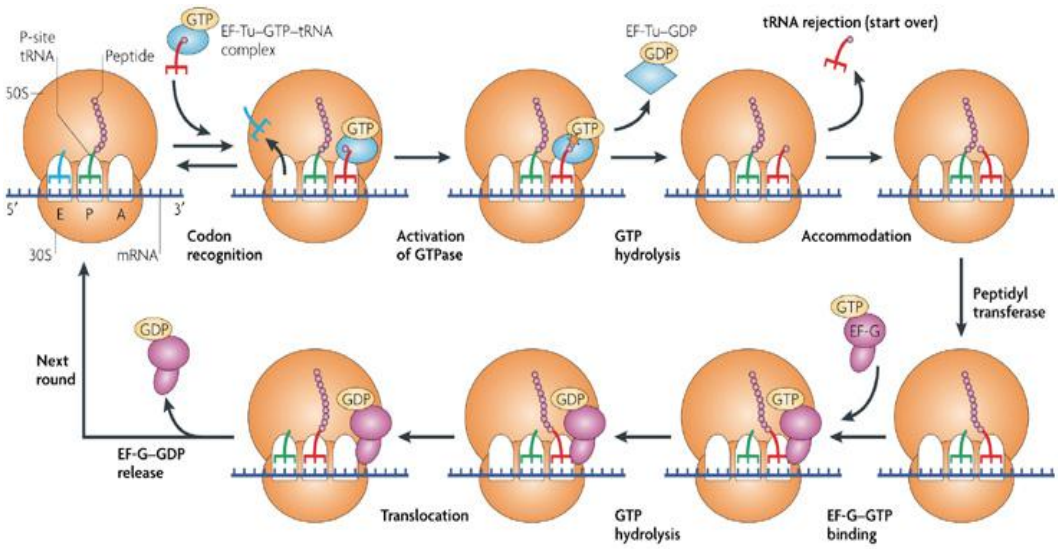


Figure 5.10: An overview of mRNA translation (Steitz, 2008).

Another protein which has been differentially expressed in treated *E. coli* is the outer membrane protein A (OmpA). OmpA main role is to maintain integrity to the membrane through physical linkage between the outer membrane and the peptidoglycan layer. It is also known that OmpA is important in the conjugation of bacteria (Ried and Henning, 1987). The depletion of this protein might cause the bacterial cell membrane to lose its integrity and thus cell lysis occurs.

There was not much information regarding stringent starvation protein A (SspA) in *P. aeruginosa* but there are other information regarding this protein in *E. coli*. SspA is needed during acid-induced stress to enable the survival of *E. coli* in human gut. SspA apparently inhibits stationary-phase accumulation of histone-like nucleoid-structuring (H-NS). H-NS has a role in the formation of nucleoid structure and is a regulator where it derepresses multiple stress defense systems including acid stress and nutrient starvation. In mutant SspA, H-NS regulon is altered causing acid sensitive and hypermotile phenotypes. H-NS is sensitive to minor changes in the environment and thus enabling the cells to response quickly. SspA and H-NS are highly conserved among many pathogenic Gram-negative bacteria (Hansen et al., 2005).

One possible reason of the reduced proteins expression in treated bacteria cell might be due to the phenolic acid from plant extracts. Phenolic acid are known to be weak organic acids (pKa 4.2). The concentration of dissociated acid might be responsible for its antimicrobial characteristic (Campos et al., 2009). The reduction of intracellular pH will lead to protoplasmic poisoning of the cytoplasm which in turn causes protein denaturation to take place. Thus the proteins in the bacteria are differentially expressed when treated with the extracts as compared to the control cell.

5.6 Investigation of Morphological Changes via Scanning Electron Microscopy (SEM)

In order to learn more about the possible mechanisms of antibacterial activity of the plant extracts, electron microscopy was employed on plant extracts treated bacteria cells. SEM was used to acquire the images of morphological damages in bacteria. After treatment with plant extracts, the bacteria was observed to have morphological changes including loss of integrity in cells, deformity in cell walls and cell lysis.

Through the use of SEM, we were able to see the cell surface of the bacteria and how it was affected after being treated with plant extracts. From the result gotten, it is clearly seen that the bacteria cell surface was greatly disrupted, deformed, pitted, shriveled, and adhesive to each other and some are heavily fragmented beyond its original shape.

It is known that the bacteria membrane and also its cytoplasm are easily affected by phenolic acid as a result of being partially lipophilic in nature. The passive diffusion of phenolic acid across the cell membrane in their undissociated form disturbs the integrity of the cell membrane structure, whereby it will acidify the cytoplasm and causes protein denaturation. There are several works that have been done to support the effect of phenolic acids toward the cell membrane of bacteria and its primary action (Campos et al., 2009). The antibacterial

compounds in *M. candidum* extract has shown to be able to damage the cell wall, cell membrane and induced cell lysis of bacteria from the observation done through SEM.

These antibacterial compounds can easily enter the bacterial cells through the damaged cell membrane and react with the bacterial DNA causing cell death. As shown in the result, it was clear to us that the bacteria were heavily affected when treated with plant extracts compared to the control. This goes to show that the extracts played a role in affecting the cell surface of the bacteria. Maintaining the integrity of its membrane helps keep most of the bacteria alive as the cell membrane acts as a shield against the harsh environment the bacteria are in such as when they are in the acidic guts of living organism. The cell membrane helps keep the content of the bacteria in tact as well (Silhavy et al., 2010). Thus, maintaining the cell membrane ensure the survival of the bacteria.

CHAPTER 6

CONCLUSION

The uses of medicinal plants have been passed down for generations as it has a lot of medicinal uses and values. A lot of research was done to discover more of its benefits and uses, as many of the commercially available antibiotics have proven to have more disadvantages than its advantages such as the appearance of antibiotic resistant bacteria.

The present study has shown that medicinal plants have antibacterial effect against various bacteria. Among the fifteen medicinal plants that were tested in this study, *M. candidum* has shown to have the best antibacterial activity, as it had effect against both Gram-positive and Gram-negative bacteria. The methanol extract of *M. candidum* had the highest activity, compared to other solvent extracts. Time-kill assay was done to evaluate the bacteriostatic and bactericidal effects of the extracts. In general, the bacteriostatic and bactericidal effect of the plant extract depends on the different concentration of the extract used, and different bacteria react differently towards the extract.

TLC-bioautography was utilized to study the antibacterial compounds of the extracts. In this study, 2, 4-bis-(1, 1-dimethylethyl)-phenol, hexadecanoic acid, benzenepropanoic acid, 1-butyl 2-(2-ethylhexyl) phthalate, 1-propanone and

2-heptanamine were found through GC-MS analysis. These compounds were known to have antibacterial activities as reported in various journals.

Further studies were done using these extracts on various experiments to determine how it affects the bacteria physically and its protein expression. There were seventeen differentially expressed proteins identified from four different bacteria. These proteins were found to have decreased in their expression levels in bacteria, when treated with plant extracts. The decreased expressions of these proteins may prove to be fatal to the bacteria, as most of these proteins are essential and very important in the survival, growth and protein biosynthesis of the bacteria.

The bacteria treated with plant extracts were also shown to undergo morphological changes when viewed under scanning electron microscope. The bacteria cell surfaces were shown to be heavily damaged or disrupted. Phenolic compounds are known to disrupt the integrity of the bacteria cell membrane thus allowing the content of the bacteria to leak.

In conclusion, *M. candidum* has the highest antibacterial activity followed by *S. barbata* and *C. formosana*. In this study, potentially significant clues were contributed toward the understanding of the mechanisms of action of these antibacterial plant extracts, by comparing the protein expressions in the treated and untreated bacteria. It is hoped that these information will allow further

understanding of how these plant extracts work on bacterial pathway and protein targets involved, and to further the development of target-specific or pathway-specific natural antibiotic.

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APPENDIX A

An example of calculation to determine the percentage of DPPH radical scavenging activity

The percentage of DPPH radical scavenging activity was measured using the following formula:

$$\% \text{ radical scavenging activity} = \frac{(A_{Blank} - A_{sample})}{A_{Blank}} \times 100\%$$

An example of a calculation for this assay is shown below.

The absorbance blank for *Melastoma candidum* is 0.737 and the absorbance of sample at 1 mg/ml is 0.357.

$$\% \text{ DPPH Radical Scavenging Activity} = \frac{(0.737 - 0.357)}{0.737} \times 100\%$$

$$= 51.56\ \%$$

Thus the percentage of DPPH radical scavenging activity is 51.56 % at 1 mg/ml.

APPENDIX B

An example of calculation to determine the percentage of ferrous ion chelating activity

The percentage of ferrous ion chelating activity was measured using the following formula:

$$\% \text{ radical scavenging activity} = \frac{(A_{Blank} - A_{sample})}{A_{Blank}} \times 100\%$$

An example of a calculation for this assay is shown below.

The absorbance blank for *Melastoma candidum* is 0.327 and the absorbance of sample at 5 mg/ml is 0.135.

$$\% \text{ Ferrous ion chelating activity} = \frac{(0.327 - 0.135)}{0.327} \times 100\%$$

$$= 58.72\ \%$$

Thus the percentage of ferrous ion chelating activity is 58.72% at 5 mg/ml.

APPENDIX C

An example of calculation to determine the percentage of NO_2 radical scavenging activity

The percentage of NO_2 radical scavenging activity was measured using the following formula:

$$\% \text{ radical scavenging activity} = \frac{(A_{Blank} - A_{sample})}{A_{Blank}} \times 100\%$$

An example of a calculation for this assay is shown below.

The absorbance blank for *Melastoma candidum* is 0.881 and the absorbance of sample at 1 mg/ml is 0.243.

$$\% NO_2 \text{ Radical Scavenging Activity} = \frac{(0.881 - 0.243)}{0.881} \times 100\%$$

$$= 72.42\ \%$$

Thus the percentage of NO_2 radical scavenging activity is 72.42 % at 1 mg/ml.

APPENDIX D

An Example of Calculation to Obtain Total Flavonoid Content

An example of a calculation for this assay is shown below.

$$\text{Average of absorbance} = \frac{\text{Absorbance 1 + Absorbance 2 + Absorbance 3}}{3}$$

$$= \frac{0.915 + 0.949 + 0.957}{3}$$

$$= 0.940$$

Average of absorbance value was multiplied by the dilution factor.

$$0.940 \times 1 = 0.940$$

The value that was obtained (i.e. 0.940) was substituted for "y" in the equation.

$$y = 0.909x$$

$$0.940 = 0.909x$$

$$x = \frac{0.940}{0.909}$$

$$= 1.03$$

0.2 ml of 10 mg/ml of *Melastoma candidum* was used in this assay. Thus, in order to get the amount of dried plant only, the concentration of the extract used (10 mg/ml) times the volume used (0.2 ml) per 1000 to convert it back to g.

= (10 mg/ml x 0.2 ml)/1000
= 0.002

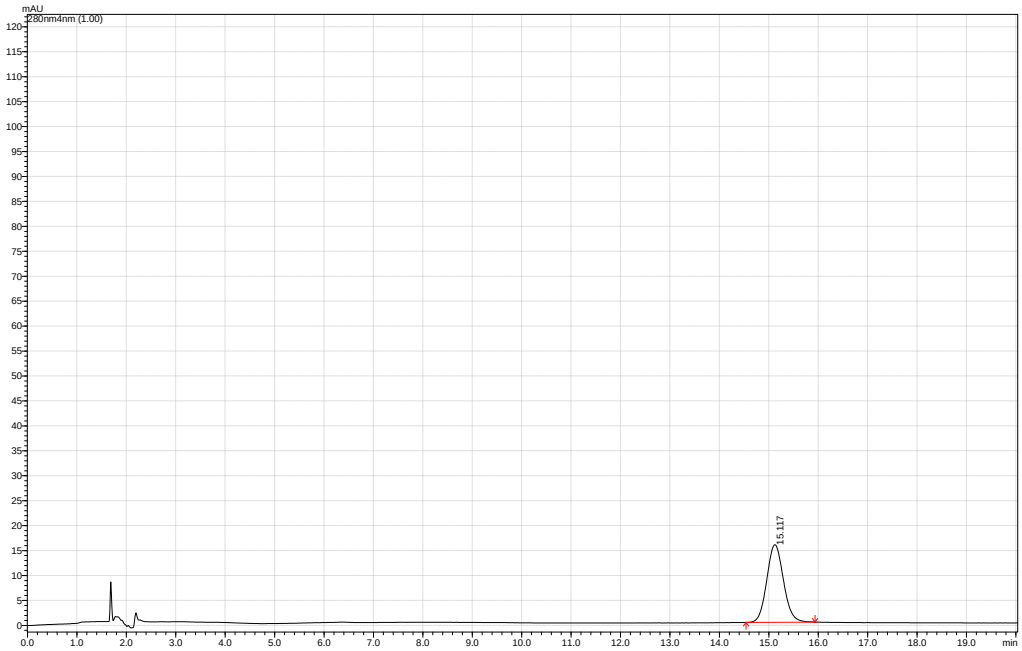
Thus in order to get the mg quercetin equivalents per gram of dried plant, the formula is as follow:

= (Value x) x (volume/amount of dried plant)
= 1.03 x (0.2 ml/ 0.002)
= 103 mg quercetin equivalents per gram of dried plant

