PHYTOCHEMICALS SCREENING AND ANTIBACTERIAL ACTIVITY OF *Andrographis paniculata*

By

TAN YEE KUAN

A project report submitted to the Department of Biological Science
Faculty of Science
Universiti Tunku Abdul Rahman
in partial fulfilment of the requirements for the degree of
Bachelor of Science (Hons) Biotechnology

April 2015

ABSTRACT

PHYTOCHEMICALS SCREENING AND ANTIBACTERIAL ACTIVITY OF *Andrographis paniculata*.

Tan Yee Kuan

The increasing of antibiotic resistance bacteria and synthetic drugs has urged the search of new antibacterial and antioxidant agents from medicinal plants. This project was carried out to study the antibacterial activity and antioxidant activity of leaf extract of *Andrographis paniculata*. In this study, the powdered leaves was subjected to sequential Soxhlet extraction by increasing polarity index of solvents (hexane, chloroform, ethyl acetate and methanol). The methanol extract gave the highest percentage yield of extraction in the sequential extraction. All the solvent extracts were then used in phytochemicals screening tests, antioxidant and antibacterial assays as well as thin layer chromatography analysis. For the phytochemicals screening test, terpenoids was found to be the most abundant compounds in chloroform, ethyl acetate and methanol extracts. The DPPH assay was carried out to determine the antioxidant activity of all solvent extracts. Hexane extract found to exhibit the highest antioxidant activity with the lowest half maximal inhibitory concentration, IC_{50} value of 2.80 mg/ml. The antibacterial activity was evaluated qualitatively through agar disc diffusion toward *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Escherichia coli*. The ethyl acetate extract showed the highest zone of inhibition value (17.0 mm) in *S. epidermidis* treatment. The potential of antibacterial activity decreases

with chloroform and hexane. The methanol extract was failed to exhibit any antibacterial activity. All the solvent extracts showed no antibacterial activity against *E. coli*. The antibacterial activity are evaluated quantitatively through minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests. The MIC values of the ethyl acetate and chloroform extracts for *S. aureus* and *S. epidermidis* were ranged from 375 μg/ml to 750 μg/ml. Both solvent extracts were bacteriostatic toward the *S. aureus* and *S. epidermidis*. In conclusion, high extraction yield does not indicates that it has high antibacterial and antioxidant activities. The ethyl acetate extract perform the best antibacterial activity and hexane extract perform the best antioxidant activity.

ACKNOWLEDGEMENT

First of all, I would like thank to my final year project supervisor, Dr Tong Kim Suan for his guidance, advice, teaching and support throughout this whole project. I am thankful for his kindness to sacrifice his own rest time to extend the lab hour for us and stay back after his office hours. Besides, his patience in explaining and giving information had help me a lot in bench work and thesis writing.

Secondly, I would like to express my sincerely gratitude toward all the lab officers from both biological science and chemistry departments for their assistance and cooperation in making this project a success.

Next, I would like to thank to all my bench- mates, Chan Pui Shi, Kek Wee Kee and Tang Man Yee for their sharing and helping throughout this project especially during bench work. Thank you for their willingness and kindness in sharing the material and apparatus and generousness in sharing information and knowledge.

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

DECLARATION

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

_____

TAN YEE KUAN

APPROVAL SHEET

This project report entitled "**PHYTOCHEMICALS SCREENING AND ANTIBACTERIAL ACTIVITY OF *Andrographis paniculata*"** was prepared by **TAN YEE KUAN** and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biotechnology at Universiti Tunku Abdul Rahman.

Approved by:

_____

(Dr TONG KIM SUAN) Date: ……..
Supervisor
Department of Biological Science
Faculty of Science
Universiti Tunku Abdul Rahman

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: _____

PERMISSION SHEET

It is hereby certified that **TAN YEE KUAN** (ID No: **11ADB05215**) has completed this final year project entitled "PHYTOCHEMICALS SCREENING AND ANTIBACTERIAL ACTIVITY OF *Andrographis paniculata*" under the supervision of Dr Tong Kim Suan from the Department of Biological Science, Faculty of Science.

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

Yours truly,

_____

(TAN YEE KUAN)

TABLE OF CONTENTS

		Page
ABSTRACTS		**ii**
ACKNOWLEDGEMENTS		**iv**
DECLARATION		**v**
APPROVAL SHEET		**vi**
PERMISSION SHEET		**vii**
TABLE OF CONTENTS		**viii**
LIST OF TABLES		**xi**
LIST OF FIGURES		**xii**
LIST OF ABBREVIATIONS		**xv**
CHAPTER		
1	INTRODUCTION	1
2	LITERATURE REVIEW	4
	2.1 Background of *Andrographis paniculata*	4
	2.1.1 Taxonomy	4
	2.1.2 Morphology	5
	2.1.3 Distribution and Habitat	5
	2.1.4 Traditional Usages	6
	2.2 Phytochemicals	6
	2.3 Antioxidant Activity	8
	2.3.1 Reactive Oxygen Species (ROS)	8
	2.3.2 Synthetic Antioxidants	11
	2.3.3 Natural Antioxidants	11
	2.4 Pathogenic Bacteria	14
	2.4.1 Gram Positive and Gram Negative Bacteria	14
	2.4.2 *Staphylococcus aureus*	15
	2.4.3 *Staphylococcus epidermidis*	17
	2.4.4 *Escherichia coli*	18

3 MATERIALS AND METHODS 20
3.1 Experimental Design 20
3.2 Materials 21
3.2.1 Plant Material 21
3.2.2 Bacterial Strains 21
3.2.3 Apparatus, Equipment, Chemicals and Consumables 22
3.3 Extraction of leaves 22
3.3.1 Plant Sample Preparation 22
3.3.2 Sequential Soxhlet Extraction of Plant Sample 22
3.3.3 Evaporation of Plant Extract 22
3.4 Preliminary Phytochemicals Screening 23
3.4.1 Alkaloids Test 23
3.4.2 Phenols (Ferric Chloride Test) 23
3.4.3 Steroids Test 23
3.4.4 Cardiac Glycosides Test 24
3.4.5 Reducing Sugars Test 24
3.4.6 Tannin Test 24
3.4.7 Terpenoids (Salkowski Test) 24
3.4.8 Saponin Test 25
3.4.9 Anthraquinones (Borntrager's Test) 25
3.4.10 Flavonoids (Alkaline Reagent Test) 25
3.5 Antioxidant Activity 25
3.6 Medium Preparation 26
3.7 Culturing Microorganism 26
3.8 Agar Disc Diffusion Assay 26
3.8.1 Test Sample Preparation 26
3.8.2 Bacterial Suspension Preparation 27
3.8.3 Inoculate Bacterial Suspension onto Mueller Hinton Agar 27
3.8.4 Preparations and Application of Antimicrobial Disc 27
3.8.5 Recording Data and Interpretating the Results 28
3.9 Minimum Inhibitory Concentration (MIC) Assay 28
3.9.1 Preparation of Inoculum 28
3.9.2 Preparation of Stock and Working Solutions 29

3.9.3 Preparation of 96-well Plates 29
3.10 Minimum Bactericidal Concentration (MBC) Assay 31
3.11 Thin Layer Chromatography (TLC) 31

4 RESULTS 32
4.1 Sequential Extraction 32
4.2 Phytochemicals Screening 33
4.3 Antioxidant Assay 34
4.4 Antibacterial Activity 37
4.4.1 Agar Disc Diffusion Assay 37
4.4.2 Minimum Inhibitory Concentration (MIC) 40
4.4.3 Minimum Bactericidal Concentration (MBC) 42
4.5 Thin Layer Chromatography (TLC) 43

5 DISCUSSION 45
5.1 Extraction of *A. paniculata* Leaves 45
5.2 Percentages Yield of Extraction 46
5.3 Phytochemicals Screening 47
5.4 Antioxidant Assay 51
5.5 Antibacterial Activity 54
5.6 Thin Layer Chromatography (TLC) 59
5.7 Future Perspectives 61

6 CONCLUSION 62

REFERENCES 63

APPENDICES 75

LIST OF TABLES

Table		Page
2.1	Taxonomy of *Andrographis paniculata*	4
2.2	The basic structures and biological activities of phytochemicals	7
2.3	The Nomenclatures of Reactive species	9
2.4	List of medicinal plants that possess antioxidant activity in leaf extract and their chemical constituents	13
2.5	The infections and diseases caused by various group of *E. coli*	19
3.1	The bacteria strains used in the antibacterial activity	21
4.1	Qualitative analysis of phytochemicals content in *A. paniculata* leaf extract prepared in different solvents	33
4.2	IC_{50} of different solvent extracts of *A. paniculata*	36
4.3	The antibacterial activities of different extracts of *A. paniculata* at different concentration through agar disc- diffusion method	39
4.4	Minimum inhibitory concentration (MIC) of chloroform, ethyl acetate extracts of *A. paniculata* and ampicillin	41
4.5	Minimum bactericidal concentration (MBC) of chloroform, ethyl acetate extracts of *A. paniculata*	43
5.1	Zone of inhibition evaluation table for agar disc diffusion assay	54
A1	The list of apparatus and equipment with their respective brand and model	75
A2	List of chemicals and consumables used in this project with their respective manufacturer	76
E1	The R_f values of hexane, chloroform, ethyl acetate and methanol extracts	87

LIST OF FIGURES

Figure		Page
2.1	*A. paniculata*	5
2.2	The targets of free radicals on proteins, lipids, sugars, DNA and RNA	10
2.3	Chemical structure of synthetic antioxidants	11
2.4	The chemical structure of some common natural antioxidant	12
2.5	The membrane structure of Gram positive and Gram negative bacteria	14
2.6	*S. aureus*	15
2.7	*S. epidermidis*	17
2.8	*E. coli* on Luria Bertani agar plate	18
3.1	The experimental design of this project	20
3.2	Design of a 96 well plate for minimum inhibitory concentration assay	30
4.1	Percentage yield of extraction of *A. paniculata* leaf obtained sing sequential extraction with different polarity index of solvents	32
4.2	Percentage of DPPH scavenging activity of different extract of *Andrographis paniculata* at various concentrations	35
4.3	Percentage of DPPH scavenging activity of quercetin (positive control) in various concentrations	36
4.4	Zone of inhibition exhibited by ethyl acetate extract	38

4.5	The inhibitory potential of ethyl acetate and chloroform extracts against *S. aureus* with an MIC value 0f 750 µg/ml shown by black circle	41
4.6	The growth of bacteria on Mueller Hinton agar from the MIC activity of ethyl acetate extract after 24 hours incubation	42
4.7	TLC images of *A. paniculata* solvent extracts viewed under short wavelength.	44
4.8	TLC images of *A. paniculata* solvent extracts viewed under long wavelength.	44
5.1	The chemical structure of andrographoloide, 14- deoxy andrographolide and neoandrographolide	48
5.2	The chemical structure of some common flavonoids isolated from *A. paniculata*	50
5.3	The reduction of the DPPH radical by antioxidant compound	52
C1	Terpenoids test	80
C2	Cardiac glycosides test	80
C3	Phenols test	81
C4	Alkaloids test	81
C5	Flavonoids test	81
C6	Saponins test	82
C7	Steroids test	82
C8	Anthtraquinones test	82
C9	Reducing sugars test	83
C10	Tannins test	83
D1	Zone of inhibition exhibited by hexane extract	84
D2	Zone of inhibition exhibited by chloroform extract	84
D3	Zone of inhibition exhibited by ethyl acetate extract	85
D4	Zone of inhibition exhibited by methanol extract	85
D5	Zone of inhibition exhibited by positive control	86

D6	Zone of inhibition exhibited by acetone, hexane, ethyl acetate, chloroform, methanol, 10% DMSO	86
D7	The inhibitory activity of ethyl acetate and chloroform extracts against *S. epidermidis* on 96 well plate	86
D8	The MBC of chloroform extract	86

LIST OF ABBREVIATIONS

agr	accessory gene regulatory
ATCC	american type culture collection
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
Ca^{2+}	calcium ion
CFU	colony forming unit
DG	dodecyl gallate
DMSO	dimethyl sulfoxide
DPPH	2, 2-diphenyl-1-picrylhydrazyl
DNA	deoxyribonucleic acid
EAEC	enteroaggregative *E.coli*
EDTA	ethylenediaminetetraacetic acid
EHEC	enterohemorrhagic *E.coli*
EIEC	enteroinvasive *E.coli*
EPEC	enteropathogenic *E.coli*
ETEC	enterotoxigenic *E.coli*
$FeCl_3$	ferric chloride
FRAP	ferric reducing antioxidant power
HC	hemorrhagic colitis
HCl	hydrochloric acid
HIV	human immunodeficiency virus
H_2O_2	hydrogen peroxide
HPLC	high performance liquid chromatography
H_2SO_4	sulphuric acid

HUS	hemolytic uremic syndrome
IC_{50}	half maximal inhibitory concentration
LB	Luria Bertani
LPS	lipopolysaccharide
MBC	minimum bactericidal concentration
MH	Mueller Hinton
MIC	minimum inhibitory concentration
NA	nutrient agar
Na^+	sodium ion
NaOH	sodium hydroxide
NMR	nuclear magnetic resonance
NPEC	nephropathogenic *E.coli*
OG	octyl gallate
PG	propyl gallate
R_f	retention factor
RNA	ribonucleic acid
ROS	reactive oxygen species
TBHQ	tertiary butyl hydroquinone
TCM	traditional Chinese medicine
TLC	thin layer chromatography
TSS	toxic shock syndrome
TSST-1	toxic shock syndrome toxin-1
UT	urinary tract infection
UV	ultraviolet
vis	visible

CHAPTER 1

INTRODUCTION

Medicinal plants have been used over the hundred years to treat various type of acute and chronic diseases (Cowan, 1999). Medicinal plants contain a lot of bioactive constituents or phytochemical compounds which are secondary metabolites that produced by plant. The major secondary metabolites including alkaloids, carbohydrates, flavonoids, tannins, terpenoids, and steroids (Edoga, Okwu and Mbaebie, 2005). Plant initially produces these phytochemical compounds to protect themselves from pathogens and predator (Poongothai, et al., 2011). However, researcher has found that these phytochemical constituents produced by plant are able to exhibit some biological activities such as, antiperiodic, antibacterial, antitumor, antidiabetic, antithrombotic, anti-inflammatory anti-HIV, antifeedant and antiviral (Jayakumar, et al., 2013).

The dramatically increases of bacteria resistance to the common use antibiotic increases the demand to search for the new, potential and alternative active compound in plants to treat the bacterial infection that cause by those antibiotic resistance bacteria (Akinpelu, et al., 2008). Besides, the discovery of new drug from the medicinal plant also can be used to replace the high cost of conventional drugs (Sibanda and Okoh, 2008). In addition, source of medicinal plant are more accessible to human beings in the developing or under developing country compared to those orthodox medicines (Ajayi, Ajibade and Oderinde, 2011). Natural drugs that produce from medicinal plants are also safer to consume, less

toxic and normally has lower side effect compared with conventional drugs which are manufactured by chemical compound.

In addition, the importance of exploitation of natural antioxidant has also increased tremendously in recent years. This is because of the recent concern on the detrimental effects of the synthetic antioxidant. There are few synthetic antioxidant had been restricted to be used as food additive due to carcinogenic namely butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT).

In Malaysia, more than 10, 000 species of medicinal plants exist in the rain forest (Ali, et al., 1995). One of the famous medicine plant is *Andrographis paniculata* which called "Hempedu bumi" in Malaysia. Many researchers in India, Thailand and other countries keen to study the antibacterial and antioxidant activities of *A. paniculata*. However, their results are not confidentially consistent because of the phytochemical compounds that present in the plant might be changed due to the growth environment of *A. paniculata* (Dada-adegbola, Olajide and Ajayi, 2014).

In addition, most of the study done on *A. paniculata* are mainly purchased from the manufacture company or collected from the herb garden. There is no sufficient study has been done on the wild type of *A. paniculata* to support its potential toward antibacterial and antioxidant activity.

The objectives of this project are:

1) To extract the phytochemical compounds from the leaf of *A. paniculata* that collected from Bagan Datoh, Perak by using sequential Soxhlet extraction.
2) To screen the phytochemical compounds that present in the leaf extract of *A. paniculata*.
3) To determine the antioxidant activity of the *A. paniculata* leaf extract.
4) To determine the antibacterial activity of *A. paniculata* leaf extract against Gram positive and Gram negative bacteria.
5) To perform thin layer chromatography on the leaf extract of *A. paniculata*.

CHAPTER 2

LITERATURE REVIEW

2.1 Background of *Andrographis paniculata*

Andrographis paniculata is a herbaceous plant that normally known as "King of bitter" due to its bitter property. It has several common names, it is called "Hempedu bumi" in Malaysia, "Chuan Xin Lian" in China, "Fa Thalai Chon" in Thailand, "Senshiren" in Japan and "Kalmegh" in India (Sivananthan and Elamaran, 2013; Sareer, Ahad and Umar, 2012).

2.1.1 Taxonomy

The taxonomy information of the *A. paniculata* is shown in Table 2.1.

Table 2.1: Taxonomy of *Andrographis paniculata* (Sivananthan and Elamaran, 2013)

Kingdom	**Plantae**
Subkingdom	Tracheobiota, vascular plants
Superdivision	Spermatophyta, seed plants
Division	Angiosperma
Class	Magnoliophyta, dicotyledonae
Sub-class	Gamopetalae
Order	Personales
Family	Acanthaceae
Genus	*Andrographis*
Species	*paniculata*

2.1.2 Morphology

A. paniculata usually can grow up to a height of 30 -110 cm with its green colour stem. It has a very special lanced shaped of leave and the leaves are hairless mea. Its leaves size is about 8 cm long and 2.5 cm wide (Sivananthan and Elamaran, 2013). Besides, it also has small white colour flower and yellow- brown colour seed (Kumar, et al., 2012).

Figure 2.1: *A. paniculata*

2.1.3 Distribution and Habitat

A. paniculata grows throughout the tropical and subtropical region of South-East Asia and it also can be found widely in Malaysia, India, Thailand, Indonesia and etc. This plant is highly distributed due to it can be easily cultivated and grows in many types of soil. For example, it able to grow in a special soil types which known as "serpentine soil" that contain high level of aluminum, copper and zinc where mostly other plants cannot be cultivated. Their optimum growing condition is in open or slightly shaded area with an easy access of water. Propagation is carried out by using seedling obtained from the mature pod. This

plant can be grown by many method of cultivations such as sowing seeds in rows, broadcasting or transplanting seedling (Sareer, Ahad and Umar, 2012).

2.1.4 Traditional Usages

In Malaysia, *A. paniculata* is commonly used to treat diabetes, hypertension and common cold (Sareer, Ahad and Umar, 2012). A long history for the medical use of *A. paniculata* against sore throat has been well known in Thailand (Poolsup, et al., 2004). In India, it is used to relieve griping, irregular bowel habits, and loss of appetite of children (Kumar, et al., 2012). In China, *A. paniculata* is normally used to get rid of the body heat to treat fever and to dispel toxins. This is because it is believed that it belongs to cold property herbal and acts as blood purifier in Traditional Chinese Medicine (TCM) (Sivananthan and Elamaran, 2013). Due to its "blood purifying" characteristic, it is recommended to use for the treatment of leprosy, gonorrhea, scabies, boils, skin eruptions (Kumar, et al., 2012).

2.2 Phytochemicals

Phytochemicals are secondary metabolites that synthesized from primary metabolites of the plant. Phytochemicals consist of several chemical classes such as terpenoids, phenols, flavonoids, saponins and tannins that have several biological effects on microorganisms (Yadav and Agarwala, 2011). The basic structures and biological activities of phytochemicals are shown in Table 2.2.

Table 2.2: The basic structures and biological activities of phytochemicals.

Phytochemicals	Basic Structure	Biological Activity	Citation
Terpenoids	Isoprene units (consists of five-carbons having two unsaturated bonds and possesses a branched chain) where the methyl groups are replaced by oxygen atoms	Anticarcinogenic, antiulcer, antimicrobial, antioxidant, antimalarial and antidiuretic	(Nayak, 2012)
Phenols	A aromatic ring that carry one hydroxyl functional groups	Antimicrobial, antioxidant	(Nice, 2009; Sahoo, 2012)
Flavonoids	Phenolic aromatic molecules, containing one carbonyl functional group	Anti-inflammatory, antithrombotic, antioxidant, antiviral	(Cowan, 1999)
Saponins	One or more hydrophilic glycoside moieties combined with lipophilic triterpene derivative	Antidiarrheal, anticancer, and antihelmintic	(Sahoo, 2012)
Tannins	Oligomeric compounds with multiple structure unit with free phenolic components	Antioxidant, antiperoxidative, antimicrobial, antimutagenic, antidiabetic and antiviral	(Sahoo, 2012)

2.3 Antioxidant Activity

The report of detrimental side effect of synthetic antioxidant agent has raised the need to search for more natural antioxidant from plant extract (Rajkumar, Guha and Ashok Kumar, 2010). The major role of antioxidant is to inhibit and scavenge the free radicals which are inducing the oxidative stress that cause infection and degenerative diseases to human. Antioxidant has the ability to donate electrons its hydroxyl functional groups to prevent oxidation in other compounds (Yong, 2007). Besides, antioxidant also can breakdown the hydroperoxides during lipid oxidation process into a more stable formed, alcohol (Venkatesh and Sood, 2011). Hence, antioxidant able to maintain the optimum level of oxidant (Aris, et al., 2009). Oxidative stress is caused by the low level of antioxidant or by the overproduction of reactive oxygen species (ROS) (Halliwell and Whiteman, 2004).

2.3.1 Reactive Oxygen Species (ROS)

ROS are the free radicals that contained unpaired electrons (Rajkumar, Guha and Ashok Kumar, 2010). The example of free radicals of reactive oxygen species are shown in Table 2.3. ROS can includes both oxygen radicals and non- radicals such as nitrogen or chloride species which are oxidizing agents that are easily converted into radicals. In short, oxygen radicals are belong to ROS but not all ROS are consider as oxygen radicals (Halliwell and Whiteman, 2004).

Table 2.3: The Nomenclatures of Reactive species (Halliwell and Whiteman, 2004).

