



UNIVERSITI PUTRA MALAYSIA

***EVALUATION OF ANTIMICROBIAL ACTIVITY OF SELECTED
MALAYSIAN HERBS ON GRAM POSITIVE AND GRAM NEGATIVE
BACTERIA***

**BY
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EVALUATION OF ANTIMICROBIAL ACTIVITY OF SELECTED MALAYSIAN HERBS ON GRAM POSITIVE AND GRAM NEGATIVE BACTERIA

MOHAMAD LUTFI BIN ZAKARIA

ABSTRACT

Malaysia is rich in biodiversity encompassing a variety of herbs and scrubs with potential medicinal properties. *Tinospora Crispa* (Patawali), *Anacardium Occidentale* (Gajus), *Garcinia Atroviridis* (Asam gelugor) and *Hibiscus Cannabinus* (Kenaf) are some of the well-known local herbs that have been studied for their medicinal applications. For examples, *Tinospora Crispa* has been shown to inhibit *Plasmodium falciparum* (Abdul Rahman *et al.*, 1999). The acidic nature of *Garcinia Atroviridis* and *Anacardium Occidentale* has also been associated with antimicrobial and weight reducing properties (Mackeen *et al.*, 2000; Kudi 1999). Another study also indicated *Hibiscus Cannabinus* of leaf extract can also kill earthworms better than the Standard Drug, Albendazole. (Kota Chaitanya Sravanti *et al.*, 2011). Thus, this study was extended to evaluate broad spectrum-antimicrobial effects of *Tinospora Crispa*, *Anacardium Occidentale*, *Garcinia Atroviridis* and *Hibiscus Cannabinus* against Gram positive and Gram negative bacteria. Aqueous extracts of the herbs were prepared with *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 as the test organisms representing pathogenic gram positive and negative bacteria respectively. Aqueous extracts that have been serially diluted were mixed with bacterial suspension in MHB at appropriate ratio. Upon 18h-incubation at 37°C, MIC was visually determined by taking the suspension that had no trace of bacterial growth. MBC was determined by further sub-culturing the extract-bacteria-suspension onto MHA. Disc diffusion method utilized the MBC of the respective extract using 5 mm filter disc. The MIC and MBC of the respective extract for both test organisms were similar; 227.27 mg/mL for *Tinospora Crispa* and *Anacardium Occidentale*, 113.64 mg/ml for *Hibiscus Cannabinus*, and 56.82 mg/mL for *Garcinia Atroviridis*. In disc diffusion method, all extracts showed a similar inhibition zone ~ (8.67-9.67) on both test organisms. All extracts showed antibacterial activities but *Garcinia Atroviridis* could be more efficient due to its lower MBC followed by *Hibiscus Cannabinus* and others. This was supported by disc diffusion's result that although the extract's concentration used to soak the disc differed accordingly for each extract, all showed a similar size of inhibition zone. Lastly, the effect of all extracts may be bactericidal as their respective MIC and MBC are equal.