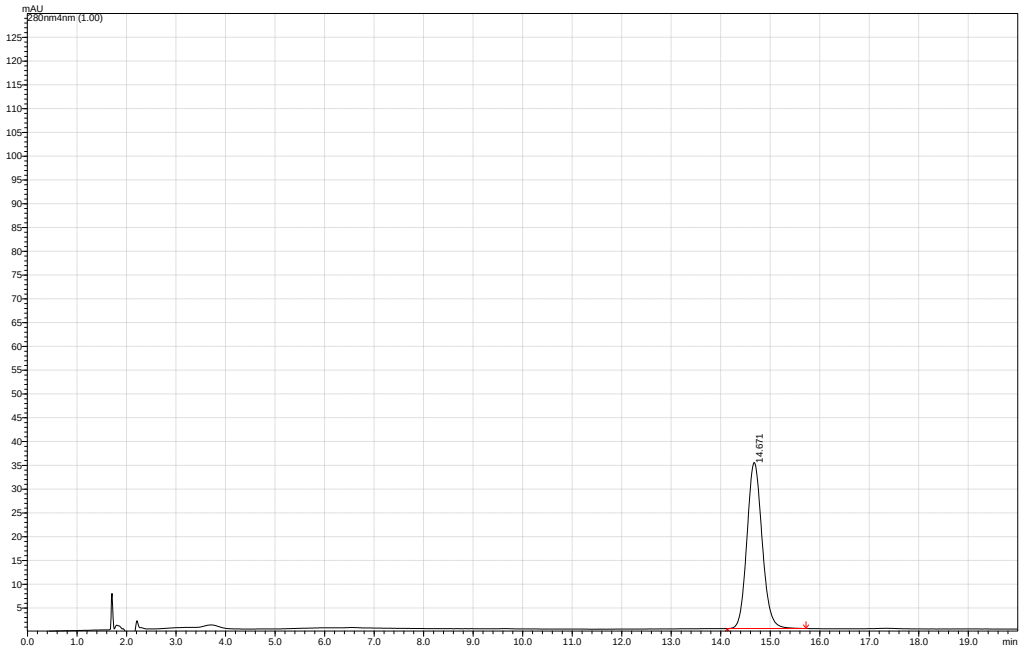
APPENDIX E

HPLC Chromatogram of Standard

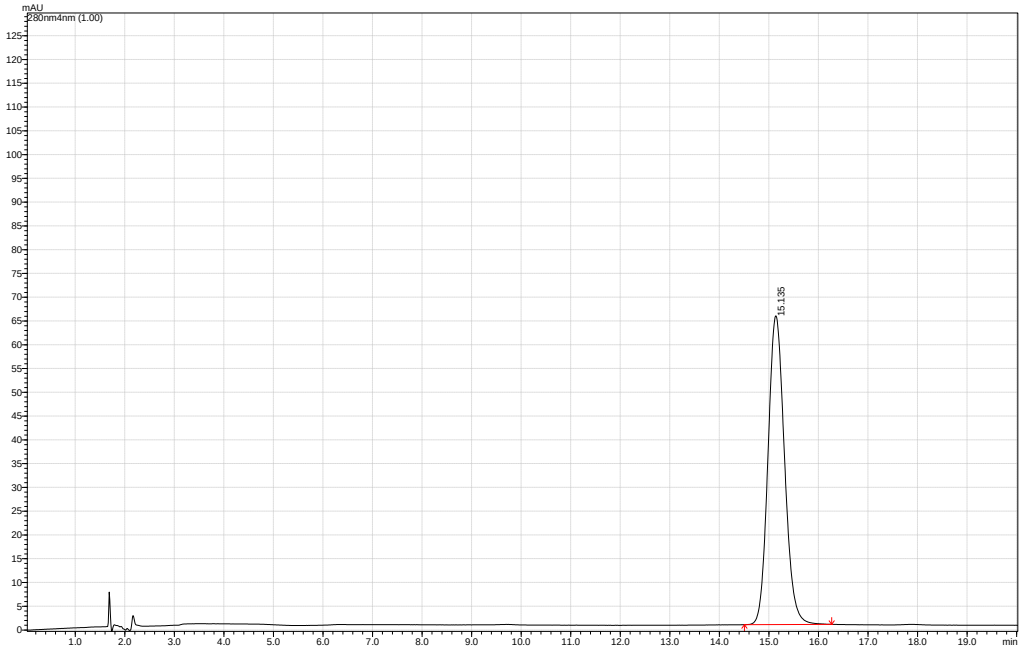
Caffeic Acid 5 ug/ml



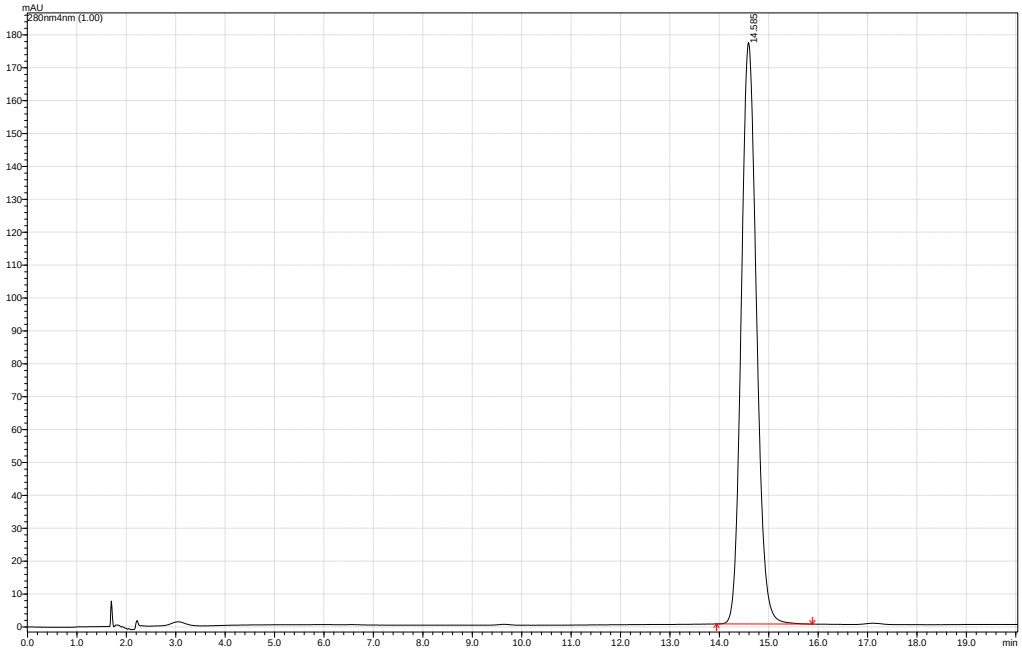
Caffeic Acid 10 ug/ml



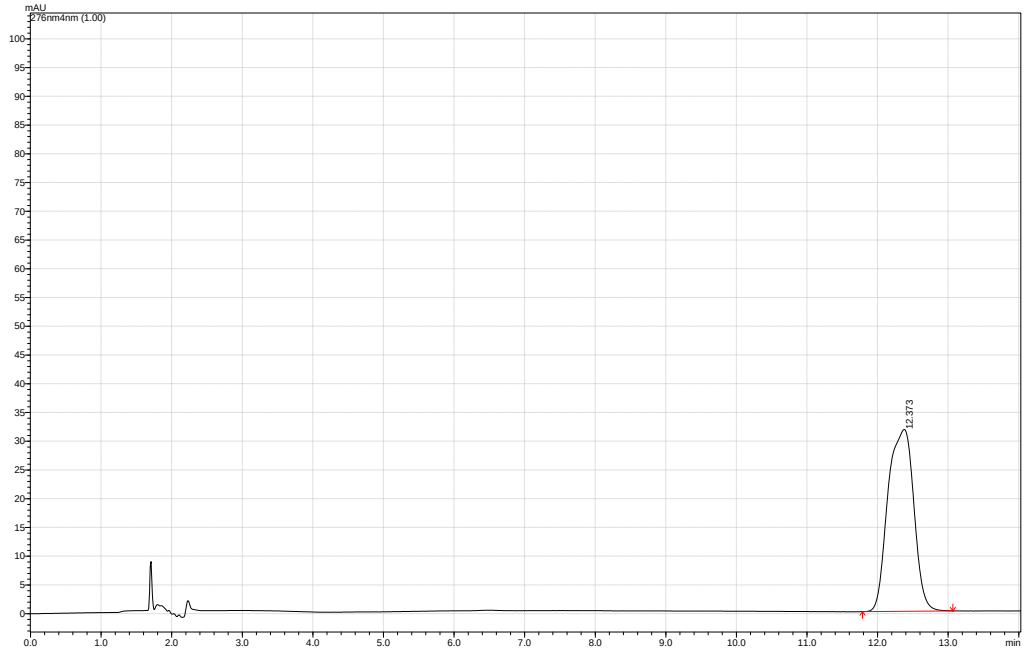
Caffeic Acid 20 ug/ml



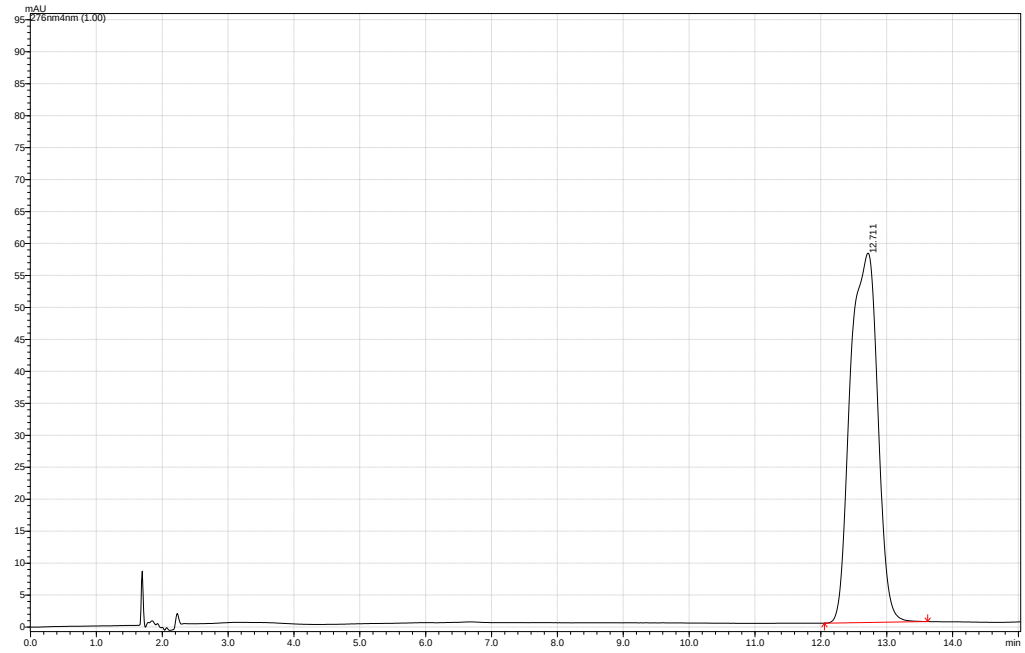
Caffeic Acid 50 ug/ml



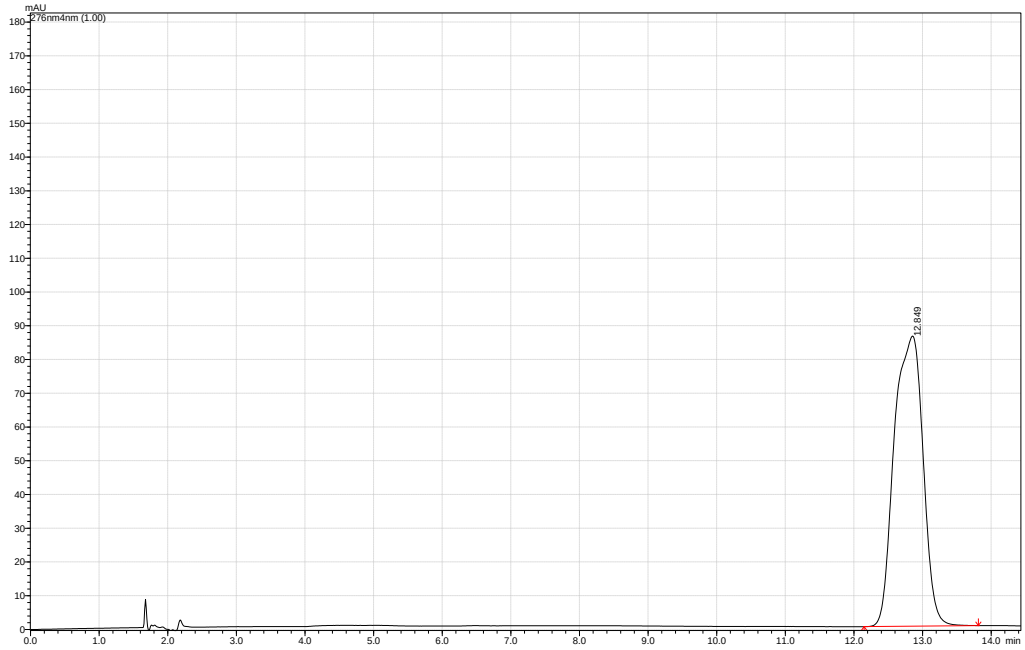
Catechin 50 ug/ml



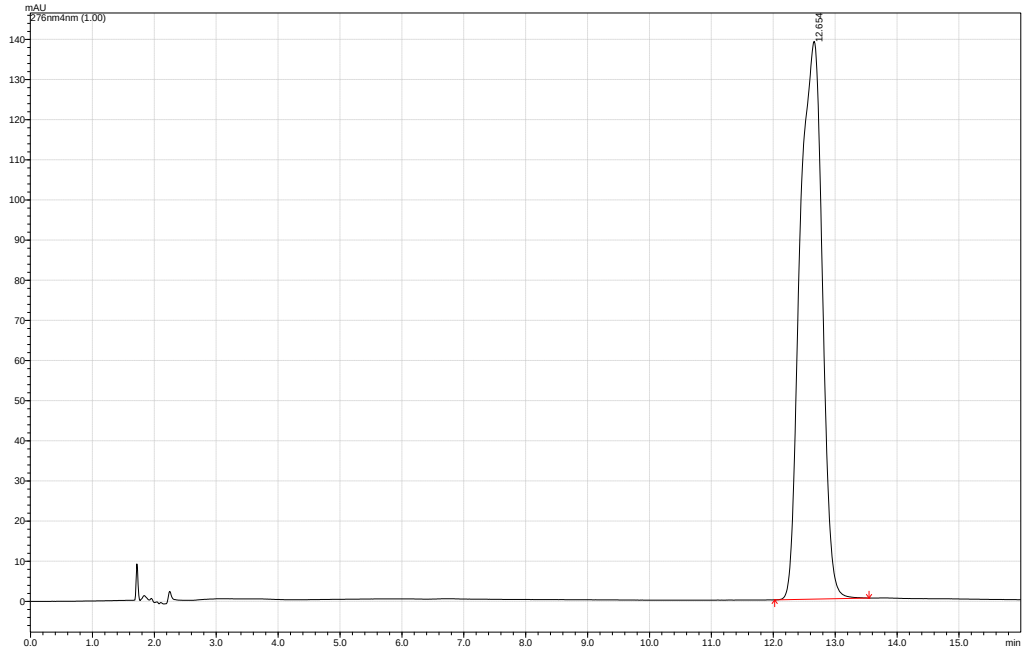
Catechin 100 ug/ml



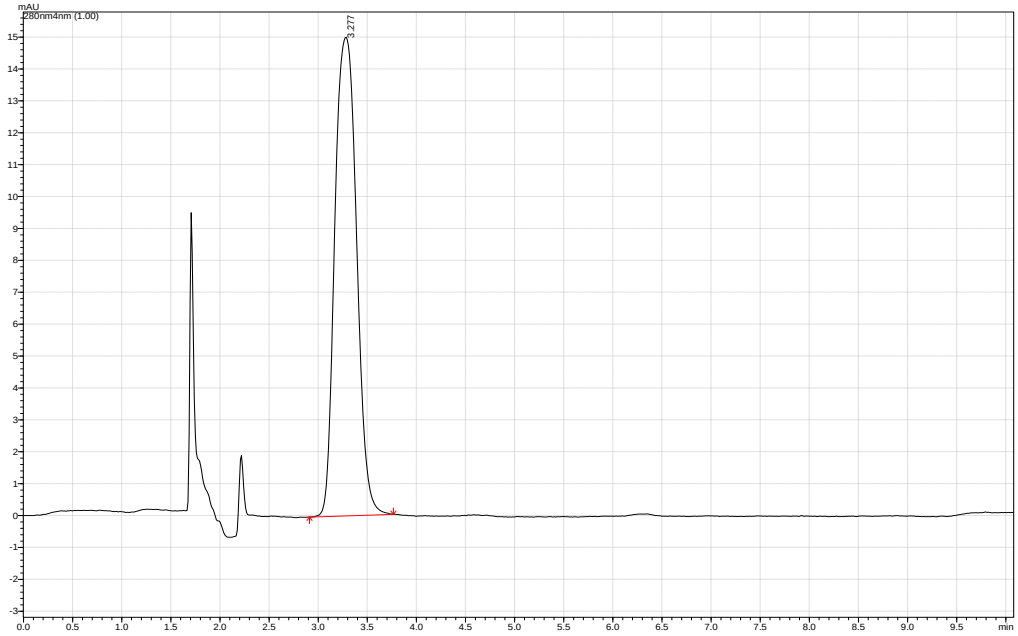
Catechin 150 ug/ml



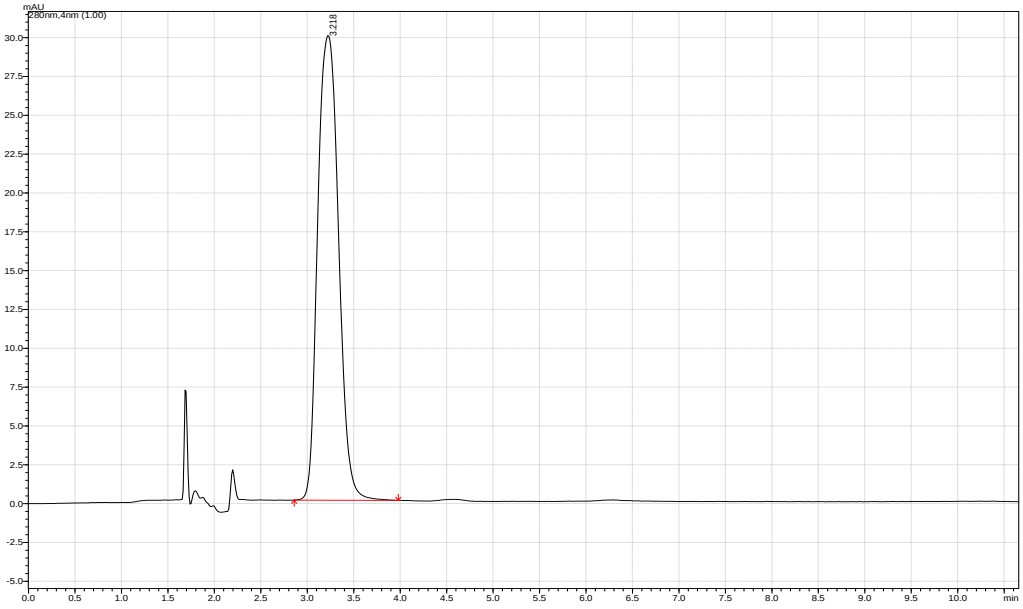
Catechin 200 ug/ml



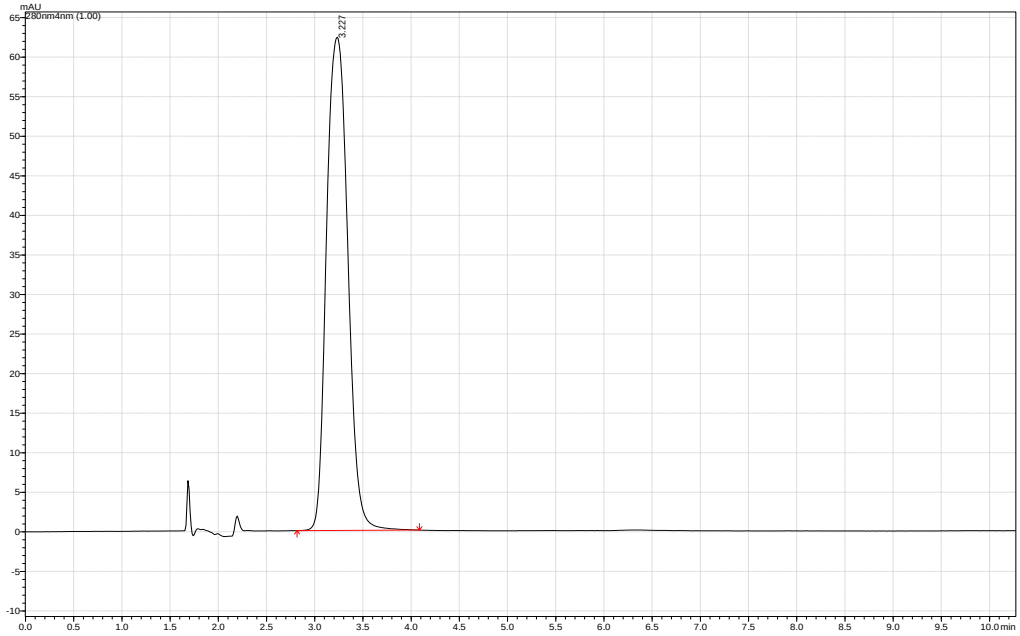
Gallic Acid 5 ug/ml



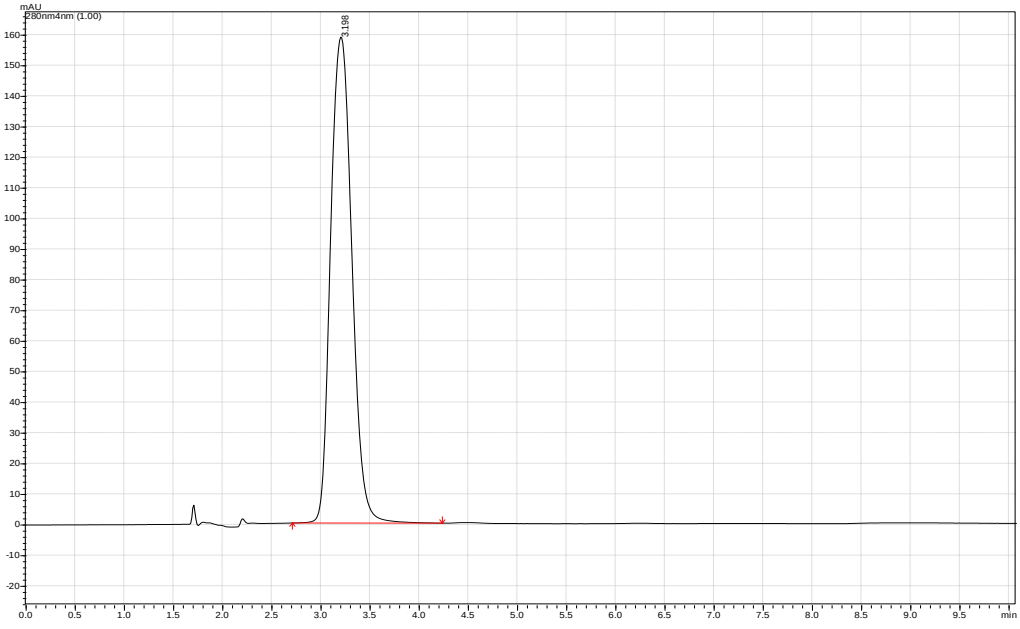
Gallic Acid 10 ug/ml



Gallic Acid 20 ug/ml