Free radicals	**Nonradicals**	**Reactive chlorine species (RCS)**	**Reactive nitrogen species (RNS)**
Superoxide, O_2^-	Hydrogen peroxide, H_2O_2	Atomic Chlorine, Cl^-	Nitric oxide, NO^-
Hydroxyl, OH^-	Hypobromous acid, HOBr	Hypochlorous acid, HOCl	Nitrogen dioxide, NO_2^-
Hydroperoxyl, HO_2^-	Hypochlorous acid, HOCl	Nitryl (nitronium) chloride, NO_2Cl	Nitrous acid, HNO_2
Peroxyl, RO_2^-	Ozone, O_3	Chloramines	Nitroxyl anion, NO
Alkoxyl, RO^-	Organic peroxides, ROOH	Chlorine gas Cl_2	Nitrosyl cation, NO^+
Carbonate, CO_3^-	Peroxynitrite, ONOO		Peroxynitrite, ONOO
Carbon dioxide, CO_2^-	Peroxynitrous acid, ONOOH		Peroxynitrous acid, ONOOH

ROS can cause damage in lipid, proteins, DNA and carbohydrates which are important biological molecules in human life (Eugine, et al., 2011). This is because ROS able to initiate the peroxidation of membrane lipids, increase the serum lipid level such as cholesterol and cause oxidative stress (Ross, 1999). The schematic diagram of the target of free radicals on the lipid, protein, DNA, RNA and sugars is shown in Figure 2.2. This has led to several diseases such as coronary artery diseases, stroke, rheumatoid arthritis, atherosclerosis, diabetes, neurological disease, inflammatory disease, Alzheimer's disease, hypertension cataracts and cancer (Guha, et al., 2011; Cui, et al., 2004).

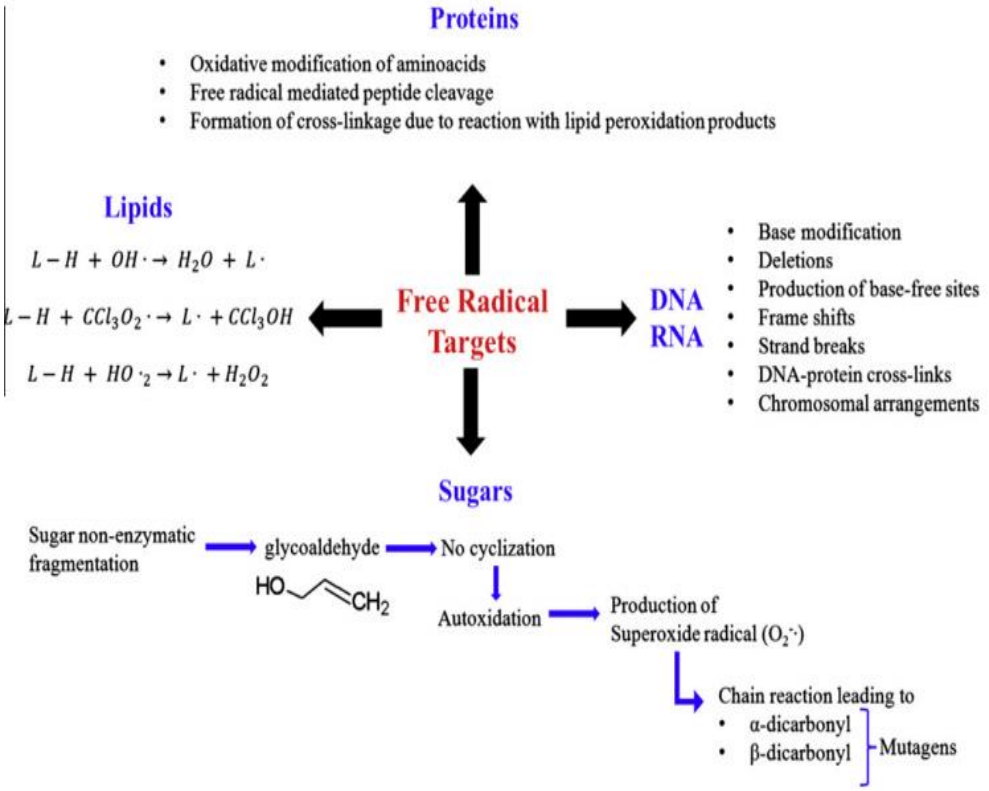


Figure 2.2: The targets of free radicals on proteins, lipids, sugars, DNA and RNA (Carocho and Ferreira, 2013).

Narayanaswamy and Balakrishnan (2011) reported that the antioxidant from the plant extract is able to inhibit the action of free radicals and protect the body from those diseases. Kahkonen, et al. (1999) also reported that the phenolic compounds of medicinal plant might act as radical scavengers and attribute in high antioxidant activity. Many studies reported that the flavonoid compounds are able to show remarkable free radical scavenging activity. Besides, they also can inhibit the oxidation of lipoprotein which able to prevent these diseases (Eugine, et al., 2011).

2.3.2 Synthetic Antioxidants

Synthetic antioxidants are antioxidants that synthesized in a chemical way and not obtain from the nature sources. The example of synthetic antioxidants and their chemical structures are shown in Figure 2.3.

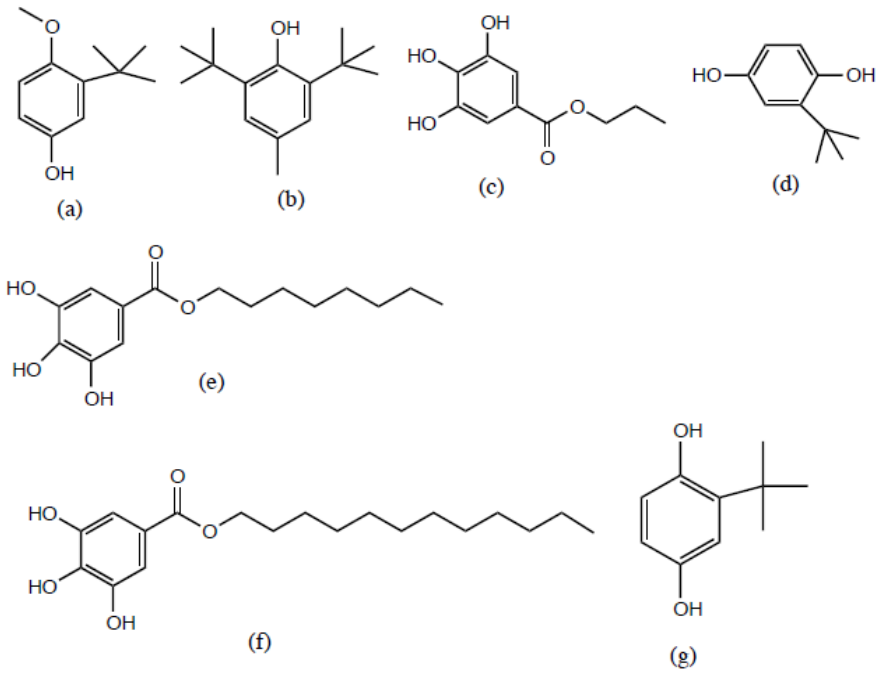


Figure 2.3: The chemical structure of synthetic antioxidants. (a) BHT, (b) BHA, (c) PG, (d) OG, (e) DG, (f) EDTA, (g) TBHQ (Venkatesh and Sood, 2011).

2.3.3 Natural Antioxidants

Natural antioxidants are commonly found in the natural sources such as plants, fungi and microorganism (Venkatesh and Sood, 2011). This is because they live under constant oxidative stress exhibited from free radicals and reactive oxygen species which are generated heat exogenously and transition metals endogenously. Thus, they have to develop antioxidant systems to control and prevent the free radicals as well as oxidative stress occurred (Brewer, 2011). The

chemical structure of some common natural antioxidants are shown in Figure 2.4, with the example of medicinal plants that contain common natural antioxidants are shown in Table 2.4.

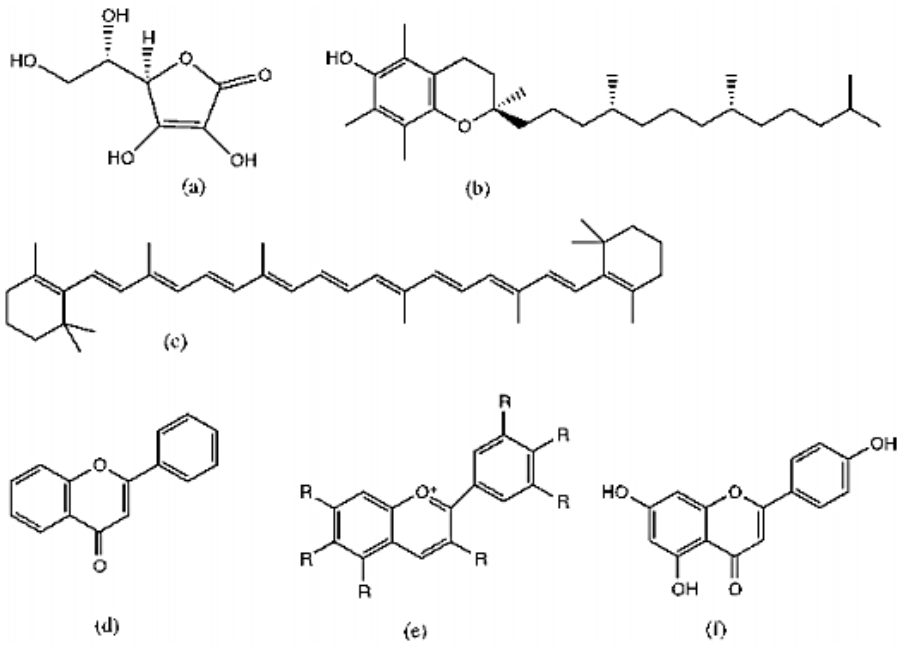


Figure 2.4: The chemical structure of some common natural antioxidant. (a) Ascorbic Acid, (b) α- tocopherol, (c) β- carotene, (d) Flavonoid, (e) Anthocyanin, (f) Polyphenol (Venkatesh and Sood, 2011).

Table 2.4: List of medicinal plants that possess antioxidant activity in leaf extract and their chemical constituents.

Plants	**Chemical Constituents**	**Citation**
Zingiber officinalis	ascorbic acid, α-tocopherol, β-carotene, β-sitosterol .	(Chanwitheesuk, Teerawutgulrag and Rakariyatham, 2004)
Ocimum sanctum	ascorbic acid, β-carotene, β-sitosterol, eugenol, tannin	(Chanwitheesuk, Teerawutgulrag and Rakariyatham, 2004)
Viscum album L.	phenolic acids, flavonoids, carotenoids	(Vicas, et al., 2009)
Catharanthus roseus	α -tocopherol,apigenin, β - carotene, γ - tocopherol, kaempferol, Luteolin menthol, limonene vincristine, Vinblastine	(Sharma, Singh and Singh, 2013).
Cotinus coggygria Scop.	flavones, aurones, chalcones	(Ivanova, et al., 2005)
Anthriscus cerefolium	flavonoids, coumarins	(Katalinic, et al., 2006)
Cichorium intybus L.	phenolic acids, flavonoids	(Katalinic, et al., 2006)
Vaccinium myrtillus L.	anthocyanin	(Trouillas, et al., 2003)

2.4 Pathogenic Bacteria

2.4.1 Gram Positive and Gram Negative Bacteria

The main different between Gram positive and Gram negative bacteria is their cell wall which is shown in Figure 2.5.

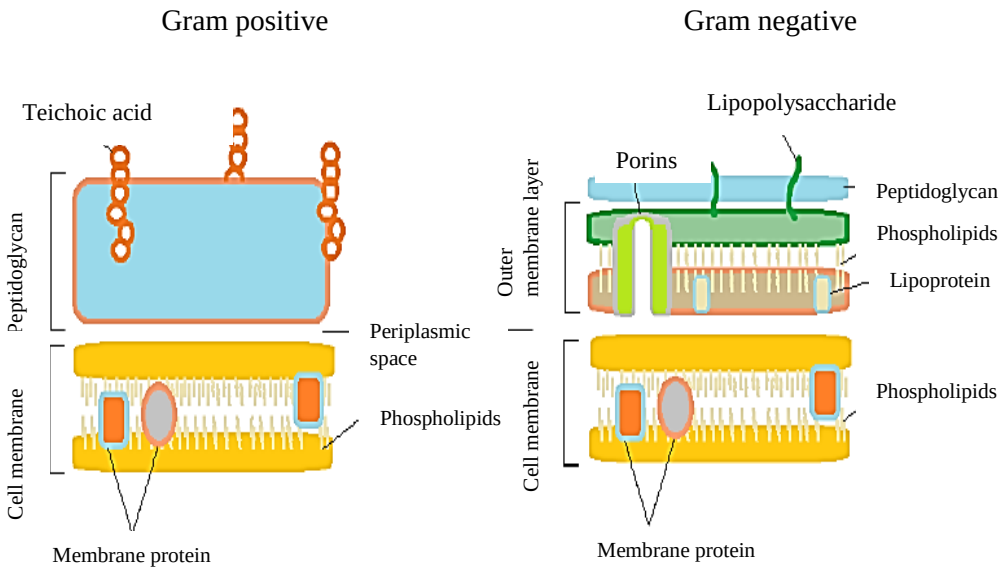


Figure 2.5: The membrane structure of Gram positive (left) and Gram negative bacteria (right) (Nice, 2012).

The Gram negative bacteria have an outer membrane which absence in the Gram positive bacteria. This characteristic enable the Gram negative bacteria to restrict most of the molecules from entering the cell wall and perisplasm and act as molecular sieve. The outer membrane of Gram negative bacteria has an important component called lipopolysaccharide (LPS). The LPS contain of lipid A which is an endotoxin that caused some infection associated with Gram negative bacteria. Besides, different species of Gram negative bacteria can be differentiate by a component of LPS which is O oligosaccharides that acts as antigen (Raetz and Whitfield, 2002). As shown in Figure 2.5, Gram positive

bacteria only have a thicker peptidoglycan layer as cell membrane. Meanwhile, Gram negative bacteria have a double phospholipid bilayer (Punapos, 2002).

2.4.2 *Staphylococcus aureus*

S. aureus is a Gram positive bacteria that belongs to the family of Micrococcaceae (Punopas, 2002). *S. aureus* is a spherical cell having a diameter about 1 µm. It is a nonmotile, nonspore forming and facultative anaerobe, so it grows by anaerobic respiration or fermentation. *S. aureus* is able to present in singly, in pairs or in irregular clusters (Harris, Foster and Richards, 2002). When viewing under the microscope, it looks like a branch of grapes (Ranpal, 2009). *S. aureus* will forms a gold- yellow colony on rich medium agar plate and hemolytic on blood agar plate as shown in Figure 2.6 (Todar, 2008a). *S. aureus* can be differentiate from the other species by showing positive results in mannitol fermentation, deoxyribonuclease and coagulase tests (Franklin and Lowy, 1998).

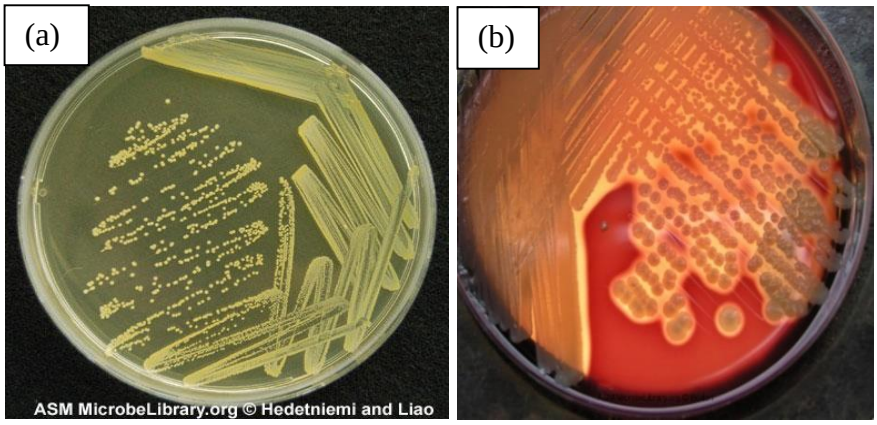


Figure 2.6: *S. aureus* on (a) Luria Bertani agar plate, (b) blood agar (Hedetniemi and Liao, 2006; Buxton, 2005).

S. aureus can cause various types of skin infections such as abscess, impetigo, carbuncles, boils, furuncles and folliculitis. These are opportunistic infections as it usually occurs due to the present of previous skin injuries like insect bites, ulcers, burns or wounds (Ranpal, 2009; Zaidan, et al., 2005; Punopas, 2002). Pyogenic abscess is the basic skin lesion that caused by *S. aureus*. In addition, *S. aureus* also able to cause a more serious infection such as toxic shock syndrome (TSS) by exhibiting some extracellular toxins. For example, Toxic Shock Syndrome toxin- 1 (TSST-1) (Harris, Foster and Richards, 2002). The early signs of TSS are high fever, rash and dehydration and it will slowly become hypotensive, multi-organs dysfunction and in shock.

Besides, *S. aureus* also can causes food poisoning when a person consuming contaminated food that contained enterotoxins produced by *S. aureus*. This staphylococcal enterotoxins are resistance to the high temperature in cooking. This staphylococcal enterotoxins are related to their cell wall protein with expression controlled by the accessory gene regulatory (agr) locus (Nice, 2012). The symptoms of food poisoning are nausea, vomit, abdominal cramping and pain (Punopas, 2002). In addition, *S. aureus* also will causes scaled skin syndrome, an exfoliative dermatitis which damage the epidermal layer of skin. This syndrome mostly occurs in the newborn and young children (Punopas, 2002).

2.4.3 *Staphylococcus epidermidis*

S. epidermidis is a Gram positive bacteria that also belongs to the genus of *Staphylococcus* and family of Micrococcaceae. It is a nonmotile cocci and facultative anaerobes. It can appear in single cell, in pairs, in tetrad or in small cluster. *S. epidermidis* has a relatively small white, opaque and flat colony and nonhemolytic on the blood agar as shown in Figure 2.7. It shows negative result in coagulase test which enable them to differentiate from the coagulase-positive *S. aureus* (Otto, 2009).

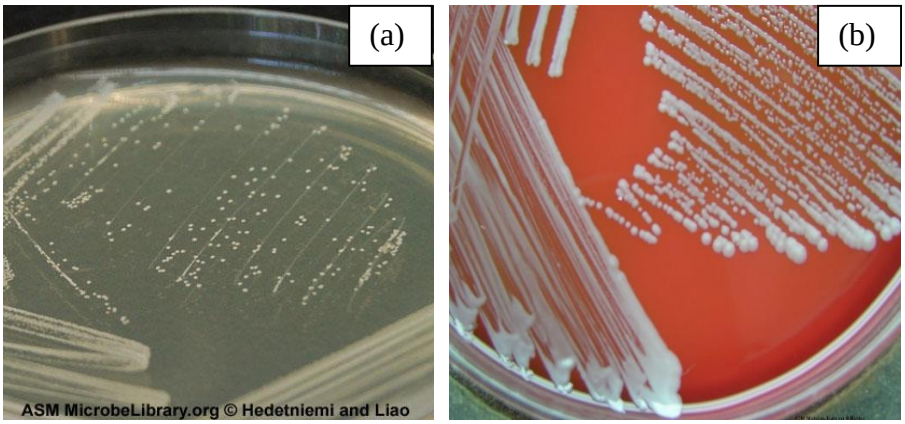


Figure 2.7: *S. epidermidis* on (a) Luria Bertani (LB) agar plate, (b) blood agar. (Hedetniemi and Liao, 2006; Buxton, 2005).

S. epidermidis is commonly found in hospital environment (Franklin, et al., 1983). It is a nosocomial pathogen that cause intravascular catheter- associated infection or other infections that associated with prosthetic heart valves, and peritoneal dialysis catheters (Rupp and Achers, 1994; Rupp, 2001). This infection occurred when the *S. epidermidis* is transferred from the skin of the patient or from the health care device to another patient's skin during device insertion (Otto, 2009). This is due to the ability of *S. epidermidis* to adhere and

proliferate on the polymer surfaces. *S. epidermidis* adheres to biomaterial surface and is followed by cell-to-cell adherence and accumulation, lastly biofilm is formed (Rupp, 2001). In addition, *S. epidermidis* are often cause urinary tract infections due to the instrumentation of the urinary tract in a hospital setting. Besides, the urinary tract infection occurred on infant or children are always caused by the *S. epidermidis* (Upadhyayula, Kambalapalli and Asmar, 2012).

2.4.4 *Escherichia coli*

E. coli is a Gram negative bacteria that belongs to the family of Enterobacteriaceae. *E. coli* is a straight rod cell with having a size of 2.0 - 6.0 µm long and 1.1 - 1.5 µm in diameter. It is a mobile, nonspore- forming, facultatively anaerobe and chemoorganotroph. It can present in singly or in pairs. They show positive results in methyl red test, catalase test and indole test, but negative results in oxidase and citrate tests (Huang, et al., 2001; Brooks, et al., 2004). *E. coli* is forming milky white colonies on the LB agar as shown in Figure 2.8.

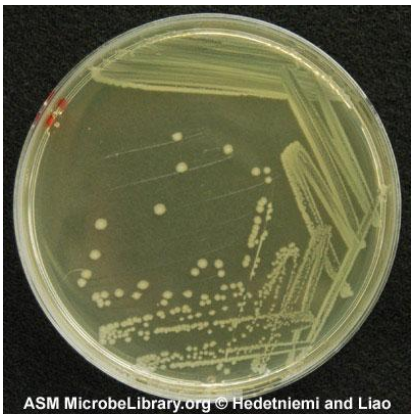


Figure 2.8: *E. coli* grow on Luria Bertani agar plate (Hedetniemi and Liao, 2006).

E. coli is usually caused watery diarrhea in young children especially in developing country. The various type of infections and diseases caused by *E. coli* are summarized in Table 2.5.

Table 2.5: The infections and diseases caused by various group of *E.coli.*

Group	**Infection/Disease**	**Target**	**Citation**
Enteroaggregative *E. coli* (EAEC)	Watery diarrhea	Young children in developing countries	(Ryan and Ray, 2010; Punopas, 2002).
Enterohemorrhagic *E. coli* (EHEC)	Foodborne disease, hemolytic uremic syndrome	Young children and the elderly	(World Health Organization, 2011).
Enteroinvasive *E. coli* (EIEC)	Bloody diarrhea, hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS)	Young children in developing countries	(Todar, 2012).
Enteropathogenic *E. coli* (EPEC)	Watery diarrhea	Infants	(Punopas, 2002).
Enterotoxigenic *E. coli* (ETEC)	Watery diarrhea	Traveler in endemic areas	(Punopas, 2002; World Health Organization, 2011).
Nephropathogenic *E. coli* (NPEC)	Urinary tract infection (UT), such as cystitis, pyelitis and pyelonephiritis	Women	(Ranpal, 2009).

CHAPTER 3

MATERIALS and METHODS

3.1 Experimental Design

The experimental design of this project is shown in Figure 3.1.