PENILAIAN AKTIVITI ANTIMIKROB OLEH HERBA-HERBA MALAYSIA
TERPILIH KE ATAS BAKTERIA GRAM POSITIF DAN GRAM NEGATIF

MOHAMAD LUTFI BIN ZAKARIA

ABSTRAK

Malaysia kaya dengan kepelbagaian biodiversiti meliputi pelbagai tumbuhan dengan khasiat perubatan yang berpotensi tinggi. *Tinospora Crispa* (Patawali), *Anacardium Occidentale* (Gajus), *Garcinia Atroviridis* (Asam gelugor) dan *Hibiscus Cannabinus* (kenaf) adalah beberapa tumbuhan tempatan terkenal yang telah dipelajari sebagai aplikasi perubatan. Sebagai contoh, *Tinospora Crispa* telah terbukti untuk mencegah *Plasmodium falciparum* (Abdul Rahman et al, 1999.). Sifat asid *Garcinia Atroviridis* dan *Anacardium Occidentale* juga telah dikaitkan dengan antimikrob dan berkesan mengurangkan berat badan (Mackeen et al, 2000;. Kudi 1999). Kajian lain juga menunjukkan ekstrak daun *Hibiscus Cannabinus* juga boleh membunuh cacing tanah lebih baik daripada ubat Standard, Albendazole. (Kota Chaitanya Sravanti et al, 2011.). Dengan demikian, kajian ini telah diperincikan untuk menilai kesan spektrum antimikrob yang luas ke atas *Tinospora Crispa*, *Anacardium Occidentale*, *Garcinia Atroviridis* dan *Hibiscus Cannabinus* terhadap bakteria Gram positif dan Gram negatif. Ekstrak tumbuhan berbentuk akueus diuji ke atas *Staphylococcus aureus* ATCC 25923 dan *Escherichia coli* ATCC 25922 sebagai organisma patogen yang merupakan bakteria gram positif dan negatif masing-masing. Ekstrak akueus yang telah dicairkan, dicampur dengan suspensi bakteria dalam MHB pada nisbah yang sesuai. Setelah 18 jam inkubasi pada 37°C, MIC secara visual ditentukan dengan mengambil suspensi yang tidak mempunyai pertumbuhan bakteria. MBC ditentukan dengan sub-kultur-bakteria ke atas ekstrak-suspensi dengan menggunakan MHA. Kita menggunakan kaedah 'Disc Diffusion' melalui MBC dari ekstrak masing-masing, dengan menggunakan 5 mm disc penapis. MIC dan MBC dari ekstrak masing-masing untuk kedua-dua organisma ujian adalah sama; 227.27 mg / mL untuk *Tinospora Crispa* dan *Anacardium Occidentale*, 113.64 mg / ml untuk *Hibiscus Cannabinus*, dan 56.82 mg / mL untuk *Garcinia Atroviridis*. Dalam kaedah 'Disc Diffusion', semua ekstrak menunjukkan zon terang yang sama ~ (8.67-9.67) pada kedua-dua organisma yang diuji. Semua ekstrak menunjukkan aktiviti antibakteria tetapi *Garcinia Atroviridis* didapati lebih berkesan berdasarkan kepekatan MBC yang rendah diikuti oleh *Hibiscus Cannabinus* dan lain-lain. Hal ini disokong oleh hasil difusi bahawa walaupun kepekatan ekstrak yang digunakan untuk disk berbeza sesuai untuk ekstrak masing-masing, semua menunjukkan

saiz yang sama pada zon terang. Akhir sekali, kesan dari semua ekstrak mungkin mempunyai sifat antibakteria kerana ekstrak-ekstrak itu, masing-masing mempunyai MIC dan MBC yang sama.



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Last but not least, I hope this thesis can be good source and reference in future, especially in Microbiology and Pharmacology field. I also wish student can appreciate and use this thesis as their medium on helping their project in future. Thank you.



APPROVAL

It is hereby certified that I have read this project paper entitled Evaluation of antimicrobial activity of selected Malaysian herbs on gram positive and gram negative bacteria by Mohamad Lutfi Bin Zakaria, and in my opinion it is satisfactory in term of scope, quality and presentation as a fulfillment of requirements for the course SBP 3999B.

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DECLARATION

I hereby declare that the thesis is based on my original work except for the quotations and citations, which have been duly acknowledged.

.....
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TABLE OF CONTENTS

	Page
ABSTRACT	ii
ABSTRAK	iii
ACKNOWLEDGEMENTS	v
APPROVAL	vii
DECLARATION	viii
LIST OF TABLES	xi
LIST OF FIGURES	xii
LIST OF ABBREVIATIONS	xiv
LIST OF APPENDICES	xv
CHAPTER	
1.0 INTRODUCTION	
1.1 Introduction	1
1.2 Objectives	7
2.0 LITERATURE REVIEW	
2.1 <i>Tinospora Crispa</i>	8
2.2 <i>Anacardium Occidentale</i>	9
2.3 <i>Hibiscus Cannabinus</i>	10
2.4 <i>Garcinia Atroviridis</i>	11
2.5 <i>Staphylococcus aureus</i>	13
2.6 <i>Escherichia coli</i>	14
2.7 Antibiotic Sensitivity Test	15
3.0 METHODOLOGY	
3.1 Preparation of plant extracts	17
3.2 Microorganisms and maintenance	18
3.2.1 Gram Stain Test	19
3.2.2 Mannitol Salt Agar (MSA) Test	20
3.3 Antibiotic Sensitivity Test	
3.3.1 Minimal Inhibitory Concentration	20
3.3.2 Minimal Bactericidal Concentration	21