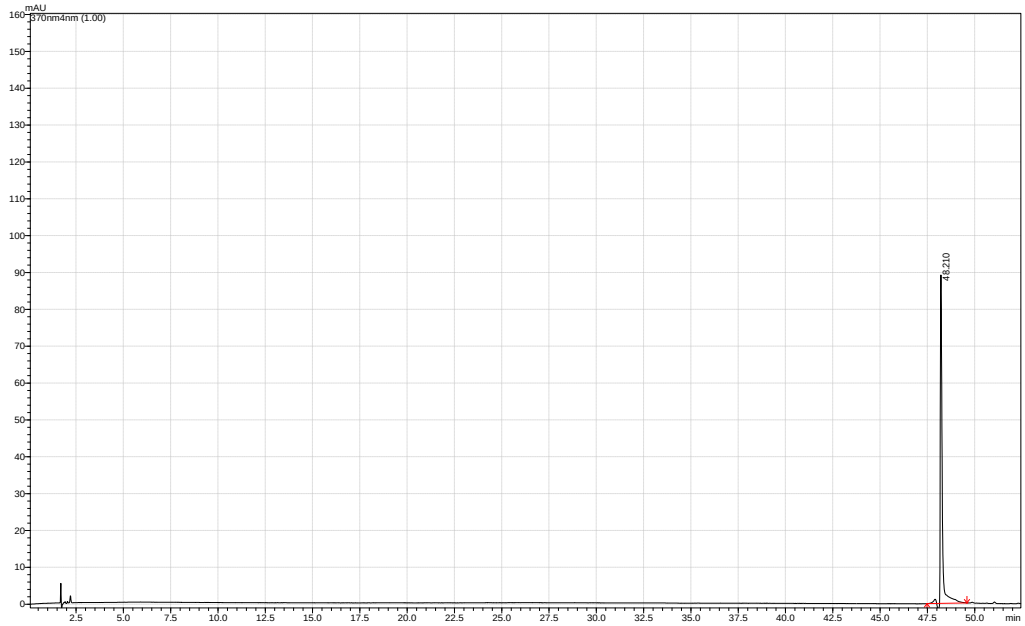


Gallic Acid 50 ug/ml

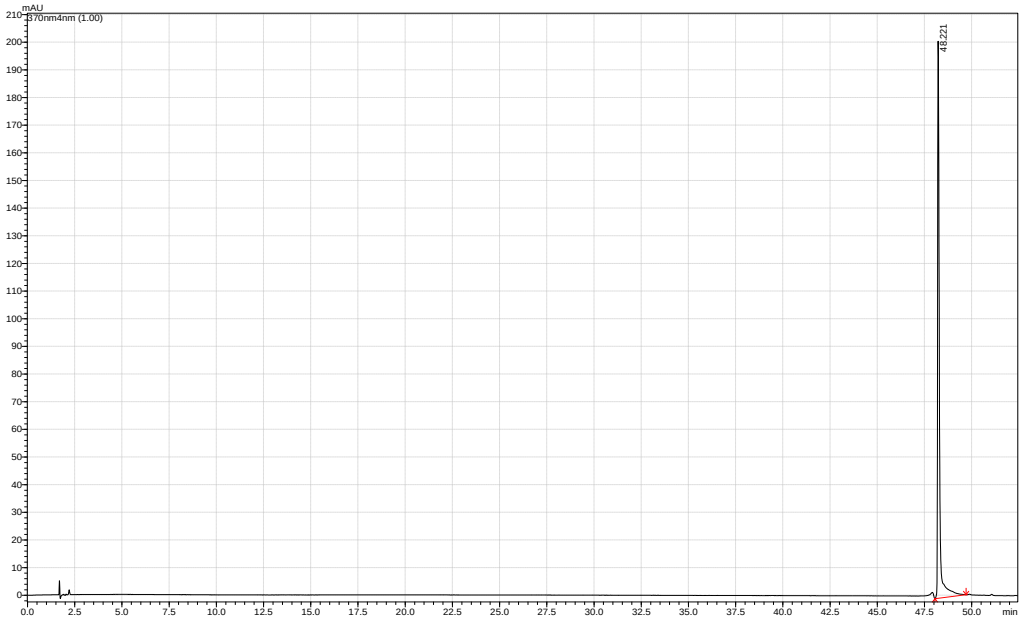


Quercetin 5 ug/ml

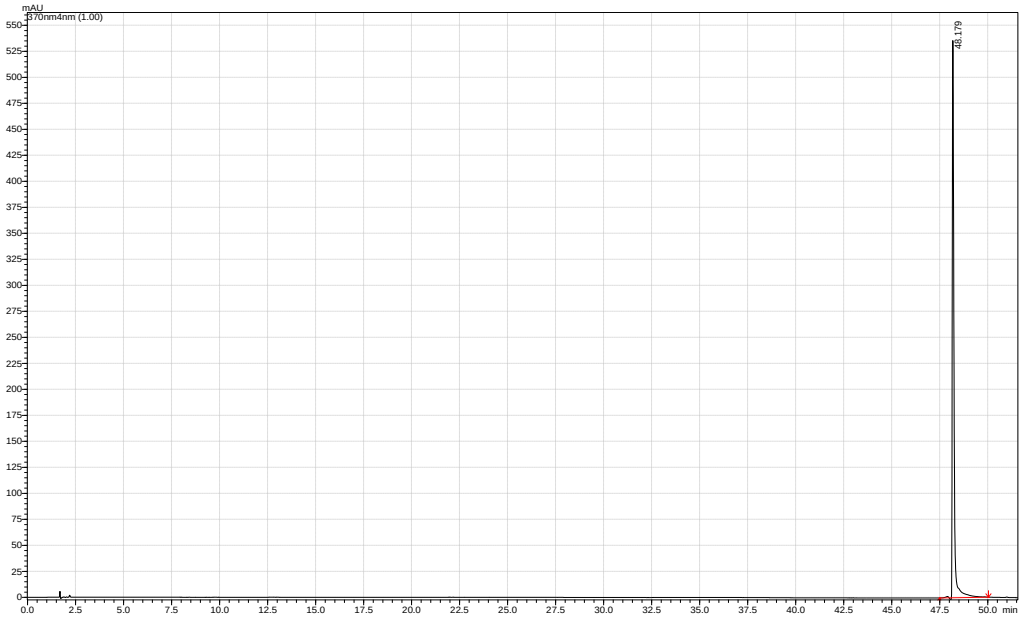
Quercetin 10 ug/ml



Quercetin 20 ug/ml



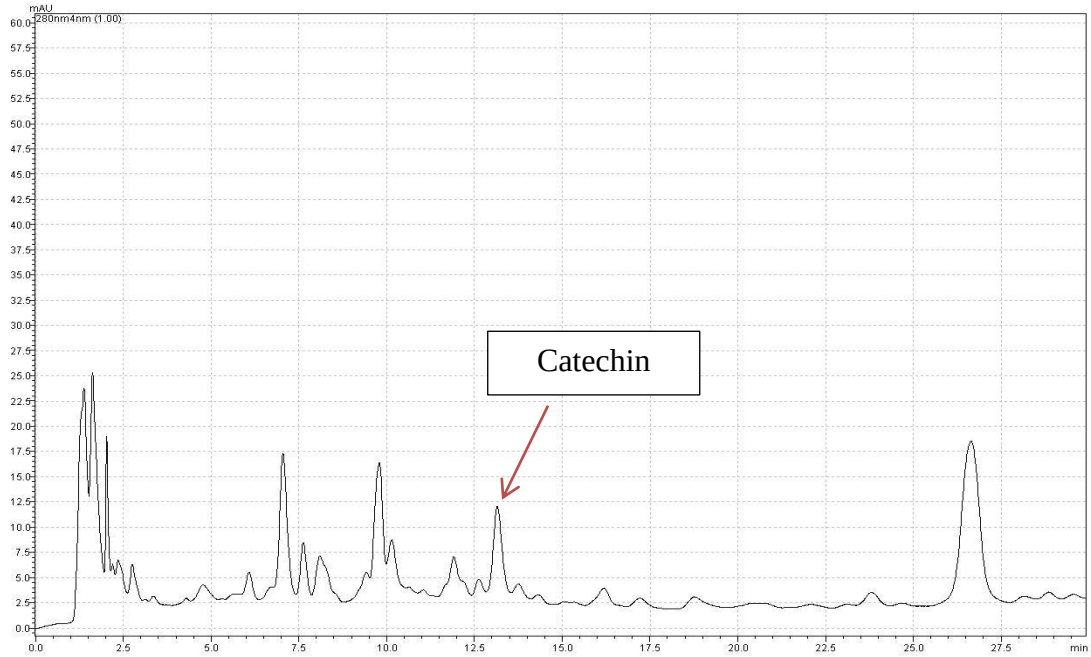
Quercetin 50 ug/ml



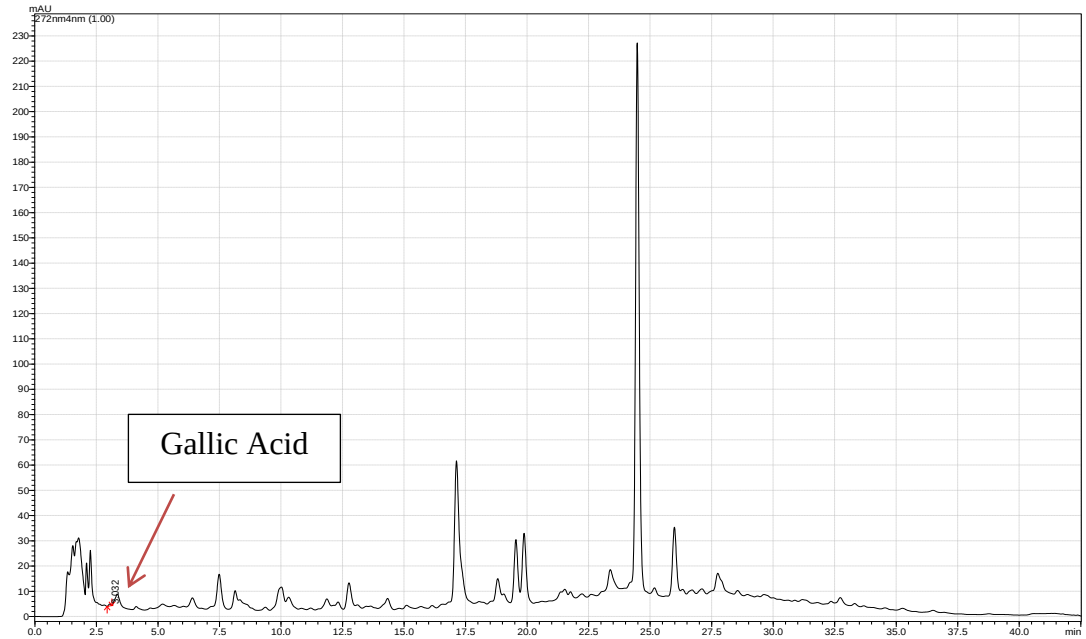
HPLC Chromatogram of Plant Extracts

Callicarpa formosana

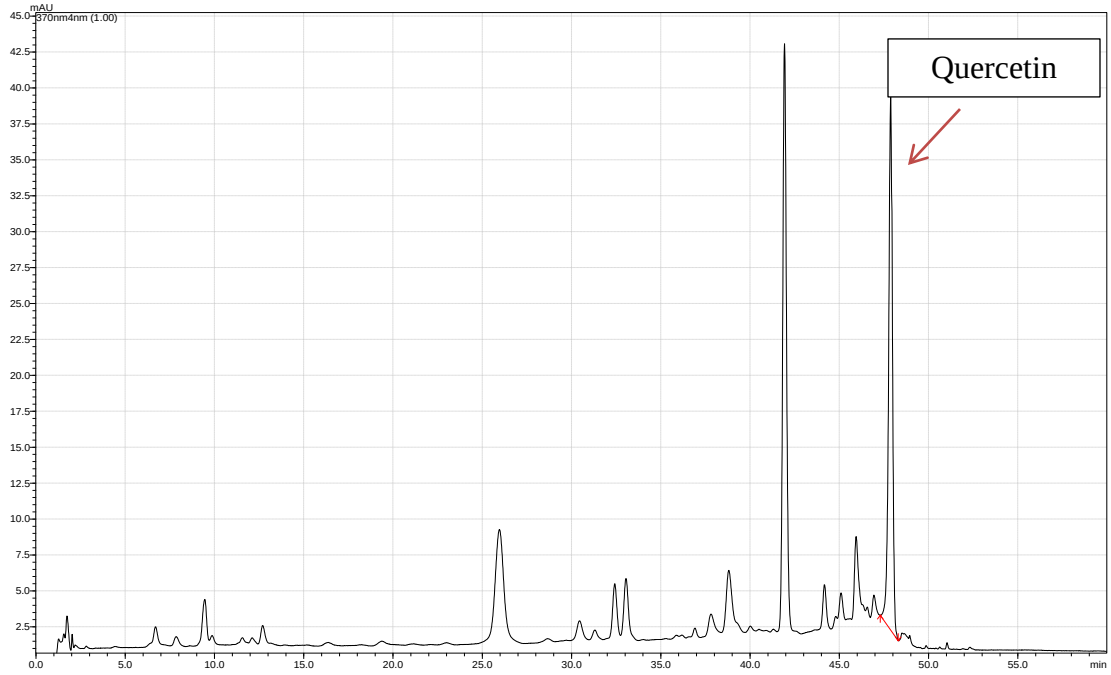
Catechin



Gallic Acid

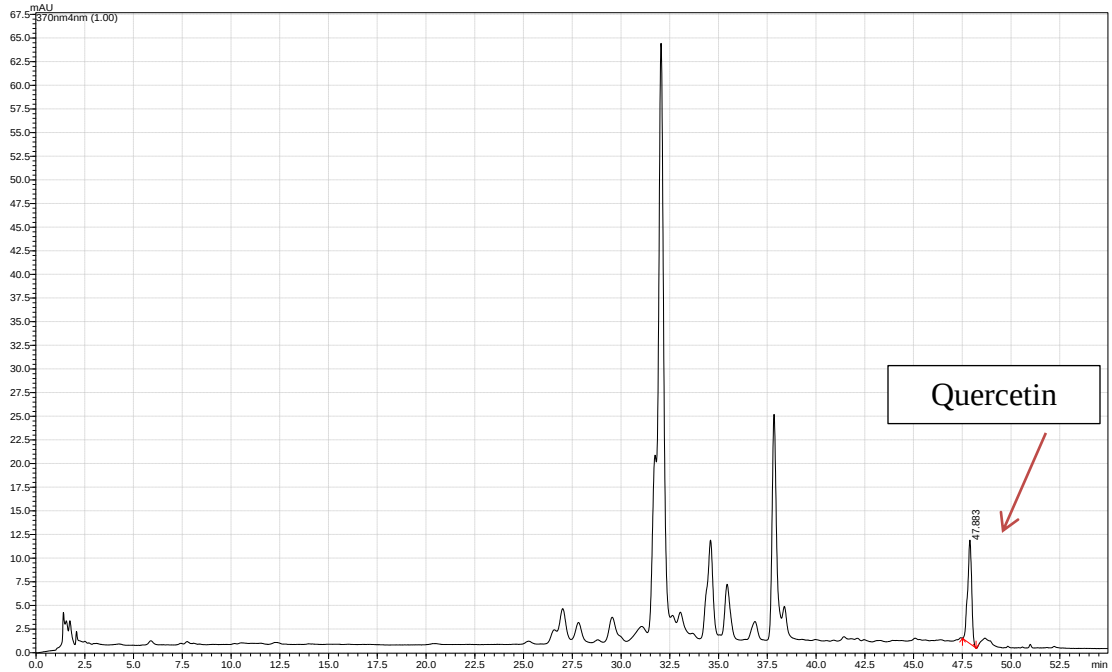


Quercetin



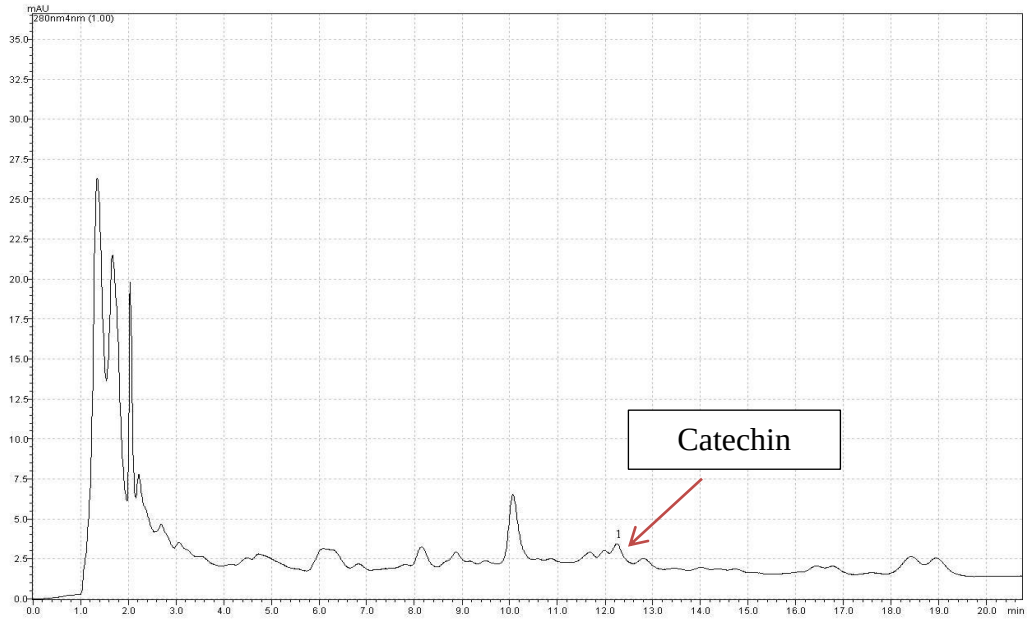
Clinacanthus nutans

Quercetin

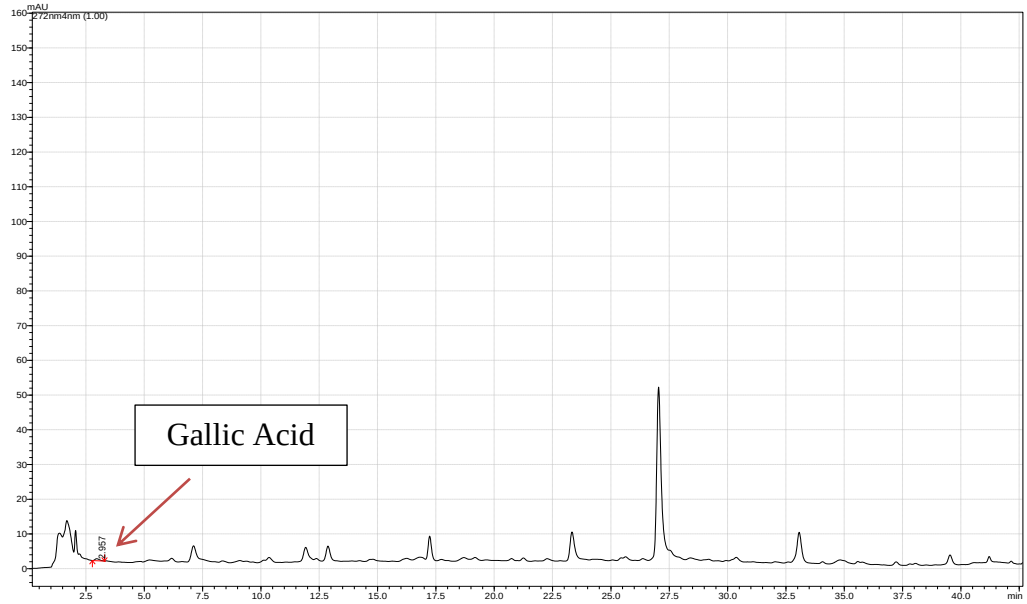


Hedyotis diffusa

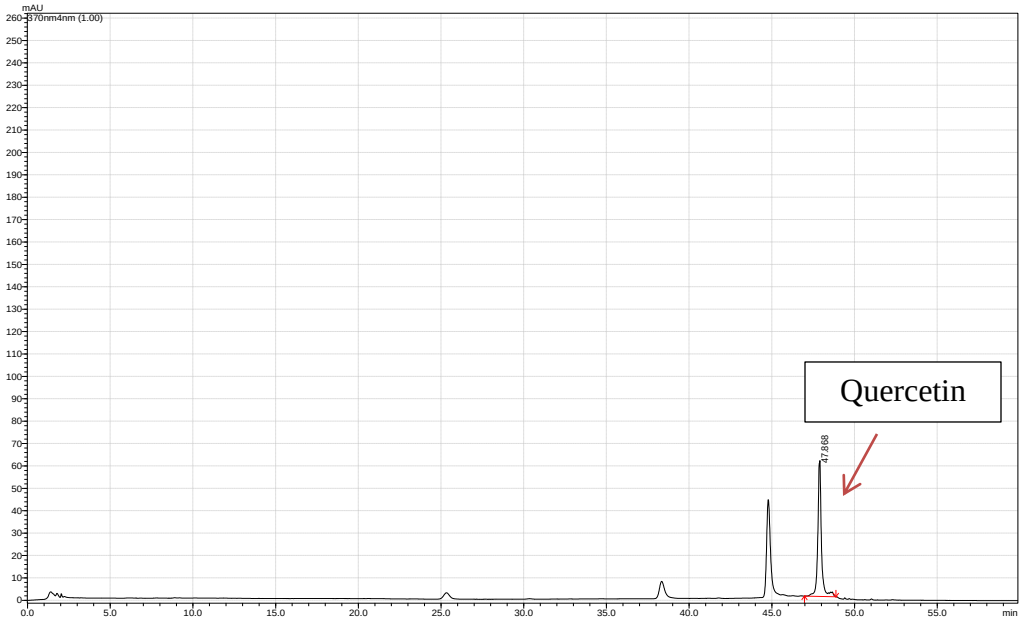
Catechin



Gallic Acid

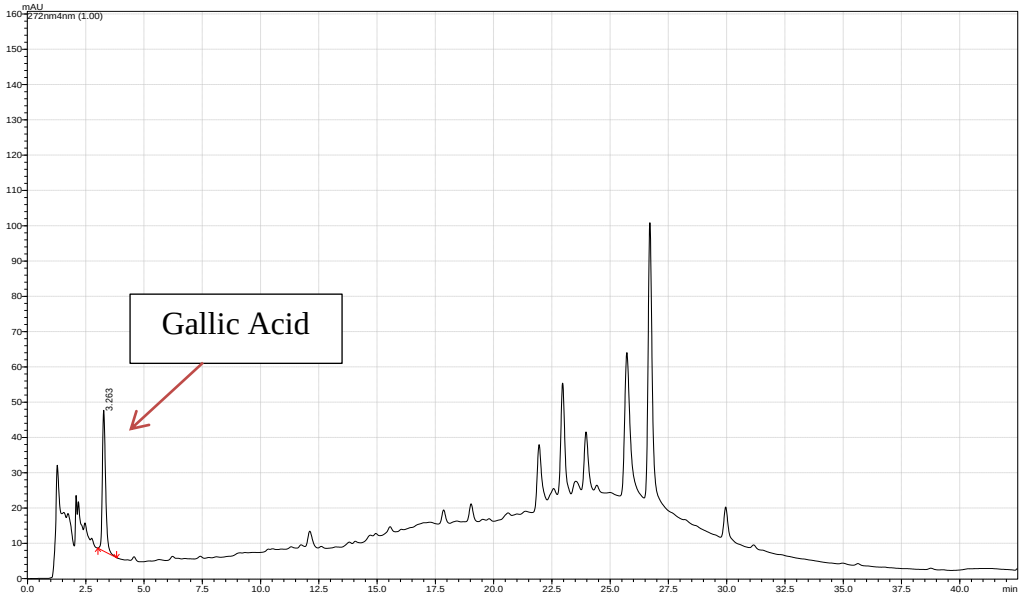


Quercetin