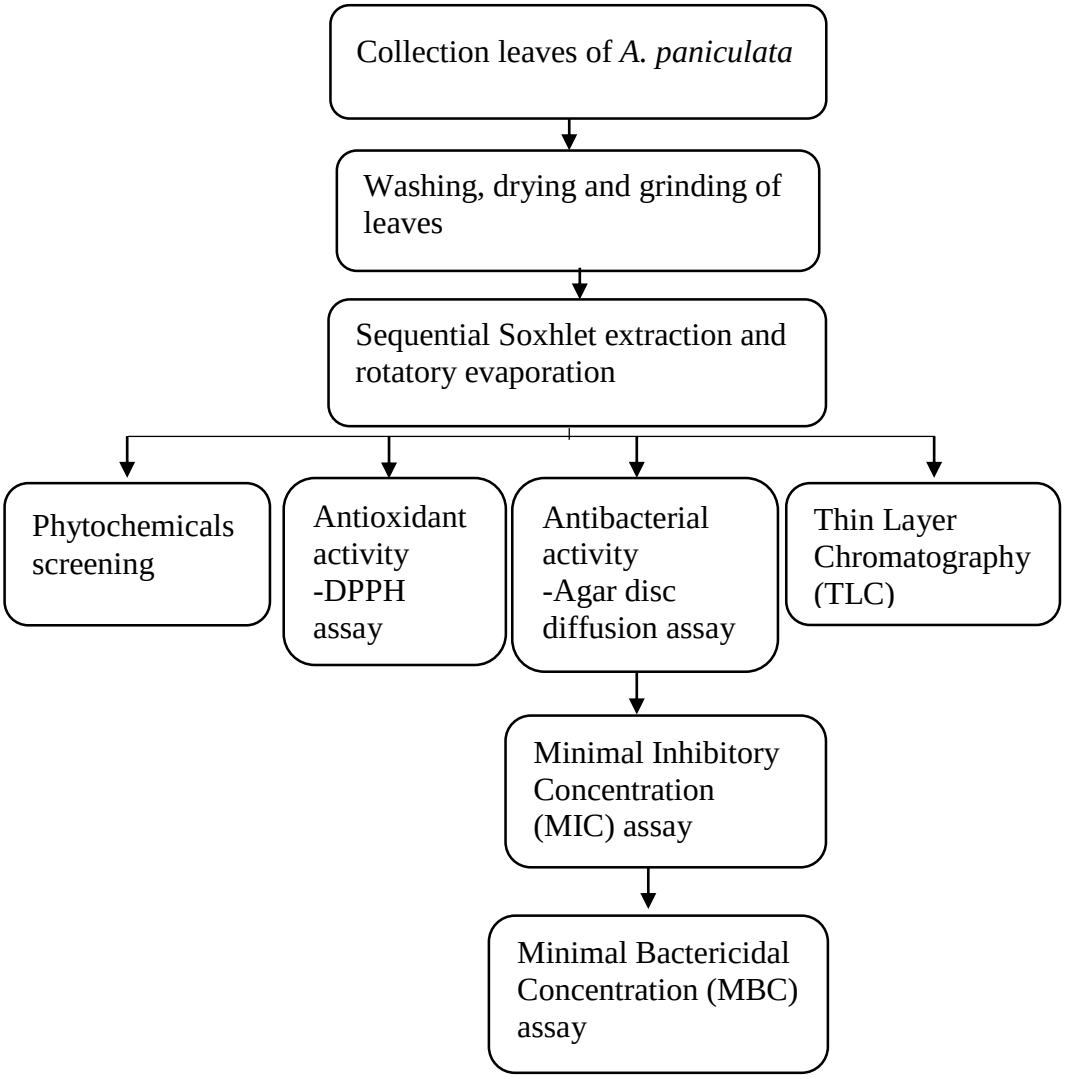


Figure 3.1: The experimental design of this project.

3.2 Materials

3.2.1 Plant Material

The leaves of *A. paniculata* were collected from three different areas in Bagan Datoh, Perak. These areas are locate at the following coordinates, ($3^0$54'31.92", $100^0$46'26.72"), ($3^0$5'4.27", $100^0$47'53.52"), and ($3^0$51'40", $100^0$48'13"). All the plant samples are wild plants which is not micro or macro propagated by human.

3.2.2 Bacterial Strains

The bacteria species that used in the antibacterial activity are shown in Table 3.1. The Gram positive bacteria are cultured on Nutrient Agar (NA) while Gram negative bacteria was cultured on Luria Bertani (LB) agar.

Table 3.1: The bacteria strains used in the antibacterial activity.

Bacteria Species	**ATCC Number**
Gram positive	
Staphylococcus aureus	6538
Staphylococcus epidermidis	12228
Gram negative	
Escherichia coli	35218

ATCC: American Type Culture Collection

3.2.3 Apparatus, Equipment, Chemicals and Consumables

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

3.3 Extraction of leaves

3.3.1 Plant Sample Preparation

The leaves of the *A. paniculata* were washed thoroughly under tap water to remove dirt and shade dried for three days. The dried plant material was then powdered using a conventional blender and stored in a closed container (Buru, et al., 2014).

3.3.2 Sequential Soxhlet Extraction of Plant Sample

Sequential Soxhlet extraction was used to extract out the phytochemical compounds contained in the leaves of *A. paniculata*. The solvents with increasing of polarity index (hexane, chloroform, ethyl acetate and methanol) was used for the extraction. A 15.0 g of powdered leaves was extracted with 200 ml of solvent by using Soxhlet extractor for 4 hours at 65 ℃ (Radha, et al., 2011).

3.3.3 Evaporation of Plant Extract

The crude solvent extracts obtained from the Soxhlet extraction were concentrated using a vacuum rotary evaporator at temperature of 40 ℃ and different pressures was applied for different solvent extracts. The pressure of evaporator for hexane, chloroform, ethyl acetate and methanol are 335, 474, 240, 337 mbar respectively. After that, the concentrated crude extracts were poured into a specimen vial (Kumoro, Hasan and Singh, 2009). The crude extracts were

completely dried in a laboratory oven at 37 ℃. All the crude extracts were then weighed to determine the percentages yield of extraction and stored in refrigerator at 4 ℃. The percentage yield of extraction was calculated by the using the following equation:

$$\text{Percentage yield of extraction (\%)} = \frac{\text{Weight of the dry concentrated crude extract}}{\text{Weight of the powdered plant sample used}} \times 100\%$$

3.4 Preliminary Phytochemicals Screening

3.4.1 Alkaloids Test

Each of the crude extract was dissolved in 1.0 ml of 1 % hydrochloric acid, HCl and treated with three drops of Dragendorff's reagent. Orange-red precipitation was indicated the presence of the alkaloid compounds (Poongothai, et al., 2011).

3.4.2 Phenols (Ferric chloride Test)

Each of the crude extract was added with 1.0 ml of 5 % ferric chloride, $FeCl_3$ solution. The blackish green color was indicated the presence of flavonoids (Hosamani, et al., 2011).

3.4.3 Steroids Test

Each of the crude extract was dissolved in 1.0 ml of chloroform. A 2.0 ml of concentrated sulphuric acid, H_2SO_4 was slowly added to form a lower layer which is in yellow colour with green fluorescence. A reddish brown colour on upper layer was interpreted as a steroid ring (Poongothai, et al., 2011).

3.4.4 Cardiac Glycosides Test

Each of the crude extract was dissolved in 1.0 ml of glacial acetic acid containing one drop of 1 % $FeCl_3$. This was followed by 2.0 ml concentrated H_2SO_4. A brown ring obtained indicates the presence of a deoxy sugar. A violet ring may appears below the ring. Meanwhile, a greenish colour ring might form in acetic acid layer and spread throughout in this layer (Akinpelu, et al., 2008).

3.4.5 Reducing Sugars Test

Each of the crude extract was mixed with 1.5 ml of distilled water to form an aqueous solution. Then, the extract was added to equal volume of boiling Fehling A and B solution in separated test tubes. The presence of reducing sugars was interpreted as the formation of brick red precipitate (Aiyelaagbe and Osamudiamen, 2009).

3.4.6 Tannins Test

Each of the crude extract was added with 2.0 ml of distilled water and boiled in a test tube and three drops of 0.1 % of $FeCl_3$ was added. The brownish green colouration indicate the presence of tannins (Akinpelu, et al., 2008).

3. 4.7 Terpenoids (Salkowski Test)

Each of the crude extract was added with 0.4 ml of chloroform. A 0.6 ml of concentrated H_2SO_4 was added, two layers will be formed. The reddish brown coloration in the interface is indicated the presence of terpenoids (Wadood, et al., 2013).

3.4.8 Saponins Test

Each of the crude extract was added with 1.0 ml of distilled water and boiled in test tube for 15 min. After cooling, the mixture was shaken and a persistent froth was indicated the present of tannin (Mojab, et al., 2003)

3.4.9 Anthraquinones (Borntrager's Test)

Each of the crude extract was taken into a dry test tube and 1.0 ml of chloroform was added and shaken for 5 min. The extract was then shaken with equal volume of 10 % ammonia solution. A pink violet or red color in the ammoniacal layer (lower layer) was indicated the presence of anthraquinone (Aiyelaagbe and Osamudiamen, 2009).

3.4.10 Flavonoids (Alkaline Reagent Test)

Each of the crude extract was added with 5.0 ml of distilled water and boiled for 5 min. Three drops of 20 % NaOH solution was added. The colour change from colourless to yellow. Then, 5 drops of 1 % of HCl was added into the mixture. The presence of flavonoids was interpreted by observing the decolorization of the yellow colour (Ajayi, Ajibade and Oderinde, 2011).

3.5 Antioxidant Assay

The 1.0 ml of solvent extract at various concentrations range (2-24 mg/ml) was added to 2.85 ml of 0.61mM of DPPH reagent. At each concentration, 1.0 ml of the extract was added to 2.85 ml of methanol to serve as sample blank control. A 1.0 ml of the solvent was added to 2.85 ml of 0.61 mM of DPPH reagent to serve as blank. The reaction mixtures were stored in dark conditions for 30 min

at room temperature. The absorbance of reaction mixture was measured at 517 nm against a blank. Quercetin was used as positive control in the experiment. The percentage of inhibition was calculated by using the following formula (Sharma and Joshi, 2011):

$$\text{Percentage of inhibition} = \left[\frac{\text{Abs}_{\text{control}} - (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{sample blank}})}{\text{Abs}_{\text{control}}}\right] \times 100$$

3.6 Medium Preparation

The preparation of NA, LB agar, Mueller Hinton (MH) agar, MH broth, 0.85 % saline water, 10 % glucose and 0.5 % phenol red are listed in Appendix B.

3.7 Culturing Microorganisms

All bacteria strains from the master plate were streaked onto the respective agar plate to obtain single colonies. The agar plates were incubate at 37℃ for 24 hours (Sule, et al., 2011).

3.8 Agar Disc Diffusion Assay

3.8.1 Test Sample Preparation

A 100 mg of the solvent extracts were dissolved in 1.0 ml of solvent that used in extraction to make a concentration of 100 mg/ml. Hexane, chloroform, ethyl acetate and methanol were used as negative controls.

3.8.2 Bacterial Suspension Preparation

Few colonies from the one day old bacteria plate were inoculated into sterile 0.85 % saline water and the turbidity was adjusted to meet 0.5 McFarland standard. To meet the 0.5 McFarland standard, the absorbance reading from UV-vis spectrophotometer must be between 0.8 - 1.0 at maximum wavelength of 625 nm (Suneetha and Ravi, 2012).

3.8.3 Inoculate Bacterial Suspension onto Mueller Hinton Agar

The bacterial suspension was streaked on the MH agar by using a sterile cotton swab. The cotton swab was pressed firmly against the wall of the centrifuge tube to remove the excess fluid. The bacterial suspension was then streaked three times onto the surface of the agar in the different directions. This is to ensure that the bacterial suspension was distributed evenly on the agar (Suneetha and Ravi, 2012).

3.8.4 Preparations and Application of Antimicrobial Discs

The prepared extract solution was sterile filtered using 0.45 μm membrane filter. After that, 10 μl of the extract solution was pipetted onto a 6 mm diameter sterile disc which made by Whatman filter paper No. 1 on a glass petri dish. The disc containing the extract was allowed to dry in the laminar air flow for few minutes. The disc was then placed gently on the MH agar plates that had been inoculated with the bacteria suspension using a sterile forceps. A maximum of six discs can be placed on the MH agar plate, to avoid overlapping of the inhibition zones. In this study, there were two controls used which were solvent containing disc as the negative control and commercially antibiotic containing disc (ampicillin,

amoxicillin and gentamicin) as the positive control for *S. aureus, S. epidermidis* and *E.coli* respectively. Plate containing disc that impregnated with extracts were incubated at 37 ℃ for 24 hours (Sule, et al., 2011).

3.8.5 Recording Data and Interpreting the Results

The results were observed after 24 hours and the diameter of inhibition zone was measured by using a ruler in mm (Suneetha and Ravi, 2012). The study were done in triplicate and mean values were taken. Data was presented in the table format.

3.9 Minimum Inhibitory Concentration (MIC) Assay

The chloroform and ethyl acetate extracts of *A. paniculata* were chosen to determine the MIC and MBC on the *S. aureus* and *S. epidermidis* due to both solvent extracts exhibited the higher antibacterial activities.

3.9.1 Preparation of Inoculum

The bacteria were subcultured on the NA agar and incubated at 37 ℃ for 24 hours. Several colonies of microorganism were inoculate into sterile distilled water. The bacterial suspension were mixed evenly and then diluted to meet the turbidity of 0.5 McFarland standard. A further 1:20 dilutions with sterile distilled water were perform to obtain the require working suspension which was 5×10^5 CFU/ml.

3.9.2 Preparation of Stock and Working Solutions

Stock solutions of the chloroform and ethyl acetate extracts were prepared in methanol solvent at the concentration of 10 mg/ml. The working solution (3000 µg/ml) were prepared by diluting the stock solution in sterile MH broth.

3.9.3 Preparation of 96-well Plates

The test was carried out in 96-well sterile microplates. Chloroform and ethyl acetate extracts were tested in triplicate in one plate for each bacteria to avoid contamination. The design of the 96 well plate for the MIC assay is shown in Figure 3.2. All the wells received 100 µl of MH broth that supplemented with 10 % of glucose and 0.5 % of phenol red (Tekwu, Peime and Beng, 2012). 100 µl of the working solution of chloroform and ethyl acetate extracts, ampicillin (positive control) and solvent (solvent control) were added into the respective well. Identical serial 1:2 dilutions were performed on column 1 continued through column 8 and 100 µl of excess medium was discarded from the well in column 8. The last columns were served as blank which only contain broth that supplemented with 10 % of glucose and 0.5 % of phenol red.

A 100 µl of the bacteria suspension was added to each well except for the last row that served as blank. The column 10 was served as negative control which only contain medium and bacterial suspension. The final test concentrations of extract were ranging from 11.72 to 1500.00 µg/ml. The microplate was covered, sealed and incubated for 24 hours at 37 ℃. The well that presence in red colour was interpreted as no viability of bacteria and well with a defined yellow colour was interpreted the growth of bacteria due to the acidic metabolites that formed

by growing bacteria. In this study, the lowest concentration of the leaf extract that able to prevent the colour of medium changes from red to yellow was defined as MIC (Tekwu, Peime and Beng, 2012).

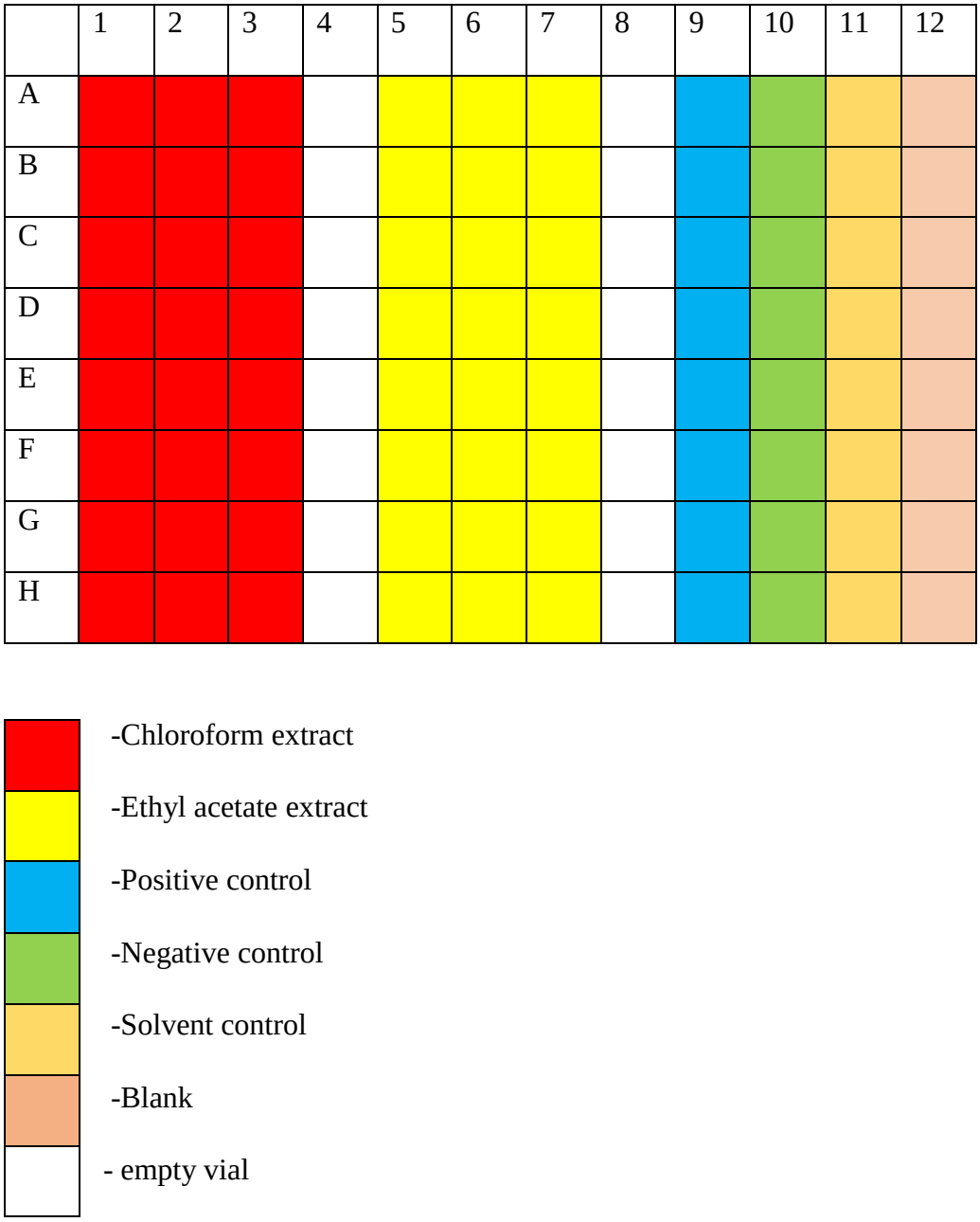


Figure 3.2: Design of a 96 well plate for MIC assay.

3.10 Minimum Bactericidal Concentration (MBC) Assay

The MBC value was determined by spreading a loop full of the culture medium from the broth of MIC assay (no showing visible sign of growing) onto fresh MH agar plates. After incubation at 37 ℃ for 24 hours, the MBC was recorded as the lowest concentration of the test sample showing no bacterial growth on the MH agar plates (Joshua and Takudzwa, 2013).

3.11 Thin Layer Chromatography (TLC)

Each extract was dissolved in respectively solvent. Each test samples was applied through a fine capillary tube onto a precoated silica gel 60 F_{254} TLC plate. The plate was developed with the mobile phase in a twin-trough chamber by using different mobile phase system for different extract. The mobile phase for hexane extract was benzene: chloroform: ethyl acetate (5:3:2 v/v). The mobile phase for chloroform extract was chloroform: ethyl acetate (4:6 v/v). The mobile phase for methanol extract was chloroform: ethyl acetate: methanol (5:3:2 v/v) (Pande, et al., 2011). The mobile phase for ethyl acetate extract was toluene: ethyl acetate: methanol (6:3:1 v/v) (Kaskoos and Ahamad, 2014). The TLC plate was observed under short wavelength (254 nm) and long wavelength (365 nm) of UV light and were photographed. The R_f value was determined by using the following formula (Gujjeti and Mamidala, 2013). :

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

CHAPTER 4

RESULTS

4.1 Sequential Extraction

Figure 4.1 shows the percentage yield of extraction for the leaves of *A. paniculata* obtained using sequential extraction with different polarity index of solvents. Based on Figure 4.1, the methanol extract gave the highest percentages yield of extraction which was 13.70 ± 0.29 % followed by chloroform (8.11 ± 0.20 %) and ethyl acetate (3.89 ± 0.38 %). The percentage yield of extraction for hexane was found to be lowest (1.68 ± 0.10 %).

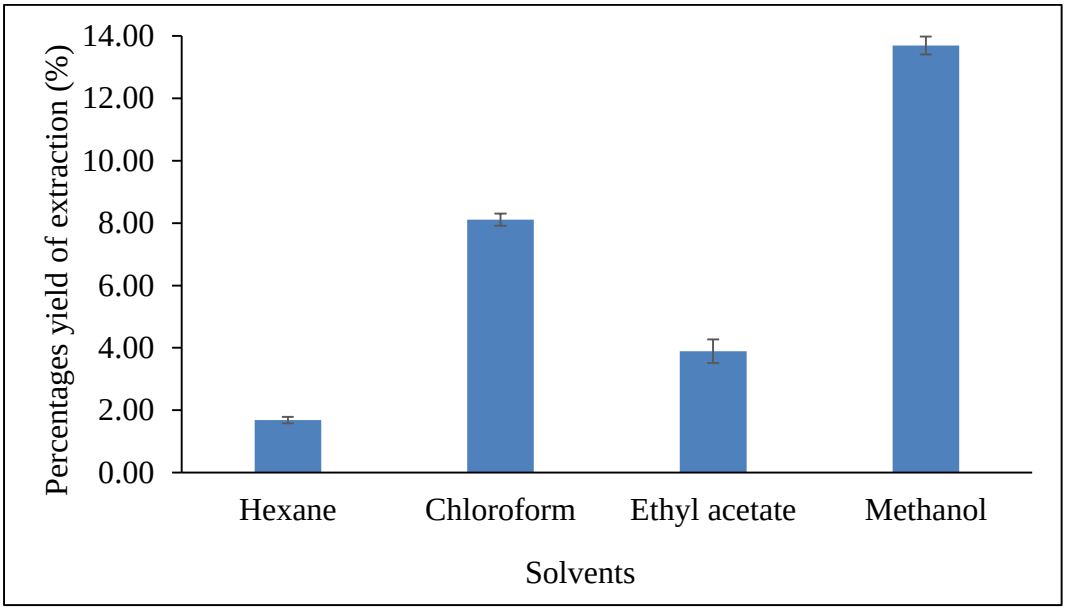


Figure 4.1: Percentage yield of extraction of *A. paniculata* leaf obtained using sequential extraction with different polarity index of solvents. Data are expressed as mean ± standard deviation (n=3).