3.3.3 Agar Disc Diffusion Method	21
4.0 RESULTS	
4.1 Minimal Inhibitory/ Bactericidal Concentration	22
4.2 Disc Diffusion Method	32
5.0 DISCUSSION	44
6.0 CONCLUSION AND RECOMMENDATIONS	47
REFERENCES	49
APPENDICES	56

LIST OF TABLES

Table		Page
4.1	MIC / MBC of Each types of Extracts for Both Test Organisms	22
4.2	Result of Statistical Analysis for Zone of Inhibition for both bacteria with 4 types of extracts.	32
4.3	Statistical Analysis using Kruskal Wallis Test for Zone of Inhibition for <i>E.coli</i> against 4 extracts of herbs.	33
4.4	Statistical Analysis using Kruskal Wallis Test for Zone of Inhibition for <i>Staphylococcus aureus</i> against 4 extracts of herbs.	33

LIST OF FIGURES

Figures	Page
3.1 A: Aqueous extract of <i>Hibiscus Cannabinus</i> B: Aqueous extracts of <i>Tinospora Crispa</i> (left) <i>Garcinia Atroviridis</i> (right) and <i>Anacardium Occidentale</i> (up)	18
4.1 The example of Minimal Inhibitory Concentration	23
4.2 Minimal Bactericidal Concentration for <i>Anacardium Occidentale</i> against <i>Staphylococcus aureus</i> ATCC 25923	24
4.3 Minimal Bactericidal Concentration for <i>Anacardium Occidentale</i> against <i>Escherichia coli</i> ATCC 25922	25
4.4 Minimal Bactericidal Concentration for <i>Tinospora Crispa</i> against <i>Staphylococcus aureus</i> ATCC 25923	26
4.5 Minimal Bactericidal Concentration for <i>Tinospora Crispa</i> against <i>Escherichia coli</i> ATCC 25922	27
4.6 Minimal Bactericidal Concentration for <i>Hibiscus Cannabinus</i> against <i>Staphylococcus aureus</i> ATCC 25923	28
4.7 Minimal Bactericidal Concentration for <i>Hibiscus Cannabinus</i> against <i>Escherichia coli</i> ATCC 25922	29
4.8 Minimal Bactericidal Concentration for <i>Garcinia Atroviridis</i> against <i>Staphylococcus aureus</i> ATCC 25923	30
4.9 Minimal Bactericidal Concentration for <i>Garcinia Atroviridis</i> against <i>Escherichia coli</i> ATCC 25922	31
4.10 Zone of Inhibition for both bacteria with 4 types of extracts	32
4.11 Disc diffusion of <i>Tinospora Crispa</i> against <i>Staphylococcus aureus</i>	

	ATCC 25923	34
4.12	Disc diffusion of <i>Anacardium Occidentale</i> against <i>Staphylococcus aureus</i> ATCC 25923	35
4.12	Disc diffusion of <i>Hibiscus Cannabinus</i> against <i>Staphylococcus aureus</i> ATCC 2592	36
4.14	Disc diffusion of <i>Garcinia Atroviridis</i> against <i>Staphylococcus aureus</i> ATCC 25923	37
4.15	Disc diffusion of Control against <i>Staphylococcus aureus</i> ATCC 25923	38
4.16	Disc diffusion of <i>Tinospora Crispa</i> against <i>Escherichia coli</i> ATCC 25922	39
4.17	Disc diffusion of <i>Anacardium Occidentale</i> against <i>Escherichia coli</i> ATCC 25922	40
4.18	Disc diffusion of <i>Hibiscus Cannabinus</i> against <i>Escherichia coli</i> ATCC 25922	41
4.19	Disc diffusion of <i>Garcinia Atroviridis</i> against <i>Escherichia coli</i> ATCC 25922	42
4.20	Disc diffusion of Control against <i>Escherichia coli</i> ATCC 25922	43