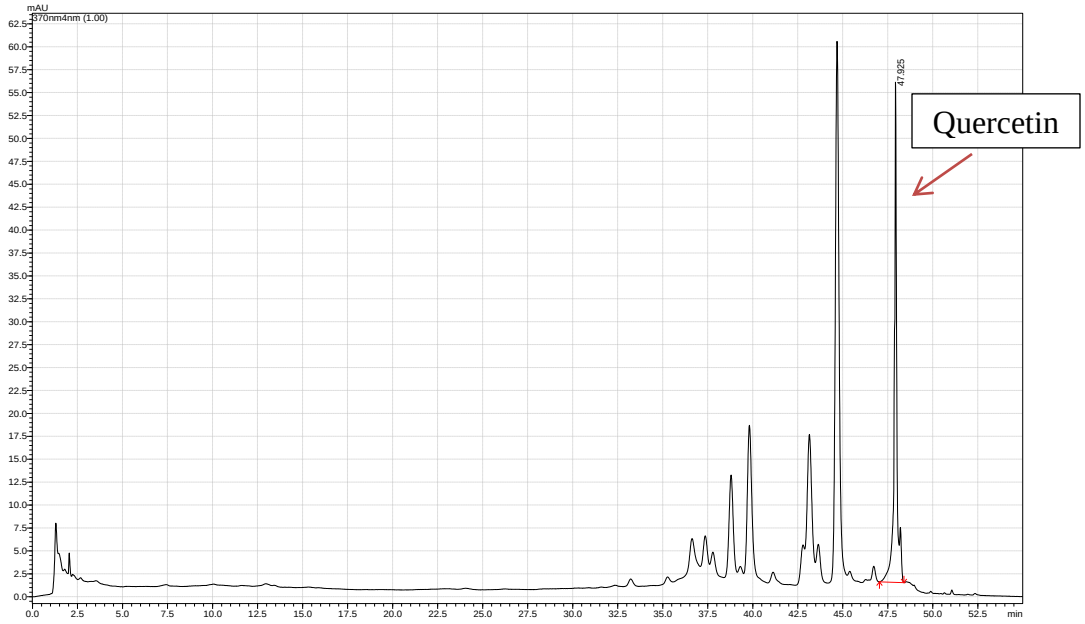


Melastoma candidum

Gallic Acid

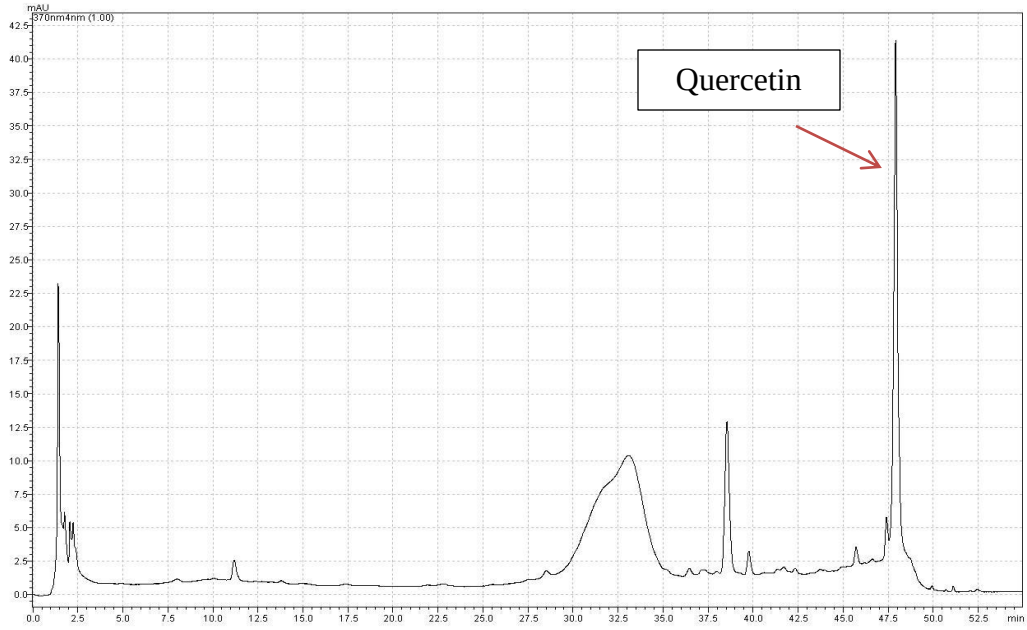


Quercetin



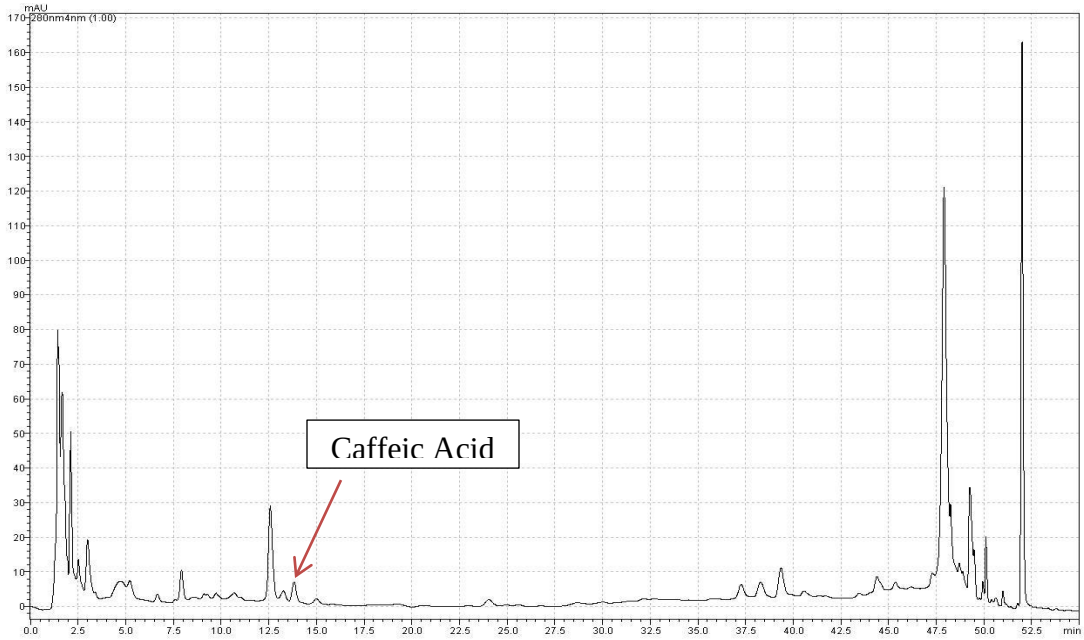
Pereskia bleo

Quercetin

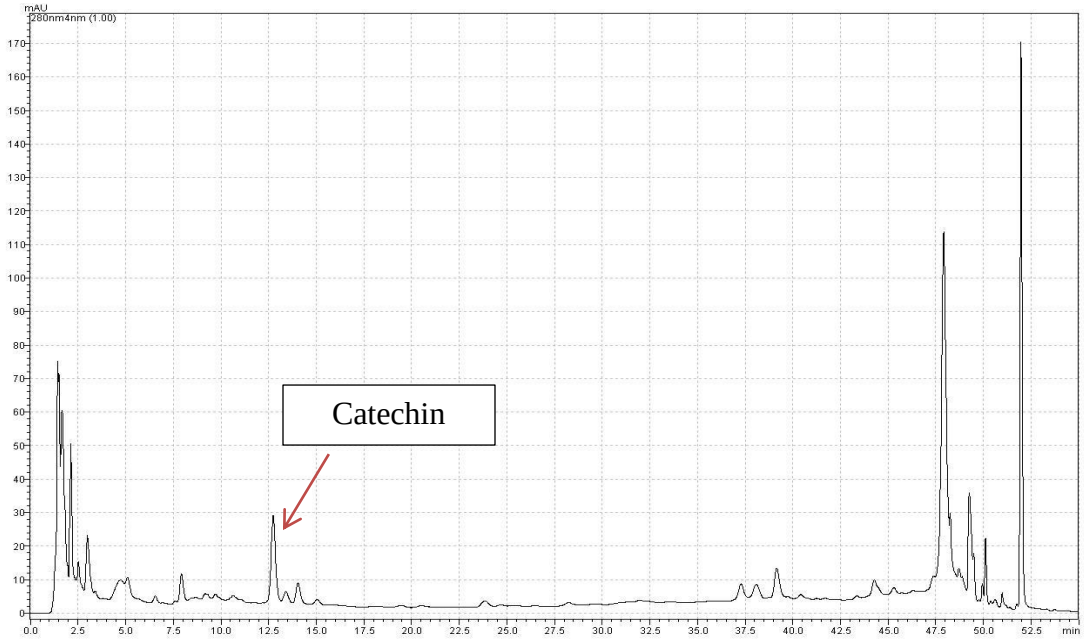


Vernonia amygdalina

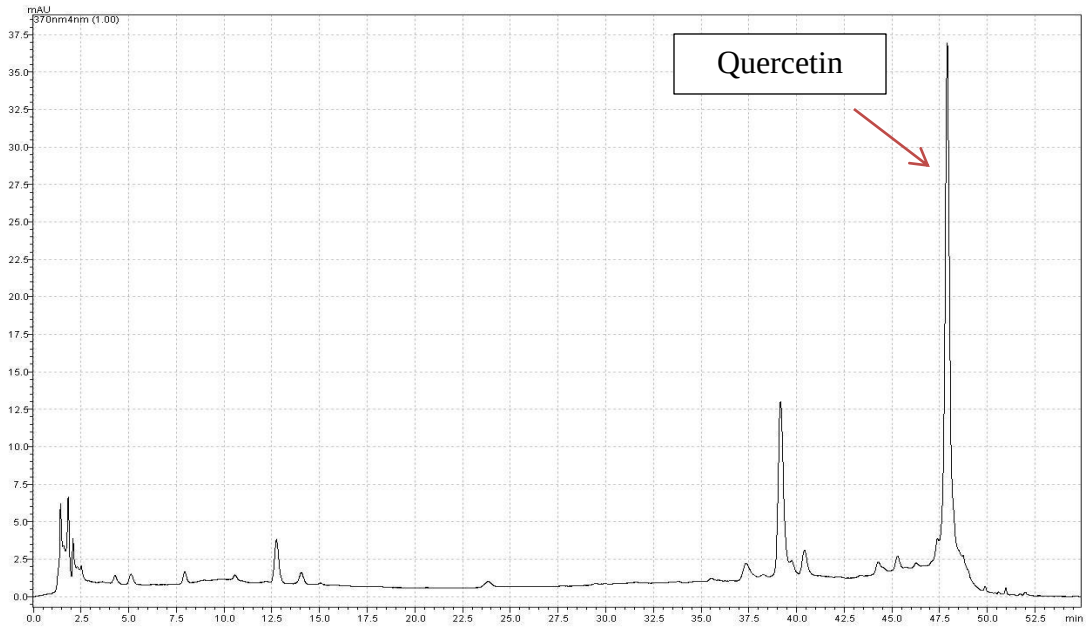
Caffeic Acid



Catechin

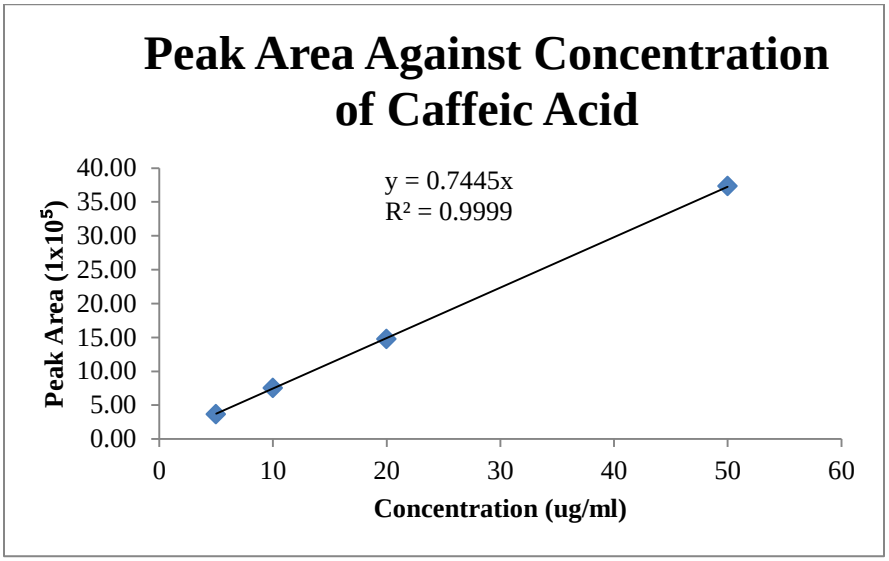


Quercetin


mAU
370nm4nm (1.00)
Quercetin
37.5
35.0
32.5
30.0
27.5
25.0
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
0.0 2.5 5.0 7.5 10.0 12.5 15.0 17.5 20.0 22.5 25.0 27.5 30.0 32.5 35.0 37.5 40.0 42.5 45.0 47.5 50.0 52.5 min

APPENDIX F

Concentration and Area for Standard Caffeic Acid from High Performance Liquid Chromatography



Concentration (ug/ml)	Area ($1x10^5$)			Average	Standard Deviation
	Injection 1	Injection 2	Injection 3		
5	3.65	3.54	3.70	3.63	0.05
10	7.44	7.60	7.43	7.49	0.06
20	13.49	15.07	15.56	14.71	0.62
50	37.92	38.34	35.64	37.30	0.84

Calculation For Concentration of Caffeic acid in Plant Extracts

Compound	Calibration Curve (ug/ml)	Calibration Parameters	
		Slope	R^2
Caffeic acid	5-50	0.7445	0.9999

From the standard curve, the concentration of the phenolic constituents were determined by substituting the area from the sample to the equation y= mx whereby area is y and x is the concentration and m is the slope. In order to get the µg per mg of dried sample, the concentration found in the HPLC were divided with the concentration of plant extract injected into the HPLC.