4.2 Phytochemicals Screening

Table 4.1 shows the qualitative analysis of phytochemicals content in *A. paniculata* leaf extracts. According to Table 4.1, the methanol extract contained the most of the phytochemicals compounds which are terpenoids, cardiac glycosides, alkaloids, flavonoids, saponins and steroids.

Chloroform and ethyl acetate extracts show only two phytochemicals compounds. Chloroform extract contained of terpenoids and phenol while ethyl acetate extract contained of terpenoids and cardiac glycosides. The hexane extract did not shows positive result in all the phytochemical screening tests. In addition, Table 4.1 shows that antraquinones, reducing sugars and tannins were absent in all of the solvent extracts. The colour changes of the extract in all the phytochemical screening tests are shown in Appendix C.

Table 4.1: Qualitative analysis of phytochemicals content in *A. paniculata* leaf extract prepared in different solvents.

Phytochemicals	**Successive Sequential Extracts**			
	Hexane	**Chloroform**	**Acetyl acetate**	**Methanol**
Terpenoids	-	+	+	+
Cardiac glycosides	-	-	+	+
Phenols	-	+	-	-
Alkaloids	-	-	-	+
Flavonoids	-	+	-	+
Saponins	-	-	-	+
Steroids	-	-	-	+
Anthraquinones	-	-	-	-
Reducing sugars	-	-	-	-
Tannins	-	-	-	-

- = negative result

+ = positive result

4.3 Antioxidant Assay

The percentage of DPPH scavenging activity of different solvent extracts with various concentrations are shown in Figure 4.2. All the solvent extracts scavenged DPPH free radical molecules in a concentration dependent manner. Hexane extract showed the highest antioxidant activity with an IC_{50} value of 2.80 mg/ml. The lower IC_{50} value indicates the solvent extract has a higher the antioxidant activity.

The methanol extract exhibited the second higher of antioxidant activity with an IC_{50} value of 5.60 mg/ml. Moreover, there was a tremendous increase of percentage of DDPH scavenging activity (52.67 $\pm$ 0.86 % to 94.66 $\pm$ 0.16 %). In contrast, hexane extract did not show the same trend as methanol extract although it gave the highest antioxidant activity (98.66 $\pm$ 0.74 %). Besides, methanol extract was able to achieve the high percentage of DPPH scavenging activity (more than 90 %) at lower concentration which was 8 mg/ml as compared to hexane extract with 16 mg/ml.

The IC_{50} value for the chloroform extract was found to be 6.40 mg/ml. The lowest antioxidant activity was obtained by the ethyl acetate extract with an IC_{50} value of 7.80 mg/ml. The quercetin was used as positive control for comparison. The IC_{50} value of quercetin was 0.05 mg/ml which is far lower than all of the solvent extracts as it has high antioxidant activity. The percentage of DPPH scavenging activity of quercetin is shown in Figure 4.3 and the IC_{50} of different solvent extracts of *A. paniculata* and quercetin is shown in Table 4.2.

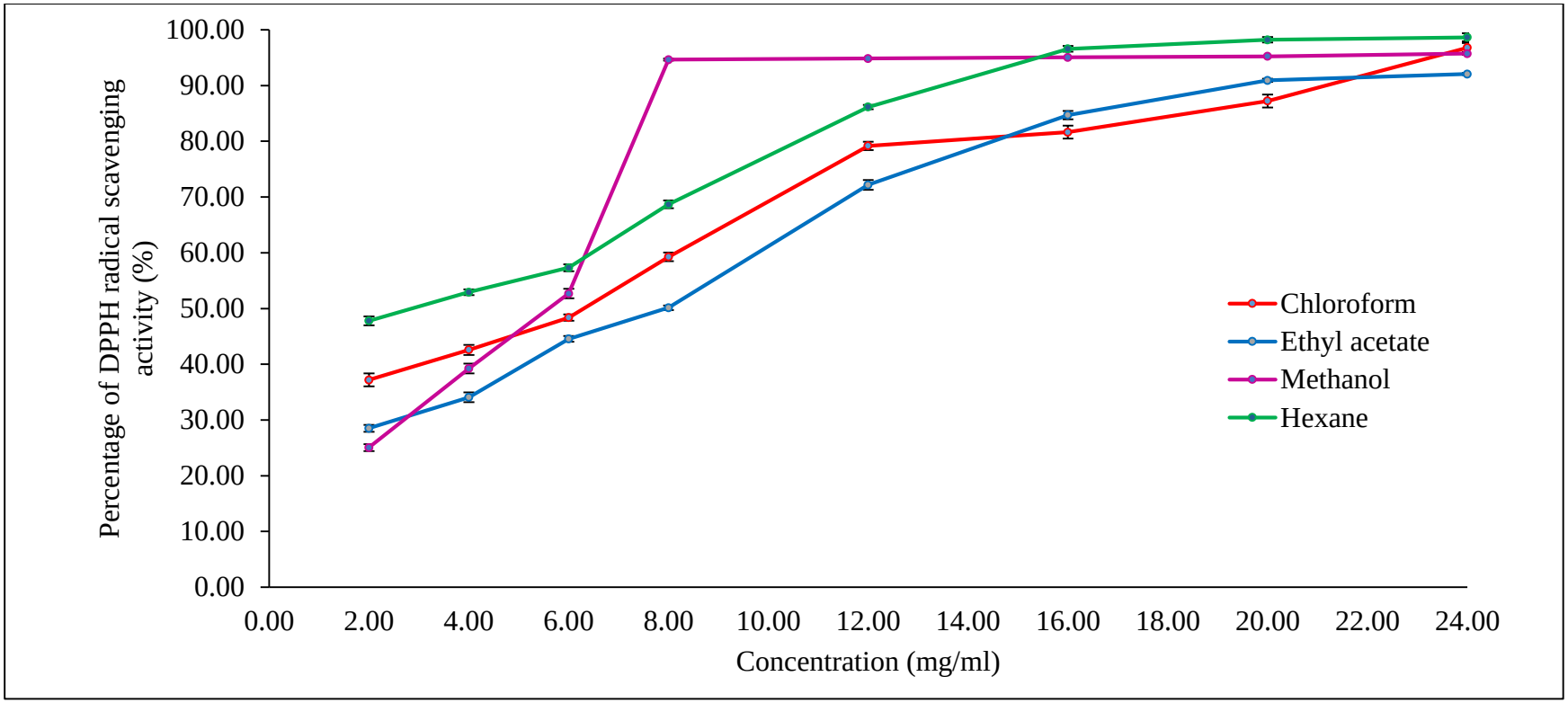


Figure 4.2: Percentage of DPPH scavenging activity of different extract of *A. paniculata* at various concentrations. Data are expressed as mean ± standard deviation.

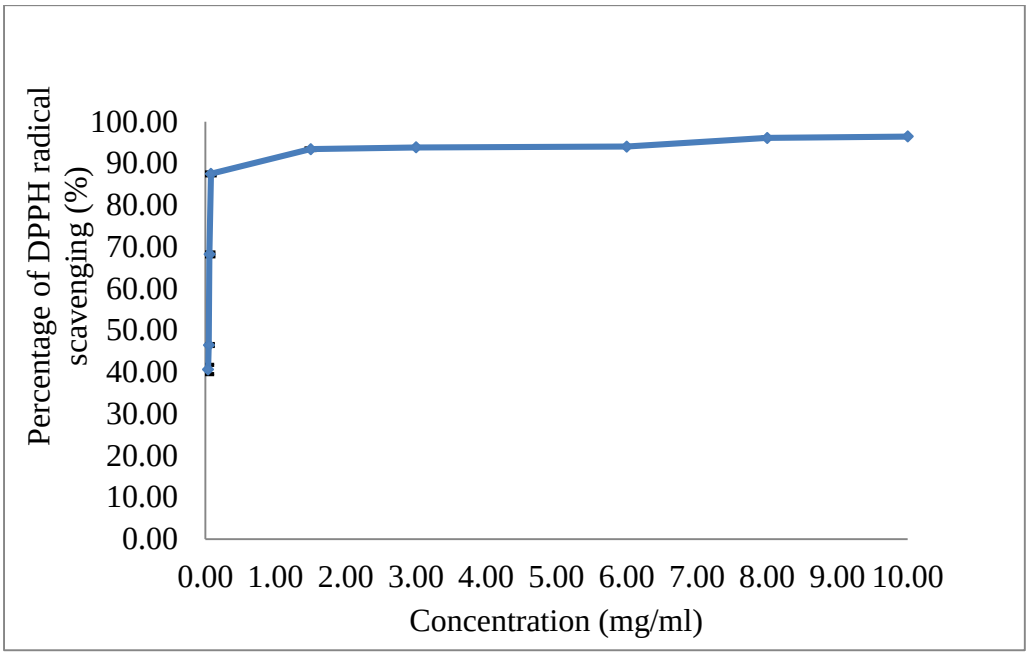


Figure 4.3: Percentage of DPPH scavenging activity of quercetin (positive control) in various concentrations. Data are expressed as mean ± standard deviation.

Table 4.2: IC_{50} of different solvent extracts of *A. paniculata*.

Solvents	**IC_{50} (mg/ml)**
Quercetin (positive control)	0.05
Hexane	2.80
Chloroform	6.40
Ethyl acetate	7.80
Methanol	5.60

4.4 Antibacterial Activity

4.4.1 Agar Disc Diffusion Assay

Agar disc diffusion was carried out to evaluate the antibacterial activity qualitatively by measuring the diameter of inhibition zone. The bigger the diameter of inhibition zone, the higher the antibacterial activity. Table 4.3 shows the antibacterial activity of different solvent extracts of *A. paniculata* with various concentrations via agar disc diffusion method. Based on Table 4.3, the ethyl acetate extract showed the highest antibacterial activity on two Gram positive bacteria which are *S. aureus* and *S. epidermidis*. The highest inhibition zone values of ethyl acetate extract against *S. aureus* and *S. epidermidis* were found to be 15.7 $\pm$0.33 mm and 17.0 $\pm$0.00 mm, respectively. Figure 4.4 shows the highest inhibition zone of ethyl acetate extract against *S. aureus* and *S. epidermidis* at concentration of 1000 µg/disc. The zone of inhibition exhibited by the solvent extract, positive control and solvent control against all the tested bacteria are shown in Figure D1 to D6 (Appendix D).

The chloroform extract showed the second highest antibacterial activities followed by hexane extract. The highest inhibition zone value of chloroform extract against *S. aureus* and *S. epidermidis* were found to be 14.0 $\pm$0.58 mm, and 15.0 $\pm$0.00 mm, respectively. The highest inhibition zone value of hexane extract against *S. aureus* was found to be 8.00 $\pm$0.00 mm at concentration of 600 µg/disc, while against *S. epidermidis* was found to be 11.0 $\pm$ 0.00 mm at concentration of 1000 µg/disc. In contrast, methanol extract did not show any antibacterial activities on both Gram positive bacteria.

According to Table 4.3, all types of solvent extracts of *A. paniculata* showed a higher antibacterial activities toward *S. epidermidis* compared with *S. aureus*. Besides, all of the extracts failed to perform inhibition action against Gram negative bacteria, *E. coli*. Ampicillin, amoxicillin and gentamicin were used as positive control for S. *aureus, S. epidermidis* and *E. coli* respectively. The inhibition zone value for ampicillin, amoxicillin and gentamicin were found to be 53.0 ± 0.00 mm, 35.0 ± 0.00 mm, and 23.0 ± 0.00 mm at various concentrations, respectively.

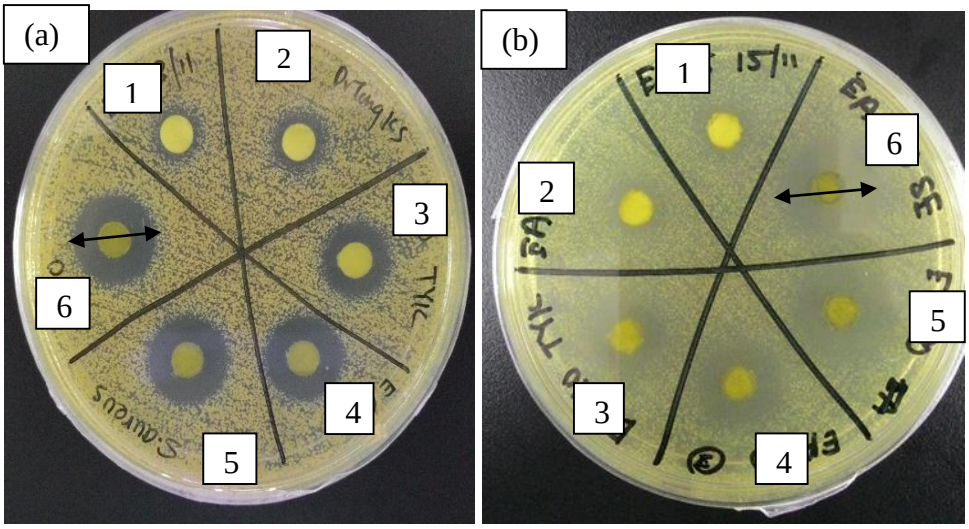


Figure 4.4: Zone of inhibition exhibited by ethyl acetate extract against (a) *S. aureus*, (b) *S. epidermidis* (1)150 µg/disc, (2) 200 µg/disc, (3) 300 µg/disc, (4) 400 µg/disc, (5) 500 µg/disc, (6) 600 µg/disc. The diameter of zone of inhibition is indicated in black arrow.

Table 4.3: The antibacterial activities of different extracts of *A. paniculata* at different concentration through agar disc- diffusion method.

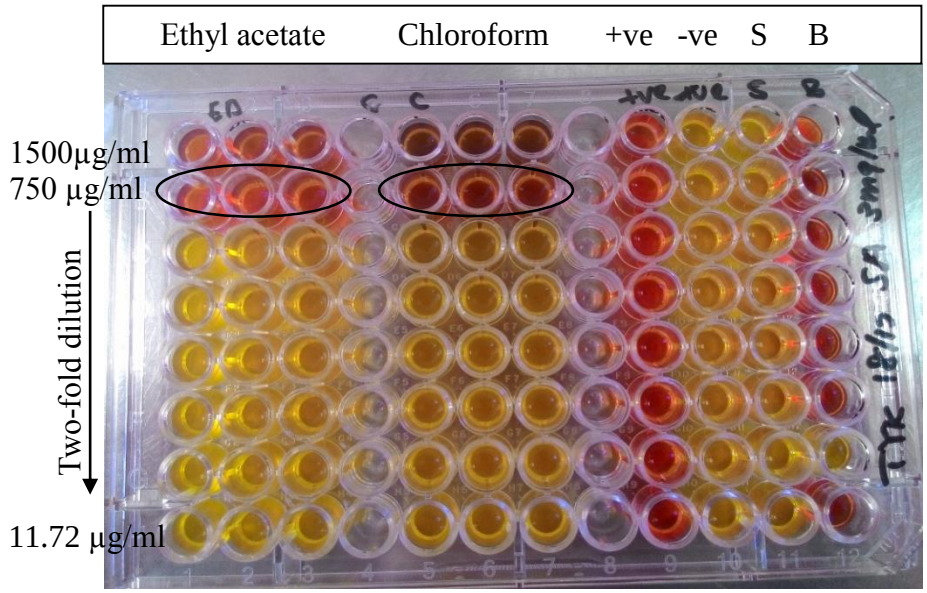
Solvent Extracts	**Concentration (µg/disc)**	**Zone of inhibition (mm)**		
		Gram positive		**Gram negative**
		S. aureus	***S. epidermidis***	***E. coli***
Hexane	150.00	-	-	-
	200.00	-	-	-
	400.00	7.70 ±0.58	-	-
	600.00	8.00 ±0.00	9.30 ±0.58	-
	800.00	8.00 ±0.00	10.00 ±0.00	-
	1000.00	8.0 0±0.00	11.00 ±0.00	-
Chloroform	150.00	11.00 ±1.00	8.30 ±0.58	-
	200.00	11.70 ±0.33	9.70 ±0.58	-
	400.00	12.70 ±0.33	11.70 ±0.58	-
	600.00	13.30 ±0.67	14.00 ±0.00	-
	800.00	13.70 ±0.88	14.30 ±0.58	-
	1000.00	14.00 ±0.58	15.00 ±0.00	-
Ethyl Acetate	150.00	9.00 ±0.00	9.00 ±0.00	-
	200.00	10.00 ±0.00	10.00 ±0.00	-
	400.00	13.00 ±0.00	11.30 ±0.58	-
	600.00	14.00 ±0.00	14.70 ±0.58	-
	800.00	14.70 ±0.33	16.00 ±0.00	-
	1000.00	15.70 ±0.33	17.00 ±0.00	-
Methanol	150.00	-	-	-
	200.00	-	-	-
	400.00	-	-	-
	600.00	-	-	-
	800.00	-	-	-
	1000.00	-	-	-
Positive control				
Ampicillin	25.00	53.00 ±0.00		
Amoxicillin	30.00		35.00 ±0.00	
Gentamacin	10.00			23.00 ±0.00

Data are expressed as mean ±standard deviation (n=3), - : no inhibition zone.

4.4.2 Minimum Inhibitory Concentration (MIC)

Figure 4.5 shows the inhibitory potential of ethyl acetate and chloroform extracts against *S. aureus* with an MIC value of 750 µg/ml shown by black circle. The lowest concentration of well contained solvent extract that remain in red colour is interpreted as the MIC value. The inhibitory activity of ethyl acetate and chloroform extracts against *S. epidermidis* on 96 well plate was shown in Figure D7 (Appendix D).

Table 4.4 shows the minimum inhibitory concentration (MIC) value of chloroform extract and ethyl acetate extracts of *A. paniculata* against *S. aureus* and *S. epidermidis*. According to Table 4.4, the MIC value of chloroform and ethyl acetate extracts treated on *S. aureus* was found to be 750.00 ± 0.00 µg/ml respectively. On the other hand, the MIC value of ethyl acetate extracts treated on *S. epidermidis* was also found to be 750.00 ± 0.00 µg/ml. The chloroform extracts exhibited a lower concentration (375.00 ± 0.00 µg/ml) for the treatment of *S. epidermidis*. Besides, the MIC value of ampicillin was determined on *S. epidermidis* (46.90 ± 0.00 µg/ml). The MIC value of ampicillin was not in the range of concentration (< 11.72 µg/ml) on the treatment of *S. aureus*.



Indicate the growth of bacteria is inhibited

Indicate the bacteria is growing

Figure 4.5: The inhibitory potential of ethyl acetate and chloroform extracts against *S. aureus* with an MIC value of 750 µg/ml shown by black circle. +ve = positive control, -ve = negative control, S = solvent control, B = blank medium.

Table 4.4: Minimum inhibitory concentration (MIC) of chloroform, ethyl acetate extracts of *A. paniculata* and ampicillin.

Solvent extracts	Minimum inhibitory concentration (µg/ml)	
	S. aureus	*S. epidermidis*
Chloroform	750.00 ± 0.00	375.00 ± 0.00
Ethyl acetate	750.00 ± 0.00	750.00 ± 0.00
Ampicillin (Positive control)	-	46.90 ± 0.00

Data are expressed as mean ± standard deviation (n=3), - represents MIC <11.72 µg/ml

4.4.3 Minimum Bactericidal Concentration (MBC)

Figure 4.6 shows the growth of *S. aureus* (gold colonies) and *S. epidermidis* (white colonies) on the Mueller Hinton agar that subculture from positive MIC test of ethyl acetate extract after 24 hours incubation. The growth of *S. aureus* (gold colonies) and *S. epidermidis* (white colonies) on the MH agar that subculture from positive MIC test of chloroform extract after 24 hours incubation was shown in Figure D8 (Appendix D). Table 4.5 shows the MBC value of chloroform, ethyl acetate extracts of *A. paniculata* on both Gram positive bacteria. Based on Table 4.5, both extracts did not show any significant bactericidal activities against *S. aureus* and *S. epidermidis*, instead of showing bacteriostatic for both bacteria.

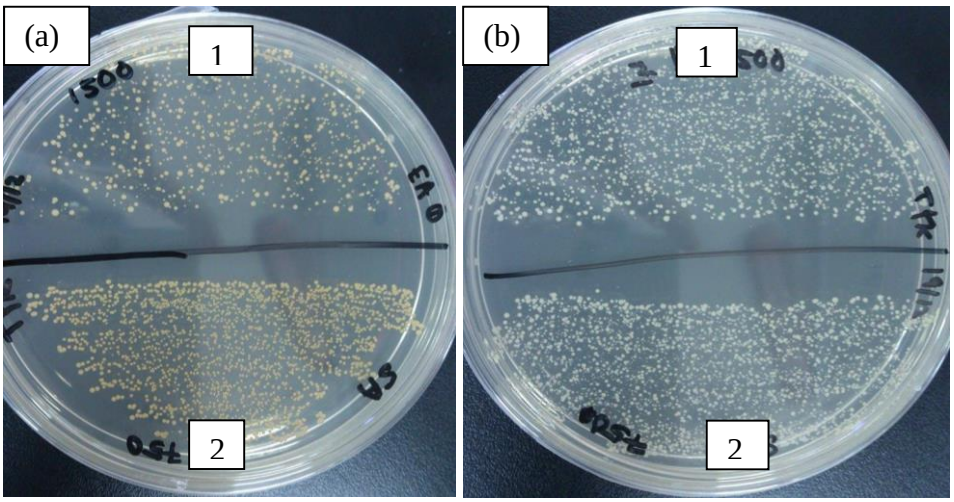


Figure 4.6: The growth of bacteria on Mueller Hinton agar from the MIC activity of ethyl acetate extract after 24 hours incubation (a) *S. aureus*, (b) *S. epidermidis*, (1) 1500 µg/ml (2) 750 µg/ml.