LIST OF ABBREVIATIONS

%	Percent
°C	degree Celsius
±	Plus minus (varied)
ANOVA	Analysis of Variance
<i>H. Cannabinus</i>	<i>Hibiscus Cannabinus</i>
<i>A Occidentale</i>	<i>Anacardium Occidentale</i>
<i>T. Crispa</i>	<i>Tinospora Crispa</i>
<i>G. Atroviridis</i>	<i>Garcinia Atroviridis</i>
<i>E. coli</i>	<i>Escherichia coli</i>
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
Mg/ml	milligram per mililiter
S.E.M	Standard Error Mean
S.D	Standard Deviation
UPM	Universiti Putra Malaysia
O.D	Optical Density

LIST OF APPENDICES

Appendix		page
A	Statistical analysis results for Zone of Inhibition	54



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CHAPTER 1

INTRODUCTION

1.1 Overview

Medicinal plants are cheaper and more accessible to most of the population in the world, thus, there is need to encourage the use of medicinal plants as a potential source of new drugs (Igoli *et al.*,2000). The overall effect of any scientific study will help to variously establish and confirm the credibility of the use of herbals as an effective source of both traditional and modern medicine (Mohammed,1990). *Tinospora Crispa* is a woody and glabrous in the family Menispermaceae. It is locally known as Patawali or Seruntum. This climbing plant, wide leaves, heart shaped plant is believed to have several medicinal values. For instance, it can treat stomach discomfort, asthma, cough, bacterial infections and fever (Muhammad *et al.*, 1994). *Tinospora Crispa* is widely used in Thailand, Malaysia and Indonesia as a bitter tonic, antipyretic (Kongsaktragoon *et al.*, 1994) and oral hypoglycemic agent (Noor H *et al.*, 1989).

As for *Anacardium Occidentale*, its genus name is *Anacardium*, species *occidentale*, family *anacardiaceae* and its common name is cashew; also useful for



medicinal treatment. Its English name derives from the Portuguese name for the fruit of the cashew tree, caju, which in turn derives from the indigenous Tupi name, acajú. It is now widely grown in tropical climates for its cashew nuts and cashew apples. The leaves are spirally arranged, leathery textured, 4 to 22 cm long and 2 to 15 cm broad, with a smooth margin. The plant extract had a good in vitro antibacterial activity against *E. coli* and *P.aeruginosa* (Kudi 1999) and the bark extract of *Anacardium Occidentale* exhibited antibacterial activity in vitro against 13 to 15 microorganisms tested (Bicalho *et al.*,2001). *Anacardium Occidentale* methanol and aqueous extract also can be hypoglycemic agent (S.D. Sokeng *et al.*, 2007) and anti-inflammatory agent (M.B Patil *et al.*, 2003).

Hibiscus Cannabinus, is a plant in the Malvaceae family. *Hibiscus Cannabinus* is in the genus *Hibiscus* and is probably native to southern Asia, though its exact natural origin is unknown. The name also applies to the fibre obtained from this plant. Kenaf is one of the allied fibres of jute and shows similar characteristics. Other names include Bimli, Ambari, Ambari Hemp, Deccan Hemp, and Bimplipatum Jute. The essential oil of *Hibiscus Cannabinus* leaves has phytotoxic and fungitoxic activities (Mozaina Kobaisy *et al.*, 2001). Earlier studies have reported the haematinic and hepatoprotective activities of this plant (Agbor *et al.*, 2001; Agbor *et al.*,2002). It also has antioxidative property (Agbor *et al.*,2003). *Hibiscus Cannabinus* of leaf extract can also kill earthworms better than the Standard Drug, Albendazole. (Kota Chaitanya Sravanti *et al.*, 2011). The

methanolic extract of *Hibiscus Cannabinus* leaf exhibited significant hypoglycemic activity in streptozotocin induced diabetic rats (T. Raj Kumar *et al.*, 2011).

Garcinia Atroviridis is a medium sized fruit tree which belongs to the family Gutiferae. In Malaysia it is popularly known as 'asam gelugur' or 'asam keping'. The plant contains citric, tartaric and ascorbic acid which have antioxidant activities. However, the most important bioactive compound is Hydroxyl Citric Acid (HCA) (Seon-Min *et al.*, 2001). *Garcinia Atroviridis* extract exhibit strong antioxidant, antimicrobial, antitumour and anti-inflammatory activities (Mackeen *et al.*, 2000). . Ethno-botanical and pharmacological reports have described the significant medicinal and preservative properties of *Garcinia Atroviridis* (Burkill, 1966; Fui, 1992; Grosvenor *et al.*, 1995a, b;Mackeen *et al.*, 2000).