***Vernonia amygdalina* (20 mg/ml)**

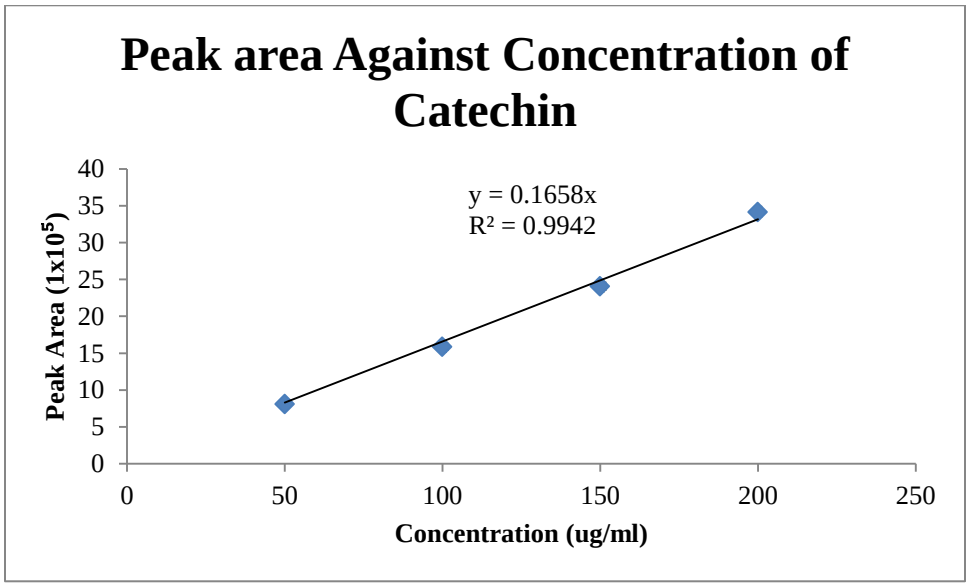
	Injection 1	Injection 2	Injection 3	Average	Standard Deviation
Peak Area	1.16	1.31	1.05	1.17	0.08
Concentration (ug/ml)	1.56	1.76	1.41	1.58	0.10

Concentration of catechin (µg per mg of dried sample)

$$= \frac{\text{1.58 ug/ml}}{\text{20 mg/ml}}$$

= 0.08 ug per mg of dried sample

Concentration and Area for Standard Catechin from High Performance Liquid Chromatography



Concentration (ug/ml)	Area ($1x10^5$)			Average	Standard Deviation
	Injection 1	Injection 2	Injection 3		
50	8.12	8.10	8.06	8.10	0.02
100	15.76	15.25	16.64	15.88	0.41
150	23.67	23.46	25.06	24.06	0.50
200	33.65	34.37	34.47	34.16	0.26

Calculation For Concentration of Catechin in Plant Extracts

Compound	Calibration Curve (ug/ml)	Calibration Parameters	
		Slope	R^2
Catechin	50-200	0.1658	0.9942

From the standard curve, the concentration of the phenolic constituents were determined by substituting the area from the sample to the equation y= mx whereby area is y and x is the concentration and m is the slope. In order to get the µg per mg of dried sample, the concentration found in the HPLC were divided with the concentration of plant extract injected into the HPLC.

***Callicarpa formosana* (20 mg/ml)**

	Injection 1	Injection 2	Injection 3	Average	Standard Deviation
Peak Area	2.31	2.00	2.29	2.20	0.10
Concentration (ug/ml)	13.93	12.06	13.27	13.26	0.55

Concentration of catechin (µg per mg of dried sample)

$$= \frac{\text{13.26 ug/ml}}{\text{20 mg/ml}}$$

= 0.66 ug per mg of dried sample

***Hedyotis diffusa* (20 mg/ml)**

	Injection 1	Injection 2	Injection 3	Average	Standard Deviation
Peak Area	0.25	0.27	0.23	0.25	0.01
Concentration (ug/ml)	1.50	1.65	1.41	1.52	0.07

Concentration of catechin (µg per mg of dried sample)

= 1.52 ug/ml
20 mg/ml

= 0.08 ug per mg of dried sample

***Vernonia amygdalina* (20 mg/ml)**

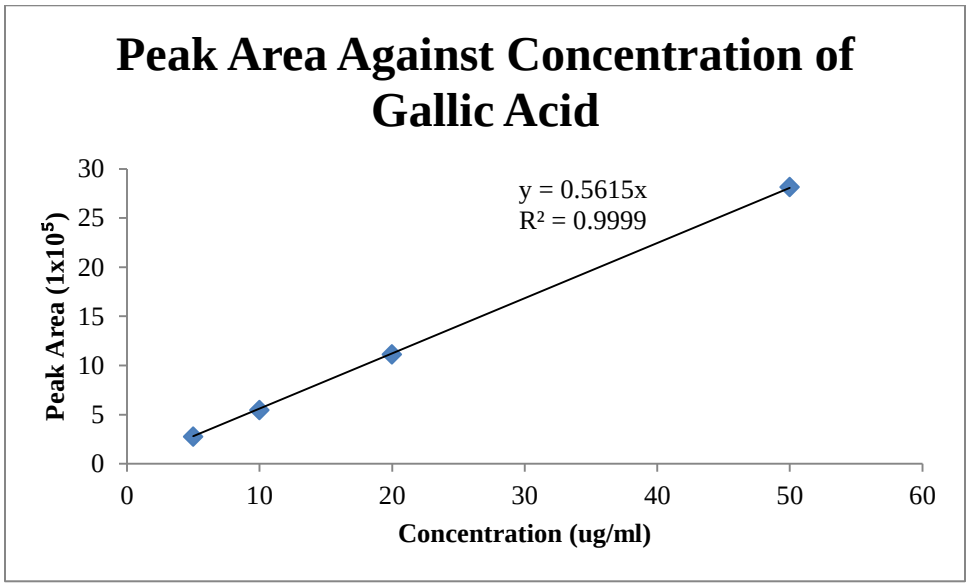
	Injection 1	Injection 2	Injection 3	Average	Standard Deviation
Peak Area	3.90	4.79	4.39	4.36	0.26
Concentration (ug/ml)	23.51	28.88	26.48	26.29	1.55

Concentration of catechin (µg per mg of dried sample)

= 26.29 ug/ml
20 mg/ml

= 1.31 ug per mg of dried sample

Concentration and Area for Standard Gallic Acid from High Performance Liquid Chromatography



Concentration (ug/ml)	Area ($1x10^5$)			Average	Standard Deviation
	Injection 1	Injection 2	Injection 3		
5	2.81	2.72	2.74	2.81	0.03
10	5.35	5.49	5.51	5.35	0.05
20	11.25	11.19	10.93	11.25	0.10
50	27.74	29.11	27.63	27.74	0.47

Calculation For Concentration of Gallic acid in Plant Extracts

Compound	Calibration Curve (ug/ml)	Calibration Parameters	
		Slope	R^2
Gallic acid	5-50	0.5615	0.9999

From the standard curve, the concentration of the phenolic constituents were determined by substituting the area from the sample to the equation y= mx whereby area is y and x is the concentration and m is the slope. In order to get the µg per mg of dried sample, the concentration found in the HPLC were divided with the concentration of plant extract injected into the HPLC.

***Callicarpa formosana* (20 mg/ml)**

	Injection 1	Injection 2	Injection 3	Average	Standard Deviation
Peak Area	0.54	0.45	0.52	0.50	0.03
Concentration (ug/ml)	0.96	0.81	0.92	0.90	0.04

Concentration of gallic acid (µg per mg of dried sample)

$$= \frac{\text{0.90 ug/ml}}{\text{20 mg/ml}}$$

= 0.04 ug per mg of dried sample

***Hedyotis diffusa* (10 mg/ml)**

	Injection 1	Injection 2	Injection 3	Average	Standard Deviation
Peak Area	0.30	0.33	0.32	0.32	0.01
Concentration (ug/ml)	0.54	0.59	0.57	0.56	0.01

Concentration of gallic acid (µg per mg of dried sample)

= 0.56 ug/ml
10 mg/ml

= 0.06 ug per mg of dried sample

***Melastoma candidum* (20 mg/ml)**

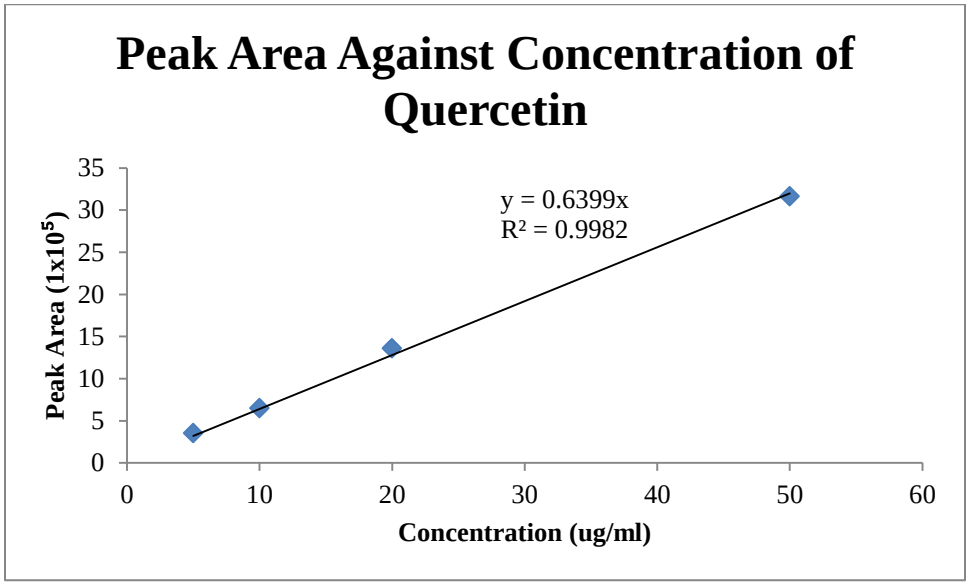
	Injection 1	Injection 2	Injection 3	Average	Standard Deviation
Peak Area	6.85	7.31	6.28	6.81	0.30
Concentration (ug/ml)	12.20	13.02	11.18	12.13	0.53

Concentration of gallic acid (µg per mg of dried sample)

= 12.13 ug/ml
20 mg/ml

= 0.61 ug per mg of dried sample

Concentration and Area for Standard Quercetin from High Performance Liquid Chromatography



Concentration (ug/ml)	Area ($1x10^5$)			Average	Standard Deviation
	Injection 1	Injection 2	Injection 3		
5	3.30	3.65	3.61	3.52	0.11
10	6.27	6.67	6.54	6.49	0.12
20	13.78	13.43	13.54	13.58	0.11
50	32.58	33.03	29.28	31.63	1.18

Calculation For Concentration of Quercetin in Plant Extracts

Compound	Calibration Curve (ug/ml)	Calibration Parameters	
		Slope	R^2
Quercetin	5-50	0.6399	0.9982

From the standard curve, the concentration of the phenolic constituents were determined by substituting the area from the sample to the equation y= mx whereby area is y and x is the concentration and m is the slope. In order to get the µg per mg of dried sample, the concentration found in the HPLC were divided with the concentration of plant extract injected into the HPLC.