Table 4.5: Minimum bactericidal concentration (MBC) of chloroform, ethyl acetate extracts of *A. paniculata*

Solvent extracts	Minimum bactericidal concentration (µg/ml)	
	S. aureus	*S. epidermidis*
Chloroform	bacteriostatic	bacteriostatic
Ethyl acetate	bacteriostatic	bacteriostatic

4.5 Thin Layer Chromatography (TLC)

The TLC image of the *A. paniculata* leaf extract viewed under short and long UV wavelength are shown in Figure 4.7 and 4.8. A total number of ten, three, six and six spots have been observed on hexane, chloroform, ethyl acetate and methanol extracts respectively.

Based on the Figure 4.7 and 4.8, hexane extract yielded the maximum spots numbers on TLC, followed by chloroform and ethyl acetate extract which yielded the same number of spots, the methanol extract yielded only three spots in TLC. All of the retention factor, R_f values of all solvent extracts was shown in Appendix E. The highest and lowest R_f value of hexane extract are 0.98 ± 0.01 and 0.10 ± 0.01 respectively. The R_f value of chloroform extract are 0.27 ± 0.05, 0.61 ± 0.04 and 0.94 ± 0.02. The highest and lowest R_f value of ethyl acetate extract are 0.98 ± 0.01 and 0.26 ± 0.05 respectively. The highest and lowest R_f value of methanol extract were 0.95 ± 0.02 and 0.26 ± 0.01 respectively. All phytochemicals in solvent extracts can be separated well using TLC method. All the R_f values for all the extracts are shown in Appendix E.

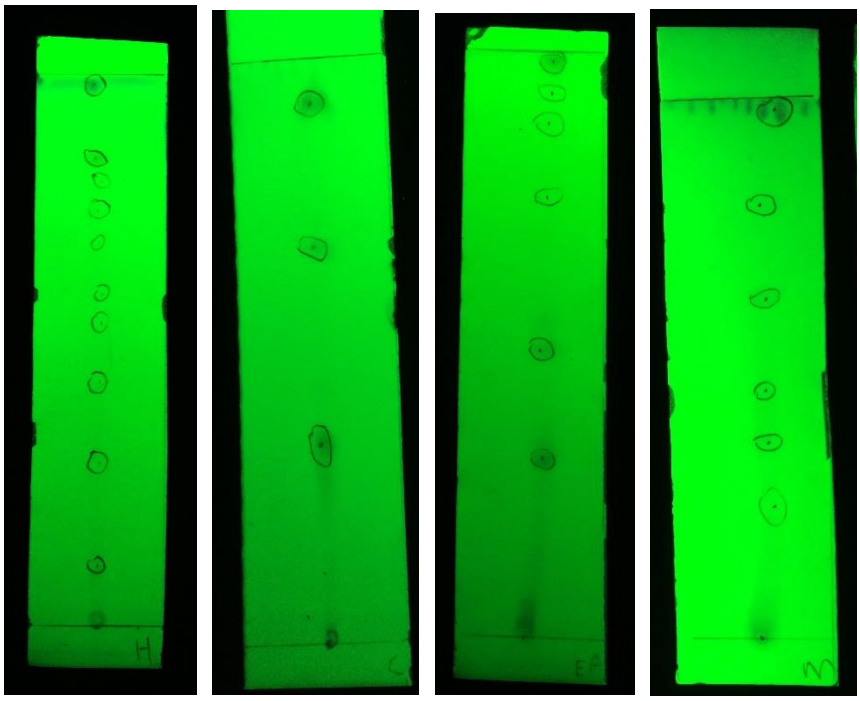


Figure 4.7: TLC images of *A. paniculata* solvent extracts viewed under short wavelength (a) hexane, (b) chloroform, (c) ethyl acetate (d) methanol

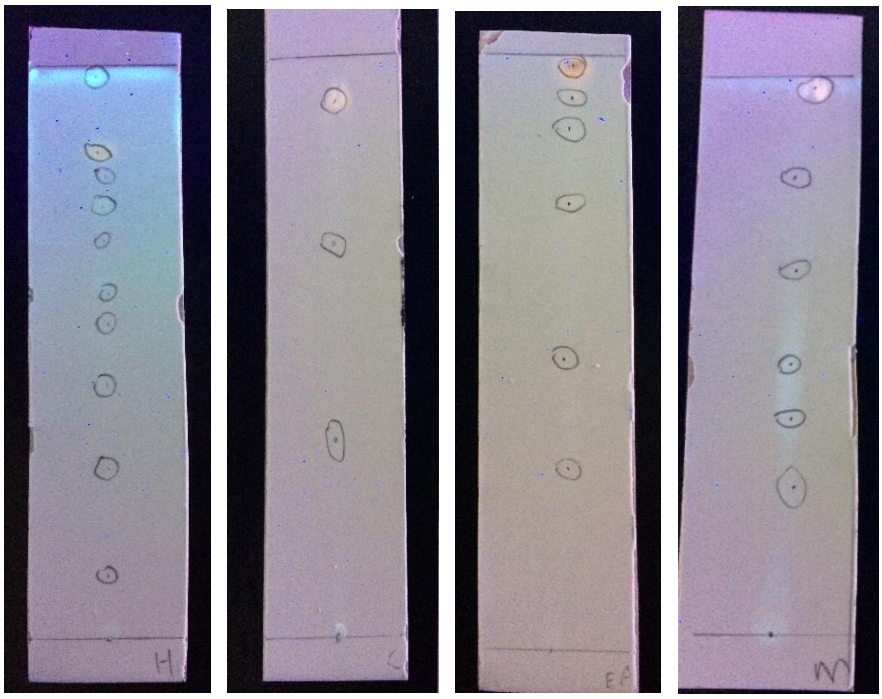


Figure 4.8: TLC images *A. paniculata* solvent extracts viewed under long wavelength (a) hexane, (b) chloroform, (c) ethyl acetate (d) methanol

CHAPTER 5

DISCUSSION

5.1 Extraction of *A. paniculata* Leaves

The leaves of *A. paniculata* were freshly collected and washed under tap water to remove the dirt or impurities and discard the leaves that were injured or microbial contaminated. The leaves of *A. paniculata* were then dried in shaded area before extraction to remove the water content in the vacuole compartment and prevent the contamination of fungus. Most of the polar secondary metabolite compounds are normally stored in the vacuole as water in the vacuole provided hydrophilic condition (Wink, 2013). In addition, it can helps to maintain a constant weight in plant since different plants have different water contents (Singh, 2014).

After dried, the leaves were grinded into small pieces using electric grinder to rupture the cell membrane and cell wall of dried leaves, so that the phytochemical compounds can be more easily to be extracted out. Besides, it can also maximizes the surface area of leaves that contact with solvent thus improve the extraction efficiency (Handa, et al., 2008).

The sequential Soxhlet extraction with increasing polarity index of solvent was used in this project. Soxhlet extraction is chosen for extraction is because it is able to save the amount of solvent used. In Soxhlet extraction, only one batch of solvent is needed as the solvent can be recycled. Sequential extraction with

increasing of polarity index of solvent able to extract out a wide range of secondary metabolites compared with single type of solvent (Tiwari, et al., 2011). Abubacker (2009) also reported that the sequential extraction with increasing of polarity index of solvent is useful to extract secondary metabolites from plants.

5.2 Percentages Yield of Sequential Extraction

The ability of a solvent to extract the bioactive compounds from plant is determined by calculating the percentage yield of extraction (Basri, et al., 2014). The percentage yield of plant extraction are mainly depend on the solvent used in the extraction. Different polarity index of solvent give different percentages yield and extract different phytochemical compounds. Nur Syukriah (2014) reported that polar solvent usually has the higher extraction yield compared to non- polar solvent. This is because the polar compounds such as polyphenols are highly extracted in polar solvent compared with non-polar solvent (Joshua and Takudzwa, 2013). This indicates that the plant usually contain more polar compounds as these compounds will be dissolved in similar polarity of solvents which apply to the "like dissolves like" principle (Pathmanathan, et al., 2010; Gupta, et al., 2012).

The methanol extract obtained the highest percentage yield of extraction, 13.70 $\pm$ 0.29 %. Meanwhile, the hexane extract obtained the lowest percentage yield of extraction, 1.68 $\pm$ 0.10 %. This result is compatible to Isha (2013) who also found the methanol is the solvent that gave the highest percentage yield of extraction of *A. paniculata* leaves extract. This can be implied that the phytochemical compounds in *A. paniculata* dissolved better in methanol solvent.

However, high percentage yield of plant extraction does not indicate that the solvent extract will perform high antibacterial activity (Pinel, Nunez and Nicoli, 2004; Armando and Dennis, 2010).

5.3 Phytochemicals Screening

In this study, 10 phytochemical screening tests have been carried out included terpenoids, cardiac glycosides, phenols, alkaloids, flavonoids, saponins, steroids, anthraquinones, reducing sugars and tannins tests. This study has revealed that terpenoids, cardiac glycosides, phenols, alkaloids, flavonoids, saponins, steroids were present in more than one solvent extracts of *A. paniculata*. In contrast, anthraquinones, reducing sugars and tannins were failed to show positive results in all solvent extracts.

In present study, terpenoids are found to be the most abundant compounds in *A. paniculata* leaves. It has been found in chloroform, ethyl acetate and methanol extracts. Terpenoids compounds have been reported that it had antibacterial, antiviral, antidiarrhoeal and antineoplastic effect (Jayashree, 2013). The terpenoids compounds exhibit antibacterial activity through the mechanism of membrane disruption by its lipophilic compounds (Cowan, 1999; Tiwari, et al., 2011). The most common terpenoid compounds that isolated from *A. paniculata* are diterpenoids lactones. One of the example of diterpenoids lactone is the andrographolide which has bitter taste, colourless and in crystal form (Okhuarobo, et al., 2014). The chemical structures of some andrographolide are shown in Figure 5.1.

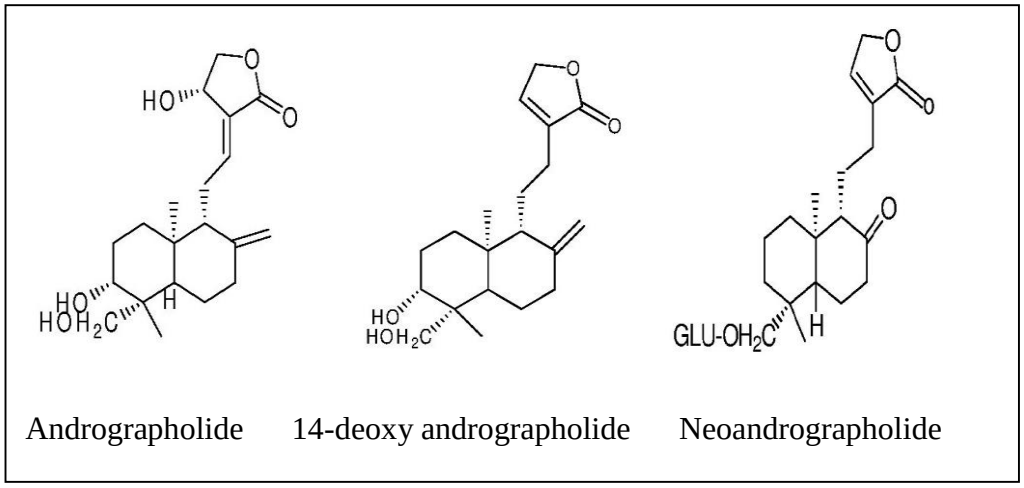


Figure 5.1: The chemical structure of andrographoloide, 14- deoxy andrographolide and neoandrographolide (Mulukuri, et al., 2011; Joselin and Jeeva, 2014; Niranjan, Tewari and Lehri, 2010).

Cardiac glycosides are usually used to treat heart diseases by strengthen heart tissues and allow it to function more efficiently (Odugbemi, 2008). They can inhibit the sodium ions, Na^+ pump and decrease the concentration of Na^+ ions in the myocytes. This will lead to the increasing of the amount of calcium ions, Ca^{2+} available for the heart muscle. Then, it will increase the cardiac output and decrease the distension of heart (Nayak, 2012). Thus, it could not be contributed to antibacterial activity. Phenolic compounds are usually contributed to the antioxidant activity due to the presence of hydroxyl functional groups in their chemical structures (Shad, et al., 2013). Besides, it has been hypothesized to inhibit carcinogenesis through electrophile trapping mechanism (Jayashree, 2013).

Alkaloids are well known with its antimicrobial activity. Some studies have been reported on the antimicrobial activity of alkaloids from several medicinal plants such as *Jatropha curcas*, *Calotropis procera*, *Carica papaya*, *Magnifera indica*

and *Psidium guajava* (Doughari, 2006; Garba and Okeniyi, 2012). The mechanism actions of alkaloids in antimicrobial activity including intercalation DNA or inhibit the biosynthesis of membrane phospholipid (Shelton, 1991). Flavonoids are hydroxylated phenolic compounds that have antimicrobial activity against a wide range of microorganisms (Yadav and Agarwala, 2011). In addition, they are potential antioxidant agent that act as free radical scavenger. The example of flavonoids that have antioxidant properties are flavonols, flavones, anthocyanins, isoflavonoids, flavanols and flavanones (Carocho and Ferreira, 2013).

The common flavonoids that have been isolated from *A. paniculata* and their chemical structures are shown in Figure 5.2 (Niranjan, Tewari and Lehri, 2010; Akhbar, 2011). Besides, flavonoids also exhibit the property of anti-allergic, antifungal, antiviral, anticancer and anti-inflammatory properties (Sharma, et al., 2011; Peteros and Uy, 2010).

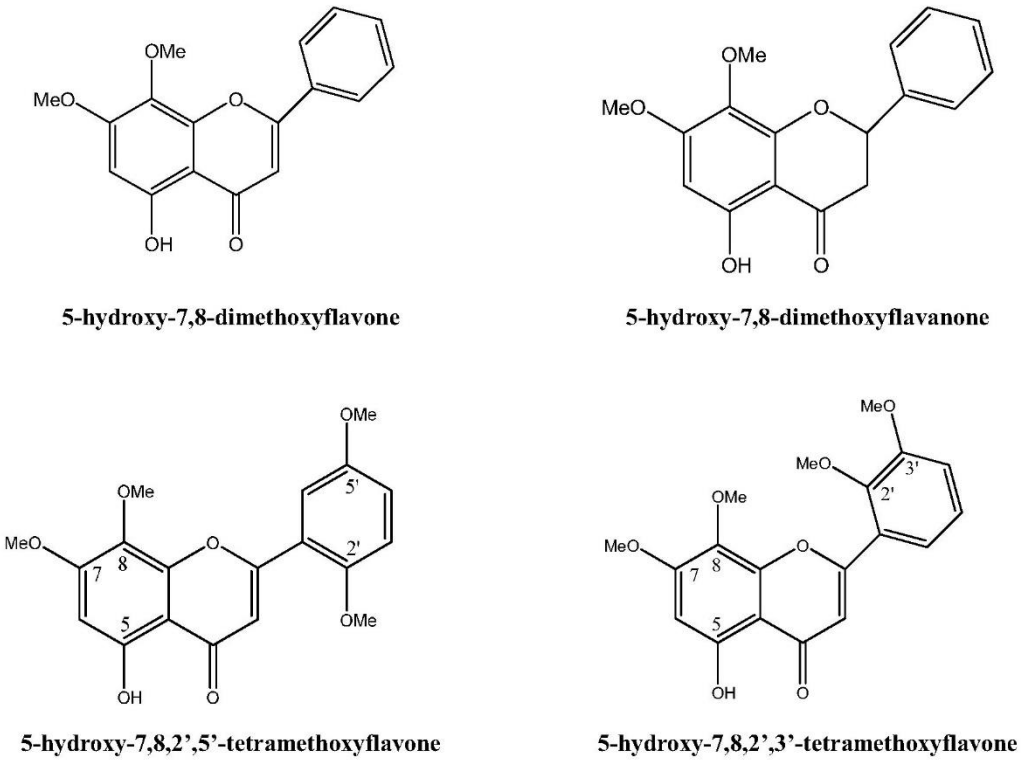


Figure 5.2: The chemical structure of some common flavonoids isolated from *A. paniculata* (Chao and Lin, 2010).

Saponins mostly are soap forming compounds that also have antimicrobial property. Their action of mechanism is causing the leakage of the protein or certain enzyme from the bacteria cell (Mohamed Sham Shihabudeen, 2010; Jayashree, 2013). In addition, saponins also can be used in anticancer, antifungal, antioxidant and weight loss treatment (Radha, Sermakkani and Thangapandian, 2011). Steroids also have been reported that it exhibits antimicrobial activity by causing the leakage on liposomes in microorganism, due to its steroidal compounds could be specifically associate with the lipid of membranes (Epand, Savage and Epand, 2007).

Anthraquinone is known to have anti-parasitic, antimicrobial and antidepressant properties (Pieters and Vlietinck, 2005). The present of reducing sugars is an indication of leaf that it provides energy through the degradation of carbohydrates (Harborne and Williams, 2000). Tannins are polyphenol compounds that well known with its protein inhibition property. Tannins interfere the process of protein synthesis by binding to the proline rich protein (Yadav and Agarwala, 2011; Shimada, 2006). Besides, high concentration of tannins also show antimicrobial and antifungal activities by coagulating the protoplasm of microorganisms (Radha, Sermakkani and Thangapandian, 2011). The leaf extract of *A. paniculata* did not contain tannins because it is commonly found in tree bark (Cheynier, Sarni-Manchado and Quideau, 2012).

In short, majority of phytochemical compounds namely terpenoids, alkaloids, saponin, steroids and anthraquinones are mainly involved in antimicrobial activity. Meanwhile, phenolic and flavonoids compounds are found to have antioxidant property.

5.4 Antioxidant Assay

DPPH assay is widely used to determine the antioxidant activity of plant extract. This assay is based on the ability of antioxidant compound to decolourise the purple colour of DPPH free radical in alcoholic solution to yellow colour by donating hydrogen atom as shown in Figure 5.3 (Sharma and Joshi, 2011; Gil, et al., 2000; Premanath and Devi, 2011). The decolourisation of the purple colour DPPH solution is stoichiometrically proportionated with the respect

number of electrons or hydrogen donated by antioxidant compounds (Jain, et al., 2011).

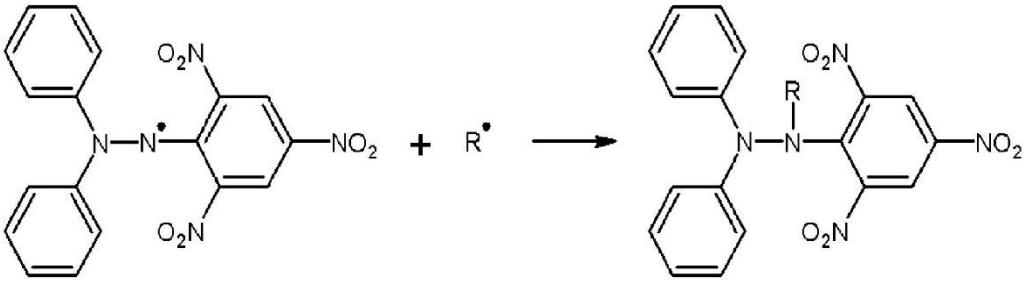


Figure 5.3: The reduction of the DPPH radical by antiradical compound (Shalaby and Shanab, 2013; Lalli, 2006)

The DPPH assay used IC_{50} value as a parameter to determine the level of antioxidant activity. The IC_{50} is defined as the concentration of plant extract that able to decrease fifty percentage of the initial absorbance of DPPH solution or scavenge fifty percentage of DPPH radicals (Aris, et al., 2009).

Gorinstein, et al. (2007) has reported that the high antioxidant activity of plant extracts were due to the presence of high phenolic and flavonoids compounds which are polar compounds in the 50 % of methanol extract. In addition, Guha (2009) has conducted a study that showed that the polar solvent extract (methanol and aqueous) possessed a higher antioxidant activity compare to non-polar extract (hexane and chloroform) over 56 different type of plants. There are also many studies have been reported that the methanol extract of *A. paniculata* showed significant DPPH radical scavenging activity and possessed high antioxidant activity (Sharma and Joshi, 2011; Malahubban, et al., 2013; Huidrom

and Deka, 2012; Eugine, et al., 2011). Similar outcome has been reported by Premanath and Devi (2011) showed that the aqueous and methanol extracts of *A. paniculata* have higher antioxidant activity compared to hexane and chloroform extracts.

However, those discussion above are not accordance with present study. Hexane extracts in this study showed the highest antioxidant activity with an IC_{50} value of 2.80 mg/ml. Besides, the methanol extract showed the second highest antioxidant activity with an IC_{50} value of 5.60 mg/ml. However, the methanol extract surprisingly able to achieve maximum scavenging activity (94.66 ± 0.16 %) at a lower concentration, 8.00 mg/ml compared to hexane extract. In contrast, hexane extract only able to achieve the maximum scavenging activity at concentration of 16 mg/ml. It needs double of the concentration of methanol extract to reach the maximum inhibition.

In addition, the scavenging activity of methanol extract is stable at high percentage of scavenging activity after the tremendous increase at 6 mg/ml, but the percentage of scavenging activity for hexane and others extracts were increased slowly after 50 %. This phenomena shows that the methanol extract is a high potential antioxidant agent. These results were compared with the positive control, quercetin which showed an IC_{50} value of 0.05 mg/ml. This comparison suggested the solvent extracts of *A. paniculata* is relatively potential antioxidant agent and compatible with the commercial antioxidant agent.

5.5 Antibacterial Activity

In this study, chloroform and ethyl acetate extracts showed a higher antibacterial activity against *S. aureus and S. epidermidis* as compared with hexane extract. Ethyl acetate extract exhibited the highest antibacterial activity on both *S. aureu*s and *S. epidermidis* by having the inhibition zone value of 15.70 ± 0.33 mm and 17.00 ± 0.00 mm, respectively. The *S. aureus* and *S. epidermidis* are sensitive toward the inhibition action of ethyl acetate extract based on the guidelines that reported by Shuddhalwar, Charde and Patil (2014) as shown in Table 5.1.

Table 5.1: Zone of inhibition evaluation table for agar disc diffusion assay (Shuddhalwar, Charde and Patil, 2014).

Category	**Diameter of zone of inhibition (mm)**
Resistance	≤10.00
Intermediate	11.00-15.00
Sensitive	≥16.00

The specific compounds that attribute to the antibacterial activity have not been determined in this study. However, preliminary screening of phytochemicals compounds of the solvent extracts had been carried out. The result of phytochemicals screening revealed that the chloroform extract contained of terpenoids and phenol, and ethyl acetate extract contained of terpenoids. Thus, terpenoid compounds are interpreted to be the potential antibacterial agents. The antibacterial activity of these two extracts may be due to the individual or combination effect of terpenoids groups. The proposed terpenoids lactone that involved in antibacterial activity are andrographolide, 1, 4 deoxyandrographolide and neoandrographolide. (Aniel, Mutyala and Rao, 2010).