***Callicarpa formosana* (20 mg/ml)**

	Injection 1	Injection 2	Injection 3	Average	Standard Deviation
Peak Area	5.58	5.64	5.09	5.50	0.17
Concentration (ug/ml)	8.72	8.82	7.96	8.50	0.27

Concentration of quercetin (µg per mg of dried sample)

$$= \frac{\text{8.50 ug/ml}}{\text{20 mg/ml}}$$

= 0.43 ug per mg of dried sample

***Clinacanthus nutans* (10 mg/ml)**

	Injection 1	Injection 2	Injection 3	Average	Standard Deviation
Peak Area	1.47	1.58	1.75	1.60	0.08
Concentration (ug/ml)	2.30	2.46	2.73	2.50	0.13

Concentration of quercetin (µg per mg of dried sample)

= 2.50 ug/ml
10 mg/ml

= 0.25 ug per mg of dried sample

***Hedoytis diffusa* (20 mg/ml)**

	Injection 1	Injection 2	Injection 3	Average	Standard Deviation
Peak Area	8.82	9.26	9.80	9.29	0.28
Concentration (ug/ml)	13.78	14.47	15.32	14.53	0.45

Concentration of quercetin (µg per mg of dried sample)

= 14.53 ug/ml
20 mg/ml

= 0.73 ug per mg of dried sample

***Melastoma candidum* (20 mg/ml)**

	Injection 1	Injection 2	Injection 3	Average	Standard Deviation
Peak Area	4.93	5.37	5.0	5.10	0.14
Concentration (ug/ml)	7.70	8.38	7.81	7.97	0.21

Concentration of quercetin (µg per mg of dried sample)

= 7.97 ug/ml
20 mg/ml

= 0.40 ug per mg of dried sample

***Pereskia bleo* (20 mg/ml)**

	Injection 1	Injection 2	Injection 3	Average	Standard Deviation
Peak Area	6.85	6.14	7.04	6.68	0.27
Concentration (ug/ml)	10.71	9.59	11.01	10.44	0.43

Concentration of quercetin (µg per mg of dried sample)

= 10.44 ug/ml
20 mg/ml

= 0.52 ug per mg of dried sample

***Vernonia amygdalina* (20 mg/ml)**

	Injection 1	Injection 2	Injection 3	Average	Standard Deviation
Peak Area	6.67	7.24	7.93	7.28	0.36
Concentration (ug/ml)	10.43	11.31	12.39	11.38	0.57

Concentration of quercetin (µg per mg of dried sample)

= $\frac{\text{11.38 ug/ml}}{\text{20 mg/ml}}$

= 0.57 ug per mg of dried sample

APPENDIX G

Protein Sequencing Data

Sequences of protein samples which matched with the sequences from the protein database are highlighted in bold red.

```
  1 MMKMKTHIVS SVTTTLLLGS ILMNPVANAA DSDINIKTGT TDIGSNTTVK
 51 TGDLVTYDKE NGMHKKVFYS FIDDKNHNKK LLVIRTKGTI AGQYRVYSEE
101 GANKSGLAWP SAFKVQLQLP DNEVAQISDY YPRNSIDTKE YMSTLTYGFN
151 GNVTGDDTGK IGGLIGANVS IGHTLKYVQP DFKTILESPT DKKVGWKVIF
201 NNMVNQNWGP YDRDSWNPVY GNQLFMKTRN GSMKAADNFL DPNKASSLLS
251 SGFSPDFATV ITMDRKASKQ QTNIDVIYER VRDDYQLHWT STNWKGTNTK
301 DKWIDRSSER YKIDWEKEEM TN
```

Protein A- Alpha Hemolysin from *S. aureus* ATCC 6538 treated with *M. candidum*

```
  1 MMKSQNKYSI RKFSVGASSI LIATLLFLSG GQAQAAEKQV NMGNSQEDTV
 51 TAQSIGDQQT RENANYQREN GVDEQQHTEN LTKNLHNDKT ISEENHRKTD
101 DLNKDQLKDD KKSSLNNKNI QRDTTKNNNA NPSDVNQGLE QAINDGKQSK
151 VASQQQSKEA DNSQDSNANN NLPSQSRTKE ALPLNKSNQT SQREIVNKTE
201 IEKVQPQQNN QANDKITNYN FNNEQEVKPQ KDEKTLSVSD LKNNQKSPVE
251 PTKDNDKKNG LNLLKSSAVA TLPNKGTKEL TAKAKGDQTN KVAKQGQYKN
301 QDPIVLVHGF NGFTDDINPS VLAHYWGGNK MNIRQDLEEN GYKAYEASIS
351 AFGSNYDRAV ELYYYIKGGR VDYGAAHAAK YGHERYGKTY EGIYKDWKPG
401 QKVHLVGHSM GGQTIRQLEE LLRNGNREEI EYQKKHGGEI SPLFKGNNDN
451 MISSITTLGT PHNGTHASDL AGNEALVRQI VFDIGKMFGN KNSRVDFGLA
501 QWGLKQKPNE SYIDYVKRVK QSNLWKSKDN GFYDLTREGA TDLNRKTSLN
551 PNIVYKTYTG EATHKALNSD RQKADLNMFF PFVITGNLIG KATEKEWREN
601 DGLVSVISSQ HPFNQAYTNA TDKIQKGIWQ VTPTKHDWDH VDFVGQDSSD
651 TVRTREELQD FWHHLADDLV KTEKVTDTKQ A
```

Protein B- Triacylglycerol lipase from *S. aureus* ATCC 6538 treated with *C. formosana*

```
  1 MPKNKILIYL LSTTLVLPTL VSPTAYADIP QKDTTAKTTS HDSKKSNDDE
 51 TSKDTTSKDI DKADNNNTSN QDNNDKKFKT IDDSTSDSNN IIDFIYKNLP
101 QTNINQLLTK NKYDDNYSLT TLIQNLFNLN SDISDYEQPR NGEKSTNDSN
151 KNSDNSIKND TDTQSSKQDK ADNQKAPKSN NTKPSTSNKQ PNSPKPTQPN
201 QSNSQPASDD KANQKSSSKD NQSMSDSALD SILDQYSEDA KKTQKDYASQ
251 SKKDKNEKSN TKNPQLPTQD ELKHKSKPAQ SFNNDVNQKD TRATSLFETD
301 PSISNNDDSG QFNVVDSKDT RQFVKSIAKD AHRIGQDNDI YASVMIAQAI
351 LESDSGRSAL AKSPNHNLFG IKGAFEGNSV PFNTLEADGN QLYSINAGFR
401 KYPSTKESLK DYSDLIKNGI DGNRTIYKPT WKSEADSYKD ATSHLSKTYA
451 TDPNYAKKLN SIIKHYQLTQ FDDERMPDLD KYERSIKDYD DSSDEFKPFR
501 EVSDSMPYPH GQCTWYVYNR MKQFGTSISG DLGDAHNWNN RAQYRDYQVS
551 HTPKRHAAVV FEAGQFGADQ HYGHVAFVEK VNSDGSIVIS ESNVKGLGII
601 SHRTINASAA EELSYITGK
```

Protein C- N-acetylmuramoyl-L-alanine amidase from *S. aureus* ATCC 6538 treated with *C. formosana*

```
  1 MMKMKTHIVS SVTTTLLLGS ILMNPVANAA DSDINIKTGT TDIGSNTTVK
 51 TGDLVTYDKE NGMHKKVFYS FIDDKNHNKK LLVIRTKGTI AGQYRVYSEE
101 GANKSGLAWP SAFKVQLQLP DNEVAQISDY YPRNSIDTKE YMSTLTYGFN
151 GNVTGDDTGK IGGLIGANVS IGHTLKYVQP DFKTILESPT DKKVGWKVIF
201 NNMVNQNWGP YDRDSWNPVY GNQLFMKTRN GSMKAADNFL DPNKASSLLS
251 SGFSPDFATV ITMDRKASKQ QTNIDVIYER VRDDYQLHWT STNWKGTNTK
301 DKWIDRSSER YKIDWEKEEM TN
```

Protein D- Alpha hemolysin from *S. aureus* ATCC 6538 treated with *C. formosana*

```
  1 MSESFAELFE ESLKSLDMQP GAIITGIVVD IDGDWVTVHA GLKSEGVIPV
 51 EQFYNEQGEL TIKVGDEVHV ALDAVEDGFG ETKLSREKAK RAESWIVLEA
101 AFAADEVVKG VINGKVKGGF TVDVNGIRAF LPGSLVDVRP VRDTTHLEGK
151 ELEFKVIKLD QKRNNVVVSR RSVLEAENSA EREALLESLQ EGQQVKGIVK
201 NLTDYGAFVD LGGVDGLLHI TDMAWKRIKH PSEIVNVGDE IDVKVLKFDR
251 ERNRVSLGLK QLGEDPWVAI KARYPEGTRV MARVTNLTDY GCFAELEEGV
301 EGLVHVSEMD WTNKNIHPSK VVQVGDEVEV QVLDIDEERR RISLGIKQCK
351 SNPWEDFSSQ FNKGDRISGT IKSITDFGIF IGLDGGIDGL VHLSDISWNE
401 VGEEAVRRFK KGDELETVIL SVDPERERIS LGIKQLEDDP FSNYASLHEK
451 GSIVRGTVKE VDAKGAVISL GDDIEGILKA SEISRDRVED ARNVLKEGEE
501 VEAKIISIDR KSRVISLSVK SKDVDDEKDA MKELRKQEVE SAGPTTIGDL
551 IRAQMENQG
```

Protein E- 30 S Ribosomal Protein S1 from *P. aeruginosa* ATCC 27853 treated with *M. candidum*

```
  1 MAAKEVKFGD SARKKMLVGV NVLADAVKAT LGPKGRNVVL DKSFGAPTIT
 51 KDGVSVAKEI ELKDKFENMG AQLVKDVASK ANDAAGDGTT TATVLAQAIV
101 NEGLKAVAAG MNPMDLKRGI DKATVAIVAQ LKELAKPCAD TKAIAQVGTI
151 SANSDESIGQ IIAEAMEKVG KEGVITVEEG SGLENELSVV EGMQFDRGYL
201 SPYFVNKPDT MAAELDSPLL LLVDKKISDI PRKCCRCWKP SPRPAVLLLI
251 VAEDVEGEAL ATLVVNNMRG IVKVAAVKAP GFGDRRKAML QDIAILTGGT
301 VISEEVGLSL EGATLEHLGN AKRVVINKEN TTIIDGAGVQ ADIEARVLQI
351 RKQIEETTSD YDREKLQERL AKLAGGVAVI KVGAATEVEM KEKKARVEDA
401 LHATRAAVEE GVVPGGGVAL VRALQAIEGL KGDNEEQNVG IALLRRAVES
451 PLRQIVANAG DEPSVVVDKV KQGSGNYGFN AATGVYGDMI EMGILDPAKV
501 TRSALQAAAS IGGLMITTEA MVAEIVEDKP AMGGMPDMGG MGGMGGMM
```

Protein F- 60 kDa Chaperonine from *P. aeruginosa* ATCC 27853 treated with *M. candidum*

```
  1 MVKELRERTG LGMMECKKAL TAAGGDIEKA IDDMRAAGAI KAAKKAGNIA
 51 AEGSIAVKIA ADNKAAVIIE VNSQTDFLAL QDDFKGFVAE SLEKAFNEKL
101 TDAAPLVEAR EEARLALVAK TGENVNIRRL TRVEGDVVGA YLHGHRIGVV
151 VNLKGGNPEL AKDIAMHVAA SNPQFLSASE VSEEAIAKEK EIFLALNADK
201 IAGKPENIVE NMVKGRISKF LAEASLVEQP FVKNPEVKVG DLAKQAGAEI
251 VSFVRYEVGE GIEKAEVDFA AEVAAQVAAT KQ
```

Protein G- Elongation Factor Ts from *P. aeruginosa* ATCC 27853 treated with *M. candidum*

```
  1 MSELNEKLAT AWEGFTKGDW QNEVNVRDFI QKNYTPYEGD ESFLAGATEA
 51 TTTLWDKVME GVKLENRTHA PVDFDTAVAS TITSHDAGYI NKQLEKIVGL
101 QTEAPLKRAL IPFGGIKMIE GSCKAYNREL DPMIKKIFTE YRKTHNQGVF
151 DVYTPDILRC RKSGVLTGLP DAYGRGRIIG DYRRVALYGI DYLMKDKLAQ
201 FTSLQADLEN GVNLEQTIRL REEIAEQHRA LGQMKEMAAK YGYDISGPAT
251 NAQEAIQWTY FGYLAAVKSQ NGAAMSFGRT STFLDVYIER DLKAGKITEQ
301 EAQEMVDHLV MKLRMVRFLR TPEYDELFSG DPIWATESIG GMGLDGRTLV
351 TKNSFRFLNT LYTMGPSPEP NMTILWSEKL PLNFKKFAAK VSIDTSSLQY
401 ENDDLMRPDF NNDDYAIACC VSPMIVGKQM QFFGARANLA KTMLYAINGG
451 VDEKLKMQVG PKSEPIKGDV LNYDEVMERM DHFMDWLAKQ YITALNIIHY
501 MHDKYSYEAS LMALHDRDVI RTMACGIAGL SVAADSLSAI KYAKVKPIRD
551 EDGLAIDFEI EGEYPQFGNN DPRVDDLAVD LVERFMKKIQ KLHTYRDAIP
601 TQSVLTITSN VVYGKKTGNT PDGRRAGAPF GPGANPMHGR DQKGAVASLT
651 SVAKLPFAYA KDGISYTFSI VPNALGKDDE VRKTNLAGLM DGYFHHEASI
701 EGGQHLNVNV MNREMLLDAM ENPENIRS
```

Protein H- Formate C-Acetyltransferase from *E. coli* ATCC 35218 treated with *M. candidum*

```
  1 MRSNHFKEFK MDKKQVTDLR SELLDSRFGA KSISTIAESK RFPLHEMRDD
 51 VAFQIINDEL YLDGNARQNL ATFCQTWDDD NVHKLMDLSI NKNWIDKEEY
101 PQSAAIDLRC VNMVADLWHA PAPKNGQAVG TNTIGSSEAC MLGGMAMKWR
151 WRKRMEAAGK PTDKPNLVCG PVQICWHKFA RYWDVELREI PMRPGQLFMD
201 PKRMIEACDE NTIGVVPTFG VTYTGNYEFP QPLHDALDKF QADTGIDIDM
251 HIDAASGGFL APFVAPDIVW DFRLPRVKSI SASGHKFGLA PLGCGWVIWR
301 DEEALPQELV FNVDYLGGQI GTFAINFSRP AGQVIAQYYE FLRLGREGYT
351 KVQNASYQVA AYLADEIAKL GPYEFICTGR PDEGIPAVCF KLKDGEDPGY
401 TLYDLSERLR LRGWQVPAFT LGGEATDIVV MRIMCRRGFE MDFAELLLED
451 YKASLKYLSD HPKLQGIAQQ NSFKHT
```

Protein I- Glutamate decarboxylase from *E. coli* ATCC 35218 treated with *M. candidum*

```
  1 MENFKHLPEP FRIRVIEPVK RTTRAYREEA IIKSGMNPFL LDSEDVFIDL
 51 LTDSGTGAVT QSMQAAMMRG DEAYSGSRSY YALAESVKNI FGYQYTIPTH
101 QGRGAEQIYI PVLIKKREQE KGLDRSKMVA FSNYFFDTTQ GHSQINGCTV
151 RNVYIKEAFD TGVRYDFKGN FDLEGLERGI EEVGPNNVPY IVATITSNSA
201 GGQPVSLANL KAMYSIAKKY DIPVVMDSAR FAENAYFIKQ REAEYKDWTI
251 EQITRETYKY ADMLAMSAKK DAMVPMGGLL CVKDDSFFDV YTECRTLCVV
301 QEGFPTYGGL EGGAMERLAV GLYDGMNLDW LAYRIAQVQY LVDGLEEIGV
351 VCQQAGGHAA FVDAGKLLPH IPADQFPAQA LACELYKVAG IRAVEIGSFL
401 LGRDPKTGKQ LPCPAELLRL TIPRATYTQT HMDFIIEAFK HVKENAANIK
451 GLTFTYEPKV LRHFTAKLKE V
```