However, the methanol extract in the present study did not show any antibacterial activity toward all the tested bacteria. This might due to the other phytochemical compounds such ad flavonoids and polyphenolics compounds also present in the methanol extract. These phytochemical compounds might interfere its antibacterial activity. Those other phyto-constituents might exert antagonistic effect on the bioactive compounds that perform antibacterial activity (Basri, et al., 2014). In addition, this finding is contradicted to the finding of Sule, et al. (2011) who reported that the methanol extract of *A. paniculata* showed a high antibacterial activity against *S. aureus* and *S. epidermidis*. The methanol extract showed highest inhibition zone of 22.00 ± 0.00 and 20.67 ± 0.58 mm respectively at concentration of 1000 µg/disc.

Despite this, the present result is corroborates by the finding of Dada-adegbola, Olajide and Ajayi (2014) who also reported that the methanol extract of *A. paniculata* did not showed any antibacterial activity against 22 tested Gram positive and 27 tested Gram negative bacteria. Hence, the antibacterial activity greatly depend on the phytochemicals that consist in the plant. The phytochemicals content in the plant are constantly fluctuated with the genetic heterogeneity of a plant species. The genetic heterogeneity might be caused by the variation in soil condition, seasonal cycle, climate and weather, age of plant, sun and shade fluctuations (Joshua and Takudzwa, 2013).

The hexane extract showed the least antibacterial activity as it is a non-polar solvent which commonly used to extract essential oils and lipids. This could suggest that the antibacterial compounds that present in this plant is lipid soluble

compounds (Joshua and Takudzwa, 2013). The low antibacterial activity of hexane extract might due to their bioactive phytochemicals present in low concentration. This low antibacterial activity may seems to be insignificant. However, if hexane extract is go to further isolation and purification, the antibacterial activity might increase.

All solvent extracts of *A. paniculata* did not show any antibacterial activity against Gram negative bacteria, *E. coli*. This is because of the cell wall structure of Gram negative bacteria, contain an extra layer of outer membrane compared to Gram positive bacteria whose only have an inner cell membrane. This outer membrane is carrying the structural of lipopolysaccharide components which allows the outer membrane to exclude the drugs, antibiotics from entering the cell (Zaidan, et al., 2005). In contrast, the outer peptidoglycan layer of Gram positive bacteria are permeable to the antimicrobial chemical substances. Thus, Gram negative bacteria are less susceptible toward inhibition of the antibacterial agent that present in the solvent extracts compared to Gram positive bacteria (Nikaido, 2003).

However, this results are not in accordance with the previous study reported that the methanol extract of *A. paniculata* showed antibacterial activity against *E. coli* (Mohamed Sham Shihabudeen, Hansi Priscilla and Thirumurugan, 2010; Sharma, et al., 2011). Premanath and Devi (2011) had also reported that the hexane, chloroform and methanol extracts of *A. paniculata* showed antibacterial activity against *E. coli* through agar disc diffusion method.

MIC and MBC are used to evaluate the antibacterial activity quantitatively. MIC is mean as the lowest concentration of antibiotics, drugs or antimicrobial agents that are able to inhibit the growth of the microorganisms. Besides, the MBC is defined as the minimum concentrations of antibiotics, drugs or antimicrobial agents that are able to prevent the growth of the microorganism in the recovery medium after subculture (Andrew, 2001). Ampicillin is used as positive control in the MIC assay, as *S. aureus and S. epidermidis* are claimed to be susceptible to ampicillin. Ampicillin actively against *S. epidermidis* with MIC value of 46.90 ± 0.00 µg/ml, while the MIC value for ampicillin against *S. aureus* was not able to be detected because no viability found in the range of concentration (< 11.72 µg/ml). This indicates that the *S. aureus* are susceptible to the ampicillin and not able to grow on medium containing low concentration of ampicillin.

According to Aligiannis, et al. (2001) standard guidelines for MIC results, the plant extract that have MIC value up to 500.00 µg/ml is a strong bacterial inhibitor, MIC value between 600.00 and 1500.00 µg/ml is a moderate bacterial inhibitor and considered as weak bacterial inhibitor if the MIC value is above 1600.00 µg/ml. *S. aureus* was moderately inhibited by the chloroform and ethyl acetate extracts as they have the MIC value of 750.00 ± 0.00 µg/ml. Thus, chloroform and ethyl acetate extracts of *A. paniculata* are moderate *S. aureus* inhibitors. *S. epidermidis* was also moderately inhibited by ethyl acetate extract with a MIC value of 750.00 ± 0.00 µg/ml. However, the *S. epidermidis* was strongly inhibited by chloroform extract with MIC value (375.00 ± 0.00 µg/ml). It is lower than the standard MIC value, 500.00 µg/ml. Hence, chloroform extract is a strong bacterial inhibitor toward *S. epidermidis*.

On the other hand, chloroform and ethyl acetate extract are bacteriostatic toward *S. aureus* and *S. epidermidis*. *S. aureus* and *S. epidermidis* are able to grow back on the MH agar plate once the inhibition factor (plant extract) has been removed. The golden colour colonies of *S. aureus* and white colour colonies of *S. epidermidi*s were observed after they have recovered on the MH agar plates as shown in Figure 4.6. This indicates that the chloroform and ethyl acetate extracts are only able to inhibit the growth of the bacteria instead of terminate the growth of the bacteria.

In other words, it means that the chloroform and ethyl acetate extract of *A .paniculata* did not show any bactericidal effect on both Gram positive bacteria even at the highest tested concentration (1500 µg/ml). Bactericidal is defined as the antibacterial agent that able to kill the bacterial instead of inhibit their growth (French, 2006).

5.6 Thin Layer Chromatography (TLC)

TLC is used to provide the insight into the chemical composition present in the plant extracts. TLC works on the principles that attract like forces and repel unlike forces. It will retain the polar compounds on the TLC plate (stationary phase) that coated with a polar adsorbent, silica gel. The sample is then moved upward via the mobile phase. The mobile phase moves through the TLC plate by capillary action and assisted with gravity force (Fernand, 2003).

R_f value is used to characterize the different phytochemical compounds that present in the extract. The formula used to calculate the R_f value is mentioned in CHAPTER 3 Section 3.7. The phytochemical compound will be obtained by different R_f values, due to their polarity. This is because the polar compounds will have strong interaction with the stationary phase on TLC and travel shorter path. Meanwhile, the non-polar compounds have weaker interaction with stationary phase and travel longer path (Costanzo, 2000). Thus, the higher the R_f value, the higher the polarity of phytochemical compounds. This information is important by choosing appropriate and suitable solvent system to further isolate pure compounds from the plant extract by other advance chromatography (Sharma and Paliwal, 2013). The comparison of R_f value of a known standard to an unknown molecule would allow for partial characterization of the latter.

The highest and lowest R_f value of hexane extract are 0.98 ± 0.01 and 0.10 ± 0.01 respectively. It indicates that the hexane extract contained of both slightly polar and non-polar compounds. The R_f value of chloroform extract are 0.27 ± 0.05, 0.61 ± 0.04 and 0.94 ± 0.02, showed that the chloroform extract contained

of polar, semi polar and non- polar compounds. The ethyl acetate extract has one polar compound with a lower R_f values of 0.26 ±0.05 and one semi-polar compound with R_f values of 0.46 ± 0.02. Besides, it also contained four non-polar compounds with R_f values of 0.74 ± 0.00, 0.87 ± 0.02, 0.94 ± 0.01 and 0.97 ± 0.01.The highest and lowest R_f value of methanol extract are 0.95 ± 0.02 and 0.26 ± 0.01 respectively. All the compounds in the methanol extract are well separated as shown in the Figure 4.7 and Figure 4.8. In addition, all solvent extracts contained of very non- polar and slightly non polar compounds as they did show R_f value of 0.98 ± 0.01, 0.94 ± 0.02, 0.97 ± 0.01 and 0.95 ± 0.02 respectively.

5.7 Future Perspectives

In present study, there are only two Gram positive and one Gram negative bacteria were tested. Thus, a wide range of bacterial strains should be tested in future study to confirm the antibacterial activity of the *A. paniculata*. Besides, other microorganisms such as fungus and viral cell also should be included in the further study to explore its antifungal and antiviral properties.

Besides, the antioxidant property of plant extract should further confirm by other antioxidant assays such as by determining the ferric reducing antioxidant power (FRAP), nitric oxide radical scavenging activity and superoxide anions scavenging activity.

In addition, the bioactive phytochemical compounds should be further isolated and purified by using column chromatography techniques. The bioactive compound can be identified and structure elucidated by nuclear magnetic resonance (NMR) and high performance liquid chromatography (HPLC).

CHAPTER 6

CONCLUSION

This project discovered that the methanol extract of *A. paniculata* leaves gave the highest percentage yield of extraction. This indicated that the leaf extract of *A. paniculata* contained more polar compounds than non-polar compounds. The phytochemical screening tests have revealed that terpenoids are the most abundant components contained in the leaf extract of *A. paniculata*. Further isolation and purification of the active phytochemical compounds is needed, so that the specific bioactive compound can be served as the template for the production of new antibacterial or antioxidant agents. The antibacterial activity was evaluated qualitatively and quantitatively through agar disc diffusion assay, MIC and MBC respectively. Ethyl acetate extract are found to be the most potential antibacterial agent to inhibit the growth of *S. aureus* and *S. epidermidis*. Besides, the methanol extract of *A. paniculata* are found to be no antibacterial activity against all the tested bacteria, thus it is not suitable to use as antibacterial agent. The DPPH assay showed that hexane extract gave the highest antioxidant activity and methanol extract also can be a potential antioxidant agent as it is able to scavenge a high percentages of free radical at low concentration. Thin layer chromatography revealed that the *A. paniculata* extract contained several non-polar and slightly non polar compounds as they did show higher R_f value. In conclusion, the ethyl acetate extract perform the best antibacterial activity and hexane extract perform the best antioxidant activity.

REFERENCES

Abubakar, E. M., 2009. Efficacy of crude extracts of garlic (*Allium sativum* Linn.) against nosocomial *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniea* and *Pseudomonas aeruginosa*. *Journal of Medicinal Plants Research*, 3(4), pp. 179-185.

Agarwal, S.S. and Paridhari, M., 2013. *Herbal drug technology*. 2nd ed. India: Orient Blackswan.

Aiyelaagbe, O.O. and Osamudiamen, P.M., 2009. Phytochemical screening for active compounds in *Mangifera indica* leaves from Ibadan, Oyo State. *Plant Science Research*, 2(1), pp. 11-13.

Ajayi, I.A., Ajibade, O. and Oderinde, R.A., 2011. Preliminary phytochemical analysis of some plant seeds. *Research Journal of Chemical Sciences*, 1(3), pp. 58-62.

Akhbar, S., 2011. *Andrographis paniculata*: A review of pharmacological activities and clinical effects. *Alternative Medicine Effect*, 16(1), pp. 66-77.

Akinpelu, D.A., Adegboye, M.F., Adeloye, O.A. and Okoh, A.L., 2008. Biocidal activity of partially purified fractions from methanolic extract of *Garcinia kola* (Heckel) seeds on bacterial isolates. *Journal of Biology Resource*, 41, pp. 277-287.

Ali, A.M., El-Sharkawy, A.H., Hamid, J.A., Ismail, N.H. and Lajis, N.H., 1995. Antimicrobial activity of selected Malaysia plants. *Pertanika Journal of Tropical Agricultural Science*, 18(1), pp. 57-61.

Aligiannis, N., Kalpotzakis, E., Mitaku, S. and Chinou, I.B., 2001. Composition and antimicrobial activity of the essential oils of two *Origanum* species. *Journal of Agricultural and Food Chemistry*, 40, pp. 4168-4170.

Andrew, J.M., 2001. Determination of inhibitory concentration. *Journal of Antimicrobial Chemotherapy*, 48, pp. 5-16.

Aniel, K.O., Mutyala, N.L. and Rao, K, G.R., 2010. *In vitro* antibacterial activity in the extracts of *Andrographis paniculata* Burm. F. *International Journal of Pharm Tech Research*, 2(2), pp. 1383-1385.

Armando, C.C. and Dennis, O.O., 2010. Preliminary phytochemical and antimicrobial evaluation of the fresh and dried whole plant extracts from *Commelina benghalensis*. *Revista Colombiana de Ciencia Animal*, 2(1), pp. 104-116.

Aris, S.R.S., Mustafa, S., Ahmat, N. and Jaafar, F.M., 2009. Phenolic content and antioxidant activity of fruits of *Ficus deltoidea* var *Angustifolia* sp. *The Malaysian Journal of Analytical Sciences*, 13(2), pp. 146-150.

Basri, D.F., Saidi, N., Mahari, H., Saari, S. and Santhanam, J., 2014. Preliminary screening for antimicrobial activity of the pulp of *Canarium odontophyllum* Miq. (Dabai) fruit. *Global Journal of Pharmacology*, 8(2), pp.213-220.

Brewer, M.S., 2011. Natural antioxidants: sources, compounds, mechanisms of action, and potential applications. *Comprehensive Reviews in Food Science and Food Safety*, 10, pp. 221-247.

Brooks, G.F., Butel, J.S. and Morse, S.A., 2004. *Jawetz, Melnick, & Adelberg's medical microbiology*, 23rd ed. Boston: McGraw Hill.

Buru, A.S., Pichika, M.R., Neela, V. and Mohandas, K., 2014. *In vitro* antibacterial effects on common bacteria in wound infections with emphasis on methicillin- resistance *Staphylococcus aureus*. *Journal of Ethnopharmacology*, 153(3), pp. 587-595.

Buxton, R., 2005. Blood agar plates and hemolysis: *Staphylococcus* and other catalase positive Gram-positive cocci. [online] Available at: <http://lib.sytu.edu.cn/asm/121-Introduce.htm> [Accessed 22 March 2015].

Carocho, M. and Ferreira, I. C.F.R., 2013. A review on antioxidants, prooxidants and related controversy: natural and synthetic compounds, screening and analysis methodologies and future perspectives. *Food and Chemical Toxicology*, 51, pp.15-25.

Chanwitheesuk, A., Teerawutgulrag, A. and Rakariyatham, N., 2004. Screening of antioxidant activity and antioxidant compounds of some edible plants of Thailand. *Food Chemistry*, 92(3), pp. 491-497.

Chao, W.W. and Lin, B.F., 2010. Review isolation and identification of bioactive compounds in *Andrographis paniculata* (Chuanxinlian). *Chinese Medicine*, 5(17), pp. 1-15.

Cheynier, V., Sarni-Manchado, P. and Quideau, S. eds., 2012. *Recent Advances in Polyphenol Research*. West Sussex: John Wiley & Sons.

Costanzo, S., 2000. Optimization mobile phase conditions for TLC methods used in pharmaceuticals analyses. *Journal of Chromatographic Science*, 35(12), pp. 47-53.

Cowan, M.M., 1999. Plant product as antimicrobial agents. *Journal of Clinical Microbiology*, 12, pp. 564- 582.

Dada-adegbola, H.O., Olajide, O. and Ajayi, B., 2014. Comparative study of antibacterial activity of juice, acetone, methanol and ethanol leaf extract of *Andrographis paniculata* (King of Bitters). *Journal of Medical Research,* 3(1), pp. 6-12.

Doss, V.A. and Kalaichelvan, P.T., 2012. *In vitro* antimicrobial and antioxidant activity screening of *Andrographis paniculata* leaf ethanolic extract in Tamil Nadu. *International Journal of Pharmacy and Pharmaceutical Sciences*, 4(1), pp. 227-229.

Doughari, J.H., 2006. Antimicrobial activity of *Tamarindus indica* Linn. *Tropical Journal of Pharmaceutical Research,* 5(2), pp. 597-603.

Edoga, H.O., Okwu, D.E. and Mbaebie, B.O., 2005. Phytochemicals constituents of some Nigerian medicinal plants. *African Journal of Biotechnology*, 4(7), pp. 685-688.

Epand, R.M., Epand, R.F. and Savage, P.B., 2007. Bacterial lipid composition and the antimicrobial efficacy of cationic steroid compounds (Ceragenins). *Biochimica et Biophysica Acta (BBA)*, 1768(10), pp. 2500-2509.

Eugine, L.P.S., Kadar, A.S.H., Nagireddy, D., Reeta, V.R.K. and Manavalan, R., 2011. Evaluation of *in-vitro* antioxidant activity of leaf extract of *Andrographis paniculata*. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, 2(2), pp. 891-895.

Fernand, V.E. *Initial characterization of crude extracts from Phyllanthus amarus schum and Thonn and Quassia amaral using normal phase thin layer chromatography*. MSc. University of Suriname. Available at: <http://etd.lsu.edu/docs/available/etd-0407103-163959/> [Accessed 14 March 2015].

Franklin, D. and Lowy, M.D., 1998. *Staphylococcus aureus* infections. *The New England Journal of Medicine*, 339, pp. 520-532.

Franklin, D., Lowy, M.D., Scott, M. and Hammer, M.D., 1983. *Staphylococcus epidermidis* infections. *Annals of Internal Medicine*, 99(6), pp. 834-839.

French, G.L., 2006. Bactericidal agents in the treatment of MRSA infections-the potential role of daptomycin. *Journal of Antimicrobial Chemotherapy*, 58, pp. 1107-1117.

Garba, S. and Okeniyi, S.O., 2012. Antimicrobial activities of total alkaloids extracted from some Nigerian medicinal plants. *Journal of Microbiology and Antimicrobials,* 4(3), pp. 60-63.

Gil, M.I., Toma ́s-Barbera ́n, F.A., Hess-Pierce, B., Holcroft, D.M. and Kader, A.A., 2000. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *Journal of Agricultural and Food Chemistry*, 48, pp. 4581-4589.

Gorinstein, S., Oscar, J., Vargas, M., Jaramillo, N.O., Salas, I.A., Ayala, A.L.M., Avila, P.A., Toledo, P., Katrich, E. and Trakhtenberg, S., 2007. The total polyphenols and the antioxidant potentials of some selected cereals and pseudocereals. *European Food Research Technology*, 225, pp. 321-328.

Guha, G., Rajkumar, V., Mathew, L. and Kumar, R.A., 2011. The antioxidant and DNA protection potential of Indian tribal medicinal plants. *Turkish Journal of Biology*, 35, pp. 233-242.

Gujjeti, R.P. and Mamidala, E., 2013. Phytochemical screening and thin layer chromatographic studies of *Aerva Lanata* root extract. *International Journal of Innovative Research in Science, Engineering and Technology*, 2(10), pp. 5725-5730.

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

Halliwell, B. and Whiteman, M., 2004. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? *British Journal of Pharmacology*, 142(2), pp. 231-255.

Handa, S. S. et al., 2008, Extraction technologies for medicinal and aromatic plants. [Online] Available at: <http://www.unido.org/fileadmin/user_media/Publications/Pub_free/Extraction_technologies_for_medicinal_and_aromatic_plants.pdf> [Accessed 27 February 2015].

Harborne, J.B. and Williams, C.A., 2000. Advances in flavonoid research since 1992. *Phytochemistry*, 100, pp. 481-504.

Harris, L.G., Foster, S.J. and Richards, R.G., 2002. An introduction to *Staphylococcus aureus*, and techniques for identifying and quantifying *S. aureus* adhesins in relation to adhesion to biomaterials: review. *European Cells and Materials*, 4, pp. 39-60.

Hedetniemi, K. and Liao, M.K., 2006. Luria Broth (LB) and Luria Agar (LA) media and their uses: *Staphylococcus aureus*. [online] Available at: <http://www.microbelibrary.org/library/laboratory-test/3032-luria-broth-lb-and-luria-agar-la-media-and-their-uses-staphylococcus-aureus> [Accessed 22 March 2015].

Hosamani, P.A., Laksamana, H.C., Sandeepkumar, K. and Hosamani, R.C., 2011. Antimicrobial activity of leaf extract of *Andrographis paniculata* Wall. *Science Research Reporter*, 1(2), pp. 92-95.

Hossain, M.S., Urbi, Z., Sule, A. and Hafizur, M., 2014. *Andrographis paniculata* (Burm.f) Wall. Ex Nees: A review of ethnobotany, phytochemistry, and pharmacology. *The Scientific World Journal*, 2014, pp. 1-28.

Huang, Y.H., Chang, H.C. and Chang, T.C., 2001. Development of a latex agglutination test for rapid identification of *Escherichia coli*. *European Journal of Clinical Microbiology and Infectious Diseases*, 20(2), pp. 97-103.

Huidrom, S. and Deka, M., 2012. Determination of antioxidant property of *Andrographis paniculata*. *Indian Journal of Drugs and Diseases*, 1(1), pp. 12-17.

Hussain, A.Z. and Kumaresan, S., 2013. Bactericidal property of *Andrographis paniculata* and *aloe vera* extracts on *Salmonella typhi* species. *Asian Journal of Plant Science and Research*, 3(6), pp. 50-52.

Isha, A., Suyut, M.A., Yusof, N.A.T., Mastuki, S.N. and Ismail, I.S., 2013. Optimization of extraction method and qualitative FT-NMR analysis of *Andrographis paniculata. International Journal of Food Science & Technology*, 3(4), pp. 1-10.

Ivanova, D., Gerova, D., Chervenkov, T., Yankova, T., 2005. Polyphenols and antioxidant capacity of Bulgarian medicinal plants. *Journal of Ethnopharmacology*, 96, pp. 145–150.

Jain, P., Bhuiyan, M.H., Hossain, K.R. and Bachar, S.C., 2011. Antibacterial and antioxidant activities of local seeded banana fruits. *African Journal of Pharmacy and Pharmacology*, 5(13), pp. 1398-1403.

Jayakumar, T., Hsieh, C.Y., Lee, J.J. and Sheu, J.P., 2013. Experimental and clinical pharmacology of *Andrographis paniculata* its major bioactive phytoconstituent andrographolide. *Evidence-Based Complementary and Alternative Medicine*, pp. 1-12.

Jayashree, D., 2013. Phytochemicals analysis and TLC fingerprinting of methanolic extracts of three medicinal plants. *International Research Journal of Pharmacy*, 4(6), pp. 123-126.

Joselin, J. and Jeeva, S., 2014. *Andrographis paniculata*: A review of its traditional uses, phytochemistry and pharmacology. *Medicinal and Aromatic Plants*, 3(4), pp. 1-15.

Joshua, M. and Takudzwa, M., 2013. Antibacterial properties of *Mangifera indica* on *Staphylococcus aureus*. *African Journal of Clinical and Experimental Microbiology,* 14(2), pp. 62-74.

Kaskoos, R.A. and Ahamad, J., 2014. Evaluation of pharmacognostic features of aerial parts of *Andrographis paniculata* Wall. *Journal of Pharmacognosy and Phytochemistry*, 3(1), pp. 1-5.

Katalinic, V., Milos, M., Kulisic, T. and Jukic, M., 2006. Screening of 70 medicinal plant extracts for antioxidant capacity and total phenols. *Food Chemistry*, 94, pp. 550-557.

Kataky, A. and Handique, P. J., 2010. Antimicrobial activity and phytochemical estimation of micropropagated *Andrographis paniculata* (Burm.f) NEES. *Asian Journal of Science and Technology*, 5, pp. 91-94.

Cui, K., Luo, X. L., Xu, K.Y. and Ven Murthy, M.R., 2004. Role of oxidative stress in neurodegeneration: recent developments in assay methods for oxidative stress and nutraceutical antioxidants. *Progress in neuropsychopharmacology and biological psychiatry*, 28 (5), pp. 771-799.

Kumar, A., Dora, J., Singh, A., Tripathi, R., 2012. A review on king of bitter (Kalmegh). *International journal of research in pharmacy and chemistry*, 2(1), pp.116- 124.

Kumoro, A.C., Hasan, M. and Singh, H., 2009. Effects of solvent properties on the Soxhlet extraction of diterpenoid lactones from *Andrographis paniculata* leaves. *Science Asia*, 35, pp. 306-309.

Lalli, J.Y.Y., 2006. *In vitro pharmacological properties and composition of leaf essential oils and extracts of selected Indigenous pelargonium (geraniaceae) species.* MSc. University of the Witwatersrand, Johannesburg. Available at: <http://wiredspace.wits.ac.za/handle/10539/1690> [Accessed 14 March 2015].

Malahubban, M., Alimon, A.R., Sazili, A.Q., Fakurazi, S. and Zakry, F.A. Phytochemical analysis of *Andrographis paniculata* and *Orthosiphon stamineus* leaf extracts for their antibacterial and antioxidant potential. *Tropical Biomedicine*, 30(3), pp. 467-480.

Mishra, P. K., Singh, R.K., Gupta, A., Chaturvedi, A., Pandey, R., Tiwari, S.P. and Mohapatra, T.M., 2013. Antibacterial activity of *Andrographis paniculata* (Burm.f.) Wall ex Nees leaves against clinical pathogens. *International Journal of Pharmacy and Pharmaceutical Sciences*, 3(3), pp. 459-462.

Mohamed Sham Shihabudeen, H., Hansi Priscilla, D. and Thirumurugan, K., 2010. Antimicrobial activity and phytochemical analysis of selected Indian folk medicinal plants. *International Journal of Pharma Sciences and Research*, 1(10), pp. 430-434.

Mohan, M., Khanam, S. and Shivananda, B.G., 2013. Optimization of microwave assisted extraction of Andrographolide from *Andrographis paniculata* and its comparison with refluxation extraction method. *Journal of Pharmacognosy and Phytochemistry*, 2(1), pp. 342-348.

Mojab, F., Kamalinejad, M., Ghaderi, N. and Vahidipour, H.R., 2003. Phytochemical screening of some species of Iranian plants. *Iranian Journal of Pharmaceutical Research*, 2, pp. 77-82.

Mulukuri, N.V.L.S., Mondal, N.B., Prasad, M.R., Renuka, S. and Ramakrishna. K., 2011. Isolation of diterpenoid lactones from the leaves of *Andrographis paniculata* and its anticancer activity. *International Journal of Pharmacognosy and Phytochemical Research*, 3(3), pp. 39-42.

Nayak, D., 2012. *Antibacterial, antioxidant and phytochemical screening of Hibiscus rosa sinensis, Acorus calamus, Cucurbita maxiam and Moringa oliefera*. MSc. National Institute of Technology, Rourkela.

Nice, K.J., 2012. *Antimicrobial screening of secondary metabolites from Solanaceae*. PhD. Royal Holloway University of London.

Nikaido, H., 2003. Molecular basic of bacterial outer membrane permeability revisited. *Microbiology and Molecular Biology Reviews*, 67(4), pp. 593-656.

Niranjan, A, Tewari, S.K. and Lehri, A., 2010. Biological activities of *Kalmegh (Andrographis paniculata* Nees) and its active principles- a review. *Indian Journal of Natural Products and Resources*, 1(2), pp. 125-135.

Nur Syukriah, A. R., Liza, M. S., Harisun, Y. and Fadzillah, A. A. M., 2014. Effect of solvent extraction on antioxidant and antibacterial activities from *Quercus infectoria* (Manjakani). *International Food Research Journal*, 21(3), pp. 1067-1073.

Odugbemi, T., 2008. *A textbook of medicinal plants from Nigeria*. [e-book] Nigeria: Tolu Odugbemi. Available at: Google Books <books.google.com> [Accessed 16 March 2015].

Okhuarobo, A., Falodun, J.E., Erharuyi, O., Imieje, V., Falodun, A. and Langer, P., 2014. Harnessing the medicinal properties of *Andrographis paniculata* for diseases and beyond: a review of its phytochemistry and pharmacology. *Asian Pacific Journal of Tropical Disease*, 4(3), pp. 213-222.

Otto, M., 2009. *Staphylococcus epidermidis* – the "accidental" pathogen. *Nature Reviews Microbiology*, 7(8), pp. 555–567.

Pande, J.N., Biswas, M., Ghosh, L.K. and Gupta, B.K., 2011. Thin layer chromatographic studies and evaluation of analgesic activity of *Andrographis paniculata* leaf extracts in mice. *Pharmacologyonline*, 2, pp. 391-396.

Pathmanathan, M. K., Uthayarasa, K., Jeyadevan, J.P. and Jeyaseelan, E.C., 2010. *In vitro* antibacterial activity and phytochemical analysis of some selected medicinal plants. *International Journal of Pharmaceutical and Biological Archives*, 1(3), pp. 291 – 299.

Pathak, D., Alam, K., Rohilla, H., Rai, A.K. and Argawal, A., 2012. Phytochemical investigation of *Boerhavia diffusa* and *Andrographis paniculata*: a comparative study. *International Journal of Pharmacy and Pharmaceutical Sciences*, 4(4), pp. 250-251.

Peteros, N.P. and Uy, M.M., 2010. Antioxidant and cytotoxic activities and phytochemical screening of four Philippine medicinal plants. *Journal of Medicinal Plants Research*, 4(5), pp. 407-414.

Pieters, L. and Vlietinck, A.J., 2005. Bioguided isolation of pharmacologically active plant components, still a valuable strategy for the finding of new lead compounds? *Journal of Ethnopharmacology*, 100, pp. 57-6.

Pinel, M.L.M., Nunez, M.J. and Nicoli, M.C., 2004. Solvent effect on quercetin antioxidant capacity. *Food Chemistry*, 88, pp. 201-207.

Premanath, R. and Devi, N.L., 2011. Antibacterial, antifungal and antioxidant activities of *Andrographis panaiculata* Nees leaves. *International Journal of Pharmaceutical Sciences and Research*, 2(8), pp. 2091-2099.

Poolsup, C., Suthisisang, C., Prathanturarug, S., Asawamekun, A. and Chanchareon, U., 2004. *Andrographis paniculata* in the symptomatic treatment of uncomplicated upper respiratory tract infection: systematic review of randomized controlled trials. *Journal of Clinical Pharmacy and Therapeutics*, 29, pp. 37-45.

Poongothai, A., Sreena, K.P., Sreejith, K., Uthiralingam, M. and Annapoorani, S., 2011. Preliminary phytochemicals screening of *Ficus Racemosa Linn* bark. *International Journal of Pharma and Bio Sciences*, 2(2), pp. 431-434.

Punopas, K., 2002. *The study of antibacterial activity of some medicinal plants in Lamiaceae family*. MSc. Suranaree University of Technology.

Raetz, C. R. H. and Whitfield, C., 2002. Lipopolysaccharide endotoxins. *Annual Review Biochem*istry, 71, pp. 635-700.

Radha, R., Sermakkani, M. and Thangapandian, V., 2011. Evaluation of phytochemical and antimicrobial activity of *Andrographis paniculata* Nees (Acanthaceae) aerial parts. *International Journal of Pharmacy and Life Sciences*, 2(2), pp. 562-567.

Rajkumar, V., Guha, G. and Ashok Kumar, R., 2010. Evaluation of antioxidant activities of *Bergenia ciliate* rhizome. *Journal of Natural Remedies*, 4(1), pp. 38-48.

Ranpal, S., 2009. *An assessment of sStatus and antibacterial properties of Dactylorhiza hatagirea in Annapurna conservation area*. BSc. Forestry of Tribhuvan University.

Ross, R.N., 1999. Atherosclerosis--an inflammatory disease. *The New England Journal of Medicine*, 340(2), pp.115-126.

Roy, S., Rao, L., Bhuvaneswari, C., Giri, A. and Mangamoori, L. N., 2010. Phytochemical analysis of *Andrographis paniculata* extract and its antimicrobial activity. *World Journal of Microbiology and Biotechnology*, 26, pp. 85-91.

Rupp, M.E. and Archer GL., 1994. Coagulase-negative staphylococci: pathogens associated with medical progress. *Clinical Infectious Disease*, 19, pp, 231-245.

Rupp, M.E., Paul, D.F., Heilmann, C. and Gotz, F., 2001. Characterization of the importance of *Staphylococcus epidermidis* autolysin and polysaccharide intercellular adhesin in the pathogenesis of intravascular catheter– associated infection in a rat model. *The Journal of Infectious Diseases*, 183, pp, 1038-1042.

Ryan, K. J. and Ray, C.G., 2010. *Sherris: Medical Microbiology*, 5th ed. USA: McGraw Hill.

Sahalan, A.Z., Sulaiman, N., Mohammed, N., Ambia, K.M. and Lian, H. H., 2007. Antibacterial activity of *Andrographis paniculata* and *Euphorbia hirta* methanol extracts. *Jurnal Sains Kesihatan Malaysia*, 5(2), pp. 1-8.

Sahoo, R.R., 2012. *Antimicrobial and antioxidant efficacy of Ficus religiosa L. & Ficus benghalensis L. plant*. MSc. National Institute of Technology, Rourkela.

Salna, K.P., Sreejith, K., Uthiralingam, M., Mithu, A. P., John, M.M.C. and Albin, T.F., 2011. A comparative study of phytochemicals investigation of *Andrographis paniculata* and *Murraya koenigii*. *International Journal of Pharmacy and Pharmaceutical Sciences*, 3(3), pp. 291-292.

Sareer, O., Ahad, H. and Umar, S., 2012. Prophylactic and lenitive effects of *Andrographis paniculata* against common human ailments: An exhaustive and comprehensive reappraisal. *Journal of Pharmaceutical Research and Opinion*, 2(10), pp. 138-162.

Shad, M.A., Nawaz, H., Rehman, T. and Ikram, N., 2013. Determination of some biochemical, phytochemicals and antioxidant properties of *Cichorium intybus L*: a comparative study. *The Journal of Animal & Plant Sciences*, 23(4), pp. 1060-1066.

Shalaby, E.A. and Shanab, M.M., 2013. Antioxidant compounds, assays of determination and mode of action. *African Journal of Pharmacy and Pharmacology*. 7(10), pp. 528-539.

Sharma, M. and Joshi, S., 2011. Comparison of anti-oxidant activity of *Andrographis paniculata* and *Tinospora cordifolia* leaves. *Journal of Current Chemical & Pharmaceutical Sciences*, 1(1), pp. 1-8.

Sharma, M., Sharma, R.G., Rawat, R.D. and Sharma, N., 2011. Evaluation of phytochemical and antibacterial activity of hot and cold methanolic extract of leaves and whole plant of *Andrographis paniculata*. *International Journal of Chemistry Science*, 9(3), pp. 960-968.

Sharma, S.K., Singh, L. and Singh, S., 2013. A review on medicinal plants having antioxidant potential. *Indian Journal of Research in Pharmacy and Biotechnology*, 1(3), pp. 404-409.

Sharma, V. and Paliwal, R., 2013. Preliminary phytochemical investigation and thin layer chromatography profiling of sequential extracts of *Moringa oleifera* pods. *International Journal of Green Pharmacy*, 7(1), pp. 41-45.

Shelton, R.M., 1991. *Aloe vera*: its chemical and therapeutic properties. *International Journal of Dermatology*, 30, pp. 679-683.

Shimada, T., 2006. Salivary proteins as a defense against dietary tannins. *Journal of Chemical Ecology*, 32(6), pp. 1149-1163.

Shuddhalwar, P.P., Charde, V.N. and Patil, S.P., 2014. Study of phytochemicals in non- polar and polar solvent extracts of root, bark and leaf of *Limonia Acidissima* and their antibacterial efficacy. *International Journal of Researches in Biosciences, Agriculture and Technology*, pp. 217-225.

Sibanda, T. and Okoh, A.I., 2008. *In vitro* antibacterial regimes of crude aqueous and acetone extract of *Garcinia kola* seeds. *Journal of Biology Science*, 8(1), pp. 149-154.

Singh, D., 2014. *Advances in plant biopesticides*. [e-book] London: Springer. Available at: Google Books <books.google.com> [Accessed 28 February 2015].

Singh, J., Sing, S., Pratap, T. and Arya, V.K., 2012. Phytochemical analysis and antifungal activity of *Andrographis paniculata*. *Asian Journal of Pharmacy and Medical Science*, 2(3), pp. 45-47.

Sivakrishnan, S. and Kottaimuthu, A., 2014. Phytochemical evaluation of ethanolic extract of aerial parts of *Albizia procera*. *British Biomedical Bulletin*, 2(1), pp. 235-241.

Sivananthan, M. and Elamaran, M., 2013. Medicinal and pharmacological properties of *Andrographis paniculata*. *International Journal of Biomolecules and Biomedicine*, 3(2), pp. 1-12.

Sule, A., Ahmed, Q, U., Samah, O. A. and Omar, M.N., 2011. Bacteriostatic and bactericidal activities of *Andrographis paniculata* on skin disease causing pathogenic bacteria. *Journal of Medicinal Plants Research*, 5(1), pp. 7-14.

Suneetha, G. and Ravi, V., 2012. Antimicrobial activity of *Andrographis paniculata* flower extracts. *International Journal of Research and Reviews in Pharmacy and Applied science*, 2(3), pp. 604-610.

Tanwer, B.S. and Vijayvergia, R., 2010. Phytochemical evaluation and molluscicidal activity of *Andrographis paniculata*. *Herba Polonica*, 56(4), pp. 72-75.

Tekwu, E.M., Pieme, A.C. and Beng, V.P., 2012. Investigations of antimicrobial activity of some Cameroonian medicinal plant extracts against bacteria and yeast with gastrointestinal relevance. *Journal of Ethnopharmacology*, 142, pp. 265-273.

Tiwari, P., Kumar, B., Kaur, M., Kaur, G. and Kaur, H., 2011. Phytochemical screening and extraction: a review. *An International Peer Reviewed Journal*, 1(1), pp. 98-106.

Todar, K., 2008a. *Todar's Online Textbook of Bacteriology*. [online] Available at: < http://textbookofbacteriology.net/staph.html> [Accessed 22 March 2015].

Todar, K., 2012b. *Pathogenic E. coli*. [Online] Available at: <http://www.textbookofbacteriology.net/e.coli.html> [Accessed 23 March 2015].

Trouillas, P., Calliste, C.A., Allais, D.P., Simon, A.,Marfak, A., Delage, C. and Duroux, J.L., 2003. Antioxidant, anti-inflammatory and antiproliferative properties of sixteen water plant extracts used in the Limousin countryside as herbal teas. *Food Chemistry*, 80(3), pp. 399- 407.

Upadhyayula, S., Kambalapalli, M. and Asmar, B.I., 2012. *Staphylococcus epidermidis* urinary tract infection in an infant. *Case Reports in Infectious Diseases*, 2012, pp. 1-2.

Ventakesh, R. and Sood, D., 2011. *A review of the physiological implications of antioxidant in food.* BSc. Worcester Polytechnic Institute.

Vicas, S., Prokisch, J., Rugina, D. and Socaciu, C., 2009. Hydrophilic and lipophilic antioxidant activities of Mistletoe (Viscum album) as determined by FRAP method. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, 37(2), pp. 112-116.

Wadood, A., Ghufran, M., Jamal, S.B., Naeem, M., Khan, A., Ghaffar, R. and Asnad., 2013. Phytochemical analysis of medicinal plants occurring in local area of Mardan. *Biochemistry and Analytic Biochemistry*, 2(144), pp. 1-4.

Wink, M., 2013. *An introduction to molecular biotechnology: fundamentals, methods and applications*. [e-book] United States: John Wiley & Sons. Available at: Google Books <books.google.com> [Accessed 28 February 2015].

Wongkittipong, R., Prat, L., Damronglerd, S. and Gourdon, C., 2004. Solid-liquid extraction of andrographolide from plants- experimental study, kinetic reaction and model. *Separation and Purification Technology*, 40 (2), pp. 147-154.

World Health Organization (WHO), 2011, *Enterohaemorrhagic Escherichia coli (EHEC)*. [Online] Available at: http://www.who.int/mediacentre/Factsheets /fs125/en/ [Accessed: 22 March 2015].

Xu, Y., Raymond, L., Marshall. and Mukkur, T. K. S., 2006. An investigation on the antimicrobial activity of *Andrographis paniculata* extracts and andrographolide *in vitro*. *Asian Journal of Plant Sciences*, 5(3), pp. 527-530.

Yadav, R.N.S. and Agarwala, M., 2011. Phytochemical analysis of some medicinal plants. *Journal of Phytology*, 3(12), pp. 10-14.

Yong, Y.K., 2007. *Determination of synthetic phenolic antioxidants in food items using HPLC and total antioxidants using FIA approaches*. MSc. University Sains Malaysia. Available at: <http://eprints.usm.my/8904/> [Accessed 22 March 2015].

Zaidan, M.R., R.A. Noor, A.R. Badrul, Adlin, A., Norazah, A. and Zakiah, I. 2005. *In vitro* screening of five local medicinal plants for antibacterial activity using disc diffusion method. *Tropical Biomedicine*, 22(2), pp. 165-170.

APPENDIX A

EQUIPMENTS, APPARATUS, CHEMICALS AND CONSUMABLES

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

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

Table A2: List of chemicals and consumables used in this project with their respective manufacturer.

Consumables	**Manufacturer**
n-Hexane	Qrec Asia Sdn Bhd, Malaysia
Chloroform	Qrec Asia Sdn Bhd, Malaysia
Ethyl acetate	Qrec Asia Sdn Bhd, Malaysia
Methanol	Qrec Asia Sdn Bhd, Malaysia
100% DMSO	Fisher Scientific, Malaysia
Acetone	Qrec Asia Sdn Bhd, Malaysia
Benzene	Merck KGaA, Germany
Toluene	Merck KGaA, Germany
Luria Bertani agar	CONDA, Spain
Nutrient agar	Merck KGaA, Germany
Mueller Hinton agar	Oxoid, England
Mueller Hinton broth	Becton Dickinson and company, USA
D-glucosa	SYSTERM, Malaysia
Sodium chloride	Merck KGaA, Germany
Sodium hydroxide	Merck KGaA, Germany
Phenol red	Nacalai tesque, Japan
Ampicillin	Bio Basic, Canada
95% sulphuric acid	Merck KGaA, Germany
37% hydrochloric acid	Qrec Asia Sdn Bhd, Malaysia
Acetic acid glacial	Qrec Asia Sdn Bhd, Malaysia
29% Ammonia	Merck KGaA, Germany
Dragendorff's reagent	VWR International, Ireland
Ferric chloride	R&M Chemicals, Malaysia
Fehling solution A	GENE Chemical
Fehling solution B	Labchem Sdn Bhd, Malaysia
Quercetin	Alfa Aesar, England
DPPH	Sigma- Aldrich, USA
Portable Bunsen burner	CAMPINGAZ C206, Czech Republic
Micropipette tips	Axygen, USA
96 well microplates	TPP, Switzerland

Centrifuge tube	Falcon, USA
Microcentrifuge tube	Axygen, USA
Sterile Cotton swab	Premier Diagnostics, Malaysia
Membrane filter, 0.45 µm	TPP, Switzerland
Syringe 10ml	Cellotron, Malaysia
Silica Gel Plates	Merck, USA

APPENDIX B

PREPARATION OF MEDIUM

The methods for prepare the NA, LB agar, MH agar, MH broth, 0.85 % saline water, 10 % glucose and 0.5 % phenol red are as follows:

Nutrient Agar

The NA was prepared by 20 g of nutrient agar was dissolved in 1 L of distilled water. The mixture was autoclaved at 121 ℃ for 10 minutes. The melted agar was let cool and poured into sterile petri dish. The agar was let cool and kept at room temperature for one day to seek for any contamination.

Luria Bertani Agar

The LB agar was prepared by dissolved 35 g of LB agar powder in 1 L of distilled water and adjusted the pH to 7.00 ± 0.20. Then, sterilized in an autoclave at 121 ℃ for 15 minutes before used. The sterile LB medium were cooled to 45-50 ℃, mixed well and poured into plate. The agar was let cool and kept at room temperature for one day to seek for any contamination.

Mueller Hinton Agar

The MH Agar was prepared by dissolved 34 g of the MH agar powder in 1 L of deionized water the pH is adjusted to 7.30 ± 0.10. The solution was then autoclaved at 121 ℃ for 15 minutes and poured onto the petri dish. The agar was let cool and kept at room temperature for one day to seek for any contamination.

Mueller Hinton Broth

The MH broth was prepared by suspended 21 g of the Mueller Hinton broth powder in 1 L of purified water and the pH is adjusted to 7.30 ± 0.10. The mixture was then sterilized by autoclaving at 121 ℃ for 15 minutes.

0.85 % Saline Water

The 0.85 % of saline water was prepared by dissolved 0.85 g of sodium chloride in 100 ml of distilled water and autoclaved at 121 ℃ for 15 minutes.

10 % glucose solution

The 10 % glucose solution was prepared by dissolved 1 g of the D-glucosa powder in 10 ml of distilled water. The 10 % glucose solution was then sterile filtered using 0.45 μm membrane filter.

0.5 % phenol red

The 0.5 % phenol red was prepared by dissolved 0.005 g of the phenol red powder in 1 ml of distilled water.

APPENDIX C

QUALITATIVE ANALYSIS OF PHYTOCHEMICALS CONTENT IN *A. paniculata* EXTRACTS PREPARED IN DIFFERENT SOLVENTS.