Protein J- Tryptophanase from *E. coli* ATCC 35218 treated with *M. candidum*

```
  1 MSKEKFERTK PHVNVGTIGH VDHGKTTLTA AITTVLAKTY GGAARAFDQI
 51 DNAPEEKARG ITINTSHVEY DTPTRHYAHV DCPGHADYVK NMITGAAQMD
101 GAILVVAATD GPMPQTREHI LLGRQVGVPY IIVFLNKCDM VDDEELLELV
151 EMEVRELLSQ YDFPGDDTPI VRGSALKALE GDAEWEAKIL ELAGFLDSYI
201 PEPERAIDKP FLLPIEDVFS ISGRGTVVTG RVERGIIKVG EEVEIVGIKE
251 TQKSTCTGVE MFRKLLDEGR AGENVGVLLR GIKREEIERG QVLAKPGTIK
301 PHTKFESEVY ILSKDEGGRH TPFFKGYRPQ FYFRTTDVTG TIELPEGVEM
351 VMPGDNIKMV VTLIHPIAMD DGLRFAIREG GRTVGAGVVA KVLS
```

Protein K- Elongation Factor Tu 2 from *E. coli* ATCC 35218 treated with *M. candidum*

```
  1 MAQVINTNSL SLITQNNINK NQSALSSSIE RLSSGLRINS AKDDAAGQAI
 51 ANRFTANIKG LTQASRNAND GISVAQTTEG ALNEINNNLQ RVRELTVQAT
101 NGTNSDSDLS SIQAEITQRL EEIDRVSEQT QFNGVKVLAE NNEMKIQVGA
151 NDGETITINL AKIDAKTLGL DGFNIDGAQK ATGSDLISKF KATGTDNYQI
201 NGTDNYTVNV DSGAVQNEDG DAIFVSATDG SLTTKSDTKV GGTGIDATGL
251 AKAAVSLAKD ASIKYQGITF TNKGTGAFDG SSNGTLIANI DGKDVTFTID
301 ATGKDATLKT SDPVYKNSAG QFTTTKVENK AATASDLDLN NAKKVGSSLV
351 VNGADYEVSA DGKTVTGLGK TMYLSKSEGG SPILVKEDAA KSLQSTTNPL
401 ETIDKALAKV DNLRSDLGAV QNRFDSAITN LGNTVNNLSS ARSRIEDADY
451 ATEVSNMSRA QILQQAGTSV LAQANQTTQN VLSLLR
```

Protein L- Flagellin from *E. coli* ATCC 35218 treated with *S. barbata*

```
  1 MARTTPIARY RNIGISAHID AGKTTTTERI LFYTGVNHKI GEVHDGAATM
 51 DWMEQEQERG ITITSAATTA FWSGMAKQYE PHRINIIDTP GHVDFTIEVE
101 RSMRVLDGAV MVYCAVGGVQ PQSETVWRQA NKYKVPRIAF VNKMDRMGAN
151 FLKVVNQIKT RLGANPVPLQ LAIGAEEHFT GVVDLVKMKA INWNDADQGV
201 TFEYEDIPAD MVELANEWHQ NLIESAAEAS EELMEKYLGG EELTEAEIKG
251 ALRQRVLNNE IILVTCGSAF KNKGVQAMLD AVIDYLPSPV DVPAINGILD
301 DGKDTPAERH ASDDEPFSAL AFKIATDPFV GNLTFFRVYS GVVNSGDTVL
351 NSVKAARERF GRIVQMHANK REEIKEVRAG DIAAAIGLKD VTTGDTLCDP
401 DAPIILERME FPEPVISIAV EPKTKADQEK MGLALGRLAK EDPSFRVWTD
451 EESNQTIIAG MGELHLDIIV DRMKREFNVE ANVGKPQVAY RETIRQKVTD
501 VEGKHAKQSG GRGQYGHVVI DMYPLEPGSN PKGYEFINDI KGGVIPGEYI
551 PAVDKGIQEQ LKAGPLAGYP VVDMGVRLHF GSYHDVDSSE LAFKLAASIA
601 FKEGFKKAKP VLLEPIMKVE VETPEENTGD VIGDLSRRRG MLKGQESEVT
651 GVKIHAEVPL SEMFGYATQL RSLTKGRASY TMEFLKYDEA PSNVAQAVIE
701 ARGK
```

Protein K- Elongation Factor G from *E. coli* ATCC 25922 treated with *M. candidum*

```
  1 MSELNEKLAT AWEGFTKGDW QNEVNVRDFI QKNYTPYEGD ESFLAGATEA
 51 TTTLWDKVME GVKLENRTHA PVDFDTAVAS TITSHDAGYI NKQLEKIVGL
101 QTEAPLKRAL IPFGGIKMIE GSCKAYNREL DPMIKKIFTE YRKTHNQGVF
151 DVYTPDILRC RKSGVLTGLP DAYGRGRIIG DYRRVALYGI DYLMKDKLAQ
201 FTSLQADLEN GVNLEQTIRL REEIAEQHRA LGQMKEMAAK YGYDISGPAT
251 NAQEAIQWTY FGYLAAVKSQ NGAAMSFGRT STFLDVYIER DLKAGKITEQ
301 EAQEMVDHLV MKLRMVRFLR TPEYDELFSG DPIWATESIG GMGLDGRTLV
351 TKNSFRFLNT LYTMGPSPEP NMTILWSEKL PLNFKKFAAK VSIDTSSLQY
401 ENDDLMRPDF NNDDYAIACC VSPMIVGKQM QFFGARANLA KTMLYAINGG
451 VDEKLKMQVG PKSEPIKGDV LNYDEVMERM DHFMDWLAKQ YITALNIIHY
501 MHDKYSYEAS LMALHDRDVI RTMACGIAGL SVAADSLSAI KYAKVKPIRD
551 EDGLAIDFEI EGEYPQFGNN DPRVDDLAVD LVERFMKKIQ KLHTYRDAIP
601 TQSVLTITSN VVYGKKTGNT PDGRRAGAPF GPGANPMHGR DQKGAVASLT
651 SVAKLPFAYA KDGISYTFSI VPNALGKDDE VRKTNLAGLM DGYFHHEASI
701 EGGQHLNVNV MNREMLLDAM ENPENIRS
```

Protein L- Formate C-Acetyltransferase from *E. coli* ATCC 25922 treated with *M. candidum*

```
  1 MSKEKFERTK PHVNVGTIGH VDHGKTTLTA AITTVLAKTY GGAARAFDQI
 51 DNAPEEKARG ITINTSHVEY DTPTRHYAHV DCPGHADYVK NMITGAAQMD
101 GAILVVAATD GPMPQTREHI LLGRQVGVPY IIVFLNKCDM VDDEELLELV
151 EMEVRELLSQ YDFPGDDTPI VRGSALKALE GDAEWEAKIL ELAGFLDSYI
201 PEPERAIDKP FLLPIEDVFS ISGRGTVVTG RVERGIIKVG EEVEIVGIKE
251 TQKSTCTGVE MFRKLLDEGR AGENVGVLLR GIKREEIERG QVLAKPGTIK
301 PHTKFESEVY ILSKDEGGRH TPFFKGYRPQ FYFRTTDVTG TIELPEGVEM
351 VMPGDNIKMV VTLIHPIAMD DGLRFAIREG GRTVGAGVVA KVLS
```

Protein N- Elongation Factor Tu 2 from *E. coli* ATCC 25922 treated with *M. candidum*

```
  1 MKKTAIAIAV ALAGFATVAQ AAPKDNTWYT GAKLGWSQYH DTGFIPNNGP
 51 THENQLGAGA FGGYQVNPYV GFEMGYDWLG RMPYKGDNIN GAYKAQGVQL
101 TAKLGYPITD DLDIYTRLGG MVWRADTKAN VPGGASFKDH DTGVSPVFAG
151 GVEYAITPEI ATRLEYQWTN NIGDAHTIGT RPDNGMLSLG VSYRFGQGEA
201 APVVAPAPAP APEVQTKHFT LKSDVLFTFN KATLKPEGQA ALDQLYSQLS
251 NLDPKDGSVV VLGYTDRIGS DAYNQALSER RAQSVVDYLI SKGIPADKIS
301 ARGMGESNPV TGNTCDNVKQ RAALIDCLAP DRRVEIEVKG IKDVVTQPQA
```

Protein O- Outer Membrane Protein A from *E. coli* ATCC 25922 treated with *M. candidum*

```
  1 MNTIINGSAS EADAARKQLR EELLAIAPVF GQKPYFLSDE FSLVDCYLAP
 51 LLWRLPQLGI EFSGPGAKEL KGYMTRVFER DSFLASLTEA EREMRLGRS
```

Protein P- Stringent Starvation Protein A from *E. coli* ATCC 25922 treated with *M. candidum*

Appendix H

Protein Fold Difference Between Untreated and Treated Bacteria Cells

Alpha Hemolysin *S. aureus* ATCC 6538 (treated with *M. candidum*)

	Area	Percent	Relative Density
Untreated (Control)	18389.723	71.989	1.00
Treated	7155.510	28.011	0.39

The bacteria which were treated with antibacterial plant extract have shown to have 2.56 fold reduction compared with the control.

Formate C-Acetyltransferase *E. coli* ATCC 35218 (treated with *M. candidum*)

	Area	Percent	Relative Density
Untreated (Control)	392.728	88.468	1.00
Treated	51.192	11.532	0.13

The bacteria which were treated with antibacterial plant extract have shown to have 7.69 fold reduction compared with the control.

Glutamate Decarboxylase of *E. coli* ATCC 35218 (treated with *M. candidum*)

	Area	Percent	Relative Density
Untreated (Control)	1885.870	97.257	1.00
Treated	53.192	2.743	0.03

The bacteria which were treated with antibacterial plant extract have shown to have 33.3 fold reduction compared with the control

Tryptophanase of *E. coli* ATCC 35218 (treated with *M. candidum*)

	Area	Percent	Relative Density
Untreated (Control)	251.042	89.210	1.00
Treated	30.364	10.790	0.12

The bacteria which were treated with antibacterial plant extract have shown to have 8.33 fold reduction compared with the control.

Elongation Factor Tu 2 of *E. coli* ATCC 35218 (treated with *M. candidum*)

	Area	Percent	Relative Density
Untreated (Control)	962.799	96.679	1.00
Treated	33.071	3.321	0.03

The bacteria which were treated with antibacterial plant extract have shown to have 33.3 fold reduction compared with the control.

Triacylglycerol lipase of *S. aureus* ATCC 6538 (treated with *M. candidum*)

	Area	Percent	Relative Density
Untreated (Control)	1043.627	76.647	1.00
Treated	317.971	23.353	0.30

The bacteria which were treated with antibacterial plant extract have shown to have 3.33 fold reduction compared with the control.

N-acetylmuramoyl-L-Alanine of *S. aureus* ATCC 6538 (treated with *M. candidum*)

	Area	Percent	Relative Density
Untreated (Control)	2152.113	92.920	1.00
Treated	163.971	7.080	0.08

The bacteria which were treated with antibacterial plant extract have shown to have 12.5 fold reduction compared with the control.

Alpha Hemolysin of *S. aureus* ATCC 6538 (treated with *C. formosana*)

	Area	Percent	Relative Density
Untreated (Control)	2106.184	95.218	1.00
Treated	105.778	4.782	0.05

The bacteria which were treated with antibacterial plant extract have shown to have 20 fold reduction compared with the control.

Flagellin of *E. coli* ATCC 35218 (treated with *M. candidum*)

	Area	Percent	Relative Density
Treated	2354.426	83.405	1.00
Untreated (Control)	468.456	16.595	0.20

The bacteria which were treated with antibacterial plant extract have shown to have 5 fold reduction compared with the control.

Elongation Factor G of *E. coli* ATCC 25922 (treated with *M. candidum*)

	Area	Percent	Relative Density
Untreated (Control)	1830.255	94.284	1.00
Treated	110.950	5.716	0.06

The bacteria which were treated with antibacterial plant extract have shown to have 16.7 fold reduction compared with the control.

Formate C-acetyltransferase of *E. coli* ATCC 25922 (treated with *M. candidum*)

	Area	Percent	Relative Density
Untreated (Control)	2569.012	97.135	1.00
Treated	75.778	2.875	0.03

The bacteria which were treated with antibacterial plant extract have shown to have 33.3 fold reduction compared with the control.

Elongation Factor Tu 2 of *E. coli* ATCC 25922 (treated with *M. candidum*)

	Area	Percent	Relative Density
Untreated (Control)	2817.154	94.082	1.00
Treated	117.192	5.918	0.06

The bacteria which were treated with antibacterial plant extract have shown to have 16.7 fold reduction compared with the control.

Outer Membrane Protein A of *E. coli* ATCC 25922 (treated with *M. candidum*)

	Area	Percent	Relative Density
Untreated (Control)	4590.397	86.329	1.00
Treated	726.941	13.671	0.16

The bacteria which were treated with antibacterial plant extract have shown to have 6.25 fold reduction compared with the control.

Stringent Starvation Protein A of *E. coli* ATCC 25922 (treated with *M. candidum*)

	Area	Percent	Relative Density
Untreated (Control)	4680.953	86.575	1.00
Treated	725.870	13.425	0.16

The bacteria which were treated with antibacterial plant extract have shown to have 6.25 fold reduction compared with the control.

30S Ribosomal Protein of *P. aeruginosa* ATCC 27853 (treated with *M. candidum*)

	Area	Percent	Relative Density
Untreated (Control)	961.870	89.240	1.00
Treated	115.971	10.670	0.12

The bacteria which were treated with antibacterial plant extract have shown to have 8.33 fold reduction compared with the control.

60 kDa Chaperonine of *P. aeruginosa* ATCC 27853 (treated with *M. candidum*)

	Area	Percent	Relative Density
Untreated (Control)	3674.083	98.956	1.00
Treated	38.778	1.044	0.01

The bacteria which were treated with antibacterial plant extract have shown to have 100 fold reduction compared with the control.

Elongation Factor Ts of *P. aeruginosa* 27853 (treated with *M. candidum*)

	Area	Percent	Relative Density
Untreated (Control)	4312.539	87.468	1.00
Treated	617.870	12.532	0.14

The bacteria which were treated with antibacterial plant extract have shown to have 7.14 fold reduction compared with the control.

APPENDIX I

LIST OF PUBLICATION

1. **Yong, A. L.**, Ooh, K. F., Ong, H. C., Chai, T. T. and Wong, F. C., 2015. Investigation of antibacterial mechanism and identification of bacterial protein targets mediated by antibacterial medicinal plant extracts. *Food Chemistry*, 186, pp. 32-36.

2. Wong, F. C, **Yong, A. L**., Sim, K. M, Ong, H. C. and Chai, T. T., 2014. Proteomic Analysis of Bacterial Expression Profiles Following Exposure to Organic Solvent Flower Extract of *Melastoma candidum* D Don (Melastomataceae). *Tropical Journal of Pharmaceutical Research*, 13(7), pp. 1085-1092.

3. Wong, F. C., **Yong, A. L**., Ong, H. C. and Chai, T. T., 2013. Evaluation of the antibacterial activities of selected medicinal plants and determination of their phenolic constituents. *Science Asia*, 39, pp. 591-595.