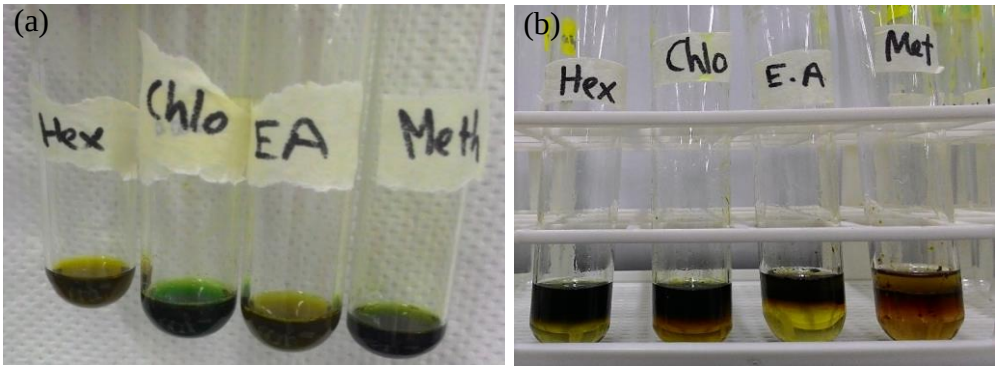


Figure C1: Terpenoids test (a) before, (b) after.

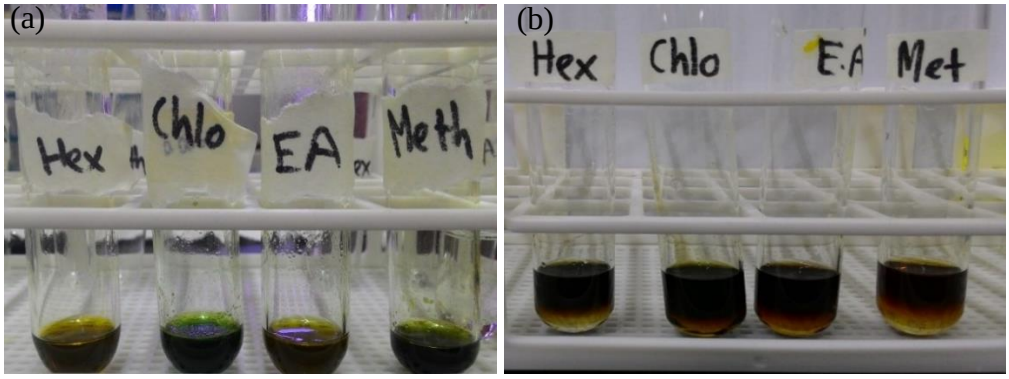


Figure C2: Cardiac glycosides test (a) before, (b) after.

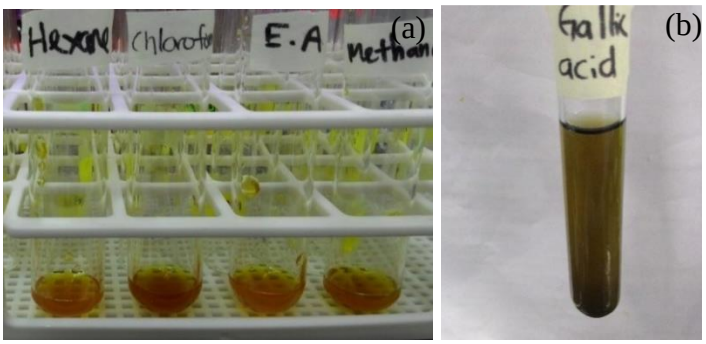


Figure C3: Phenols test (a) solvent extracts (b) positive control

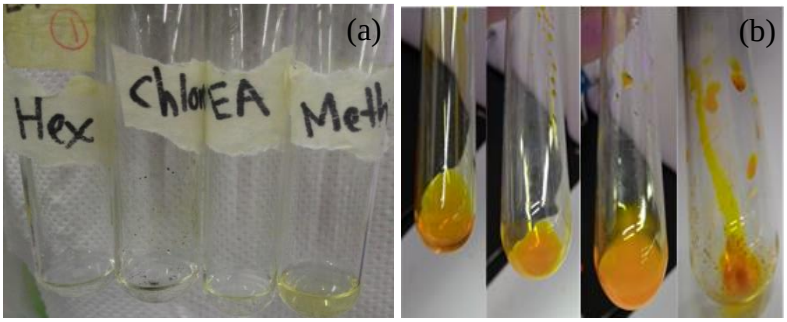


Figure C4: Alkaloids test (a) before, (b) after.

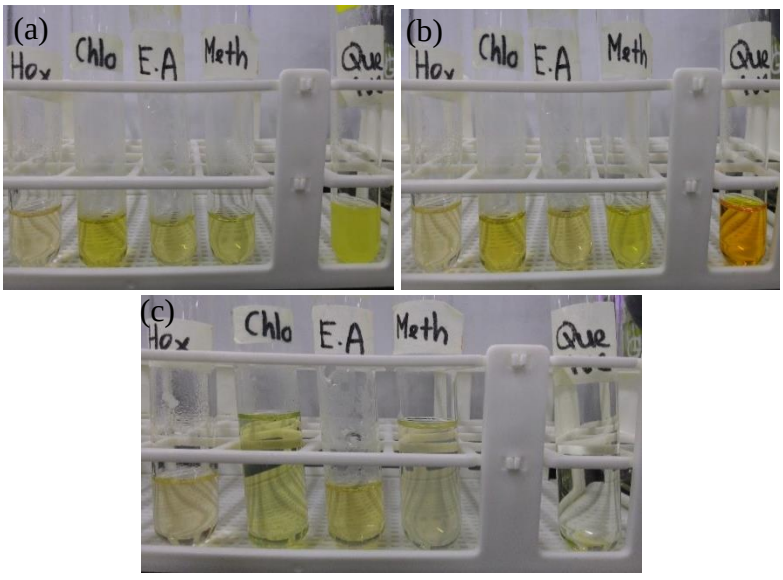


Figure C5: Flavonoids test (a) before (b) after add NaOH, (c) after add 1% HCl.

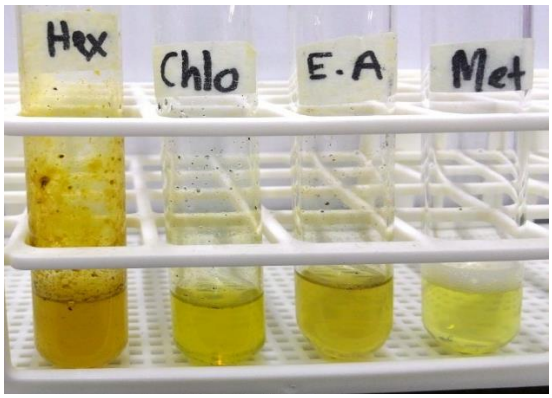


Figure C6: Saponins test.

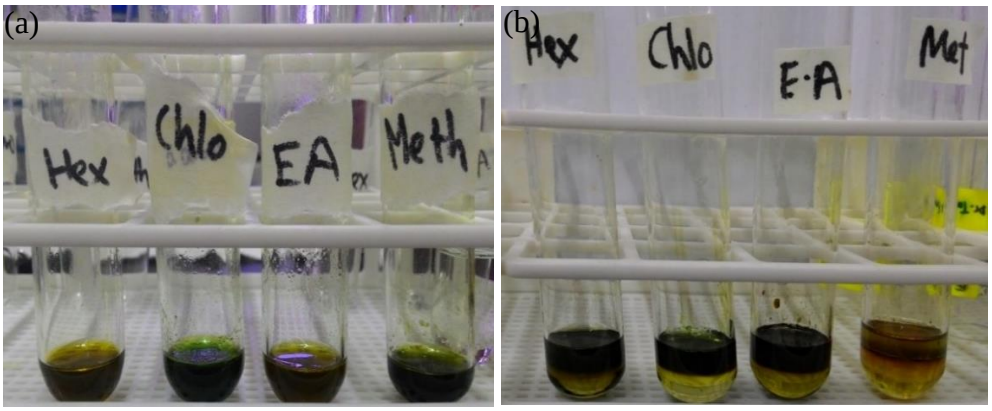


Figure C7: Steroids test (a) before, (b) after.

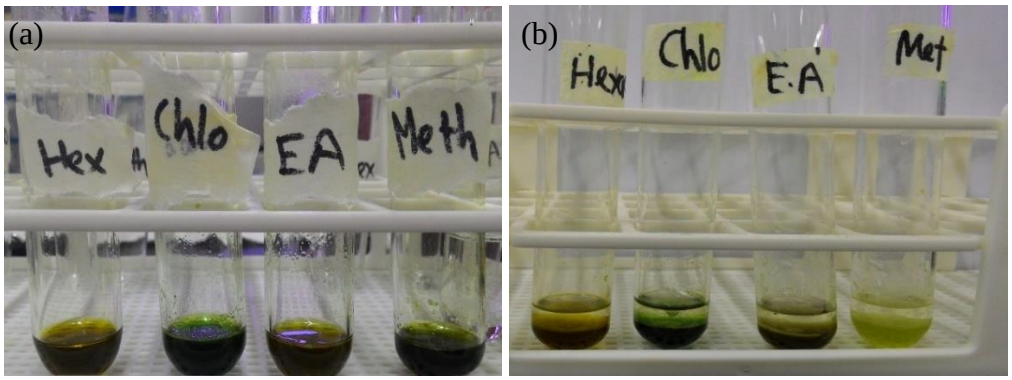


Figure C8: Anthtraquinones test (a) before, (b) after.

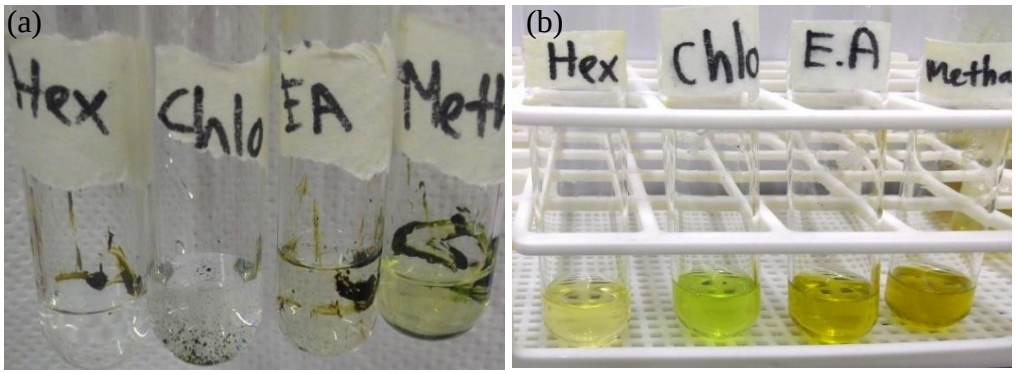


Figure C9: Reducing sugars screening test a) before, (b) after.

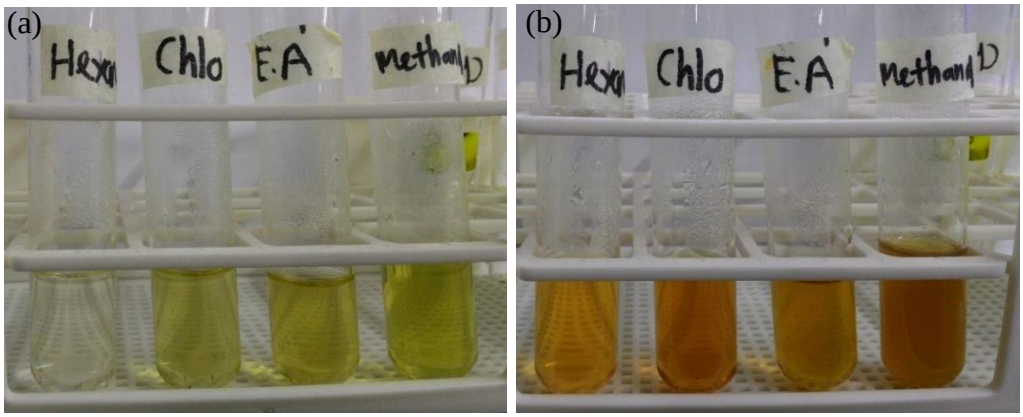


Figure C10: Tannins screening test a) before, (b) after.

Hex- Hexane extract

Chlo- Chloroform extract

EA- Ethyl acetate extract

Met- Methanol extract

APPENDIX D

ANTIBACTERIAL ACTIVITY

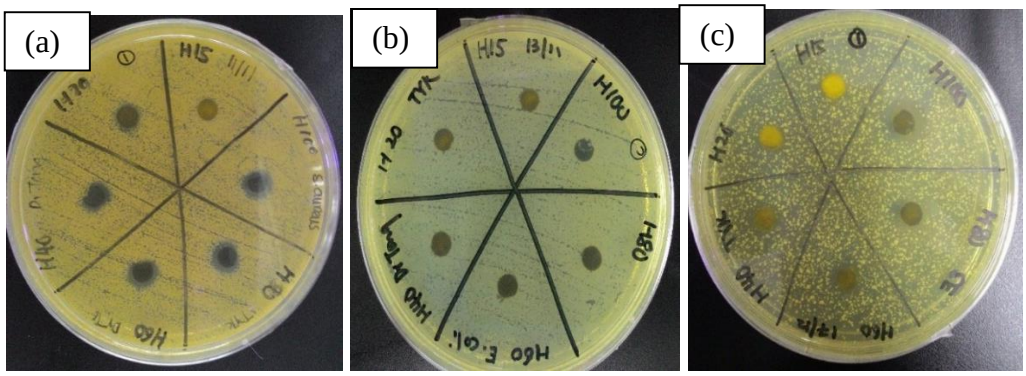


Figure D1: Zone of inhibition exhibited by hexane extract against (a) *S. aureus*, (b) *S. epidermidis*, (c) *E. coli*.

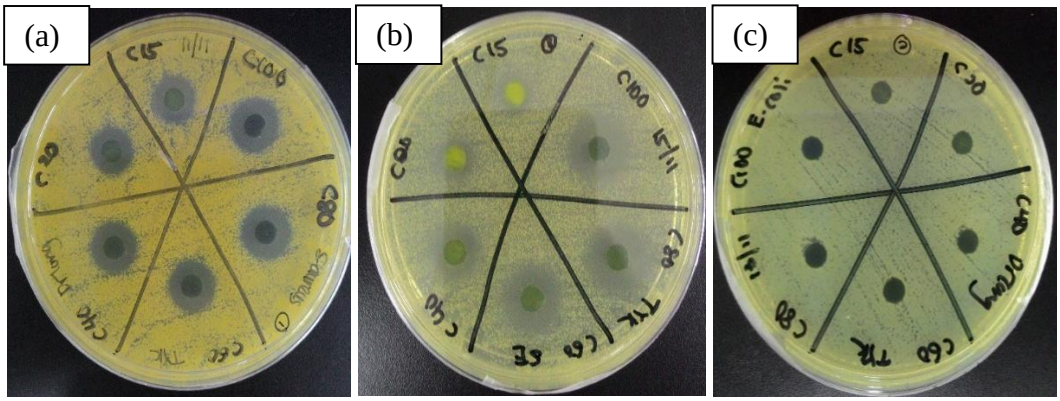


Figure D2: Zone of inhibition exhibited by chloroform extract against (a) *S. aureus*, (b) *S. epidermidis*, (c) *E. coli*.

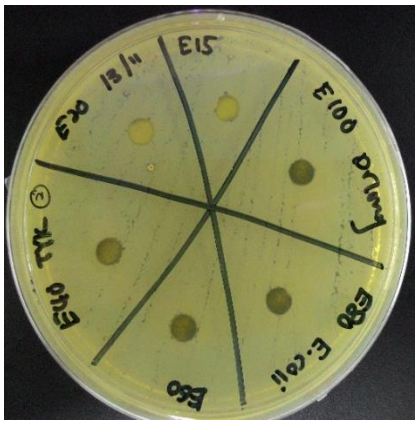


Figure D3: Zone of inhibition exhibited by ethyl acetate extract against *E. coli*.

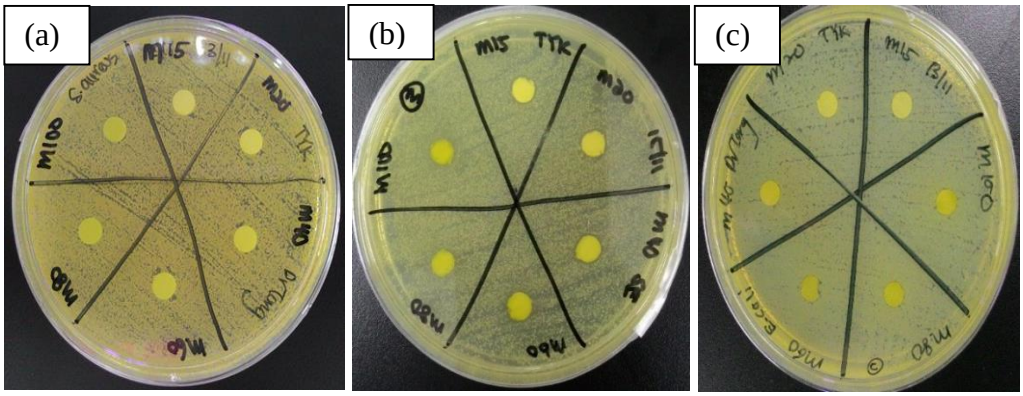


Figure D4: Zone of inhibition exhibited by methanol extract against (a) *S. aureus*, (b) *S. epidermidis*, (c) *E. coli*.

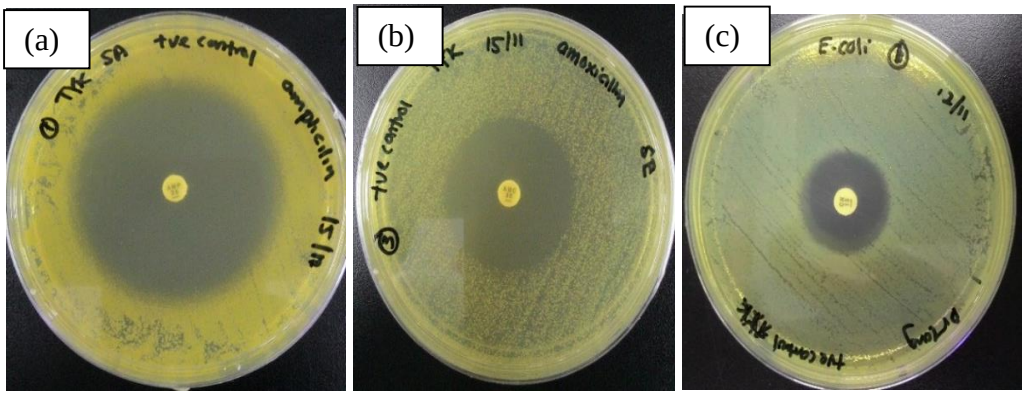


Figure D5: Zone of inhibition exhibited by positive control (a) ampicillin against *S. aureus*, (b) amoxicillin against *S. epidermidis*, (c) gentamicin against *E. coli*.

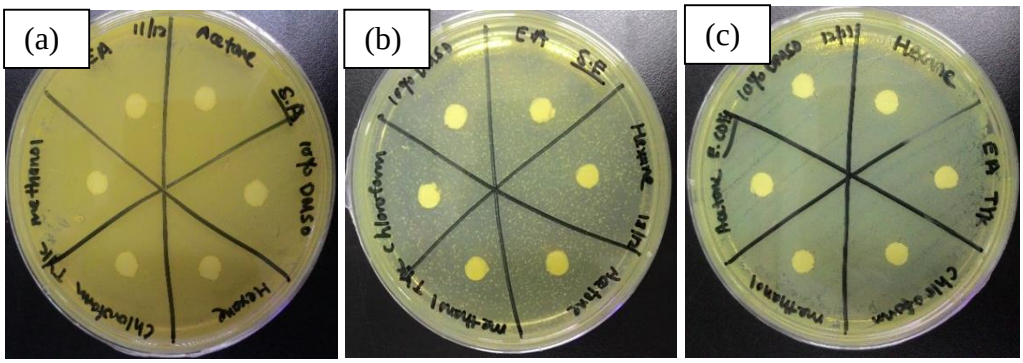


Figure D6: Zone of inhibition exhibited by acetone, hexane, ethyl acetate, chloroform, methanol, 10% DMSO against (a) *S. aureus*, (b) *S. epidermidis*, (c) *E. coli*.

Figure D7: The inhibitory activity of ethyl acetate and chloroform extracts against *S. epidermidis* on 96 well plate.

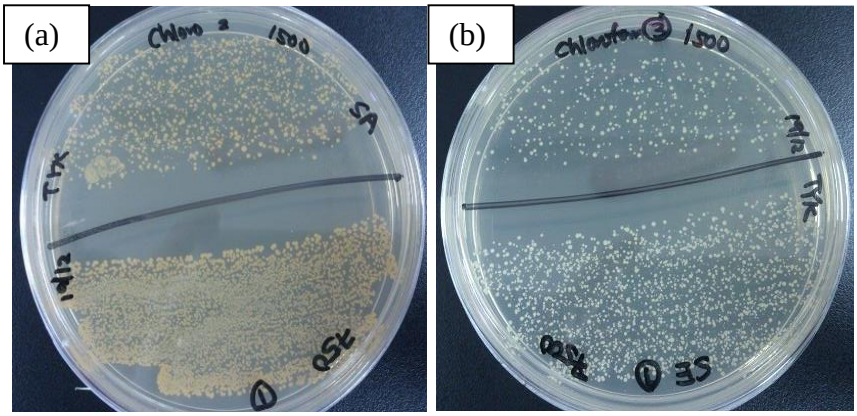


Figure D8: The MBC of chloroform extract against (a) *S. aureus*, (b) *S. epidermidis*.

APPENDIX E

THIN LAYER CHROMATOGRAPHY

Table E1: The R_f values of hexane, chloroform, ethyl acetate and methanol extracts.

R_f values			
Hexane	Chloroform	Ethyl acetate	Methanol
0.10 ± 0.01	0.27 ± 0.05	0.26 ± 0.05	0.26 ± 0.01
0.32 ± 0.03	0.61 ± 0.04	0.46 ± 0.02	0.40 ± 0.03
0.45 ± 0.01	0.94 ± 0.02	0.74 ± 0.01	0.51 ± 0.05
0.53 ± 0.01		0.87 ± 0 .02	0.68 ± 0.04
0.59 ± 0.01		0.94 ± 0.01	0.82 ± 0.02
0.69 ± 0.02		0.97 ± 0.01	0.95 ± 0.02
0.76 ± 0.02			
0.82 ± 0.01			
0.86 ± 0.01			
0.98 ± 0.01			