In order to prevent possibility of extinction of useful traditional plant, studies on traditional medicine generally are important. Furthermore, both knowledge and plant are becoming increasingly scarce and if no action is taken. To solve this problem, there are several steps need to be taken to ensure the development and perpetuation of traditional medication and medicinal plants. One of the steps taken is by conducting a research on traditional medicinal herbs based on taxonomy, natural product and its effectiveness in healing a particular illness. Nowadays, there are several attempts and experimental designs have shown these four herbs' effect as antimicrobial. Because of indiscriminate

use of commercial antimicrobial drugs or chemical commonly used in the treatment of infectious disease, multiple of this drug or chemical resistance in both human and plant pathogenic microorganism have been developed (Davis, 1994; Loper *et al.*, 1991; Service, 1995).

The searching of new antimicrobial substances from various sources like medicinal plants (Clark, 1996; Cordell, 2000) usually target structural component which trigger fast resistant within targeted organisms or targeted bacteria. Since natural product is believed not to have side effects and very effective ailments which do not develop fast resistant, approximately 80 % of the world population still depends on traditional medicine as a source of the treatment of disease. Based on literally review, the traditional medicinal plants possess both healing as well negatives side-effect, so a depth scientific research is required to discover their absolute effects. Plant-based antimicrobials have enormous therapeutic potential as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials (Iwu *et al.*, 1999).

For this study, the organism of interest which is both pathogenic and non-pathogenic is discovered based on the commonly occurring bacteria. Recently, the problem is about the resistance of bacteria towards antimicrobial agents, leads to the progressing of the new manner of treatment which leads to this study. For instance, the resistant of some strain of staphylococci to penicillin was identified almost immediately

after introduction of the penicillin. The resistance to penicillin is shown in *Staphylococcus Aureus* which is 80% of all strains of *Staphylococcus Aureus* and some strains of *Staphylococcus Aureus* have been isolated are resistant to virtually all clinically-available antibiotics. For most Gram-negative pathogens (e.g *Salmonella*, *Yersinia pestis*, *Pseudomonas*, *Shigella*), penicillin has never been effective, except for *Neisseria gonorrhoeae* because their vulnerable cell wall is protected by an outer membrane that prevent permeation of the penicillin molecule. Because of pathogens develop resistance, natural penicillin have become useless against staphylococci and must be replaced by other antibiotics. Tetracycline has become worthless as substitute to penicillin also has been so widely used and misused for decades. Today, antibiotic resistance has become a global concern (Westh H *et al.*, 2004). The emergence of multidrug-resistant pathogens has become threat to the clinical efficacy of many existing antibiotics (Bandow JE *et al.*, 2003).

There is a continuous and emergency need to develop new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and reemerging infectious diseases (Rojas A *et al.*, 1992). Thus, researchers are increasingly turning their focus to folk medicine, searching for new leads to build better drugs against microbial infections (Benkeblia N., 2004). Because of the increasing failure of chemotherapeutics and antibiotic resistance showed by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity (Colombo ML, Bosisio E., 1996, Iwu *et al.*, 1999). Nowadays, studies have suggested that several plants species show great antimicrobial effects

(Wannissorn B *et al.*, 2005). In recent years, it has been proposed that the herbal extracts may be used as natural antifungal agents to inhibit the growth of food borne pathogen (Lee S-H *et al.*,2007) and as a source of various medicinal agents (Krishnaraju AV *et al.*, 2005).

Objectives of this study is to evaluate broad spectrum-antimicrobial effects of *Tinospora Crispa*, *Anacardium Occidentale*, *Garcinia Atroviridis* and *Hibiscus Cannabinus* against Gram positive and Gram negative bacteria by using Minimal inhibitory concentration (MIC), Minimal Bactericidal concentration method (MBC) and disc diffusion method. Aqueous extracts of the herbs were prepared with *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 as the test organisms representing pathogenic gram positive and negative bacteria respectively. Aqueous extracts that have been serially diluted were mixed with bacterial suspension in MHB at appropriate ratio. Upon 18 hours incubation at 37°C, MIC was visually determined by taking the suspension that had no trace of bacterial growth. MBC was determined by further sub-culturing the extract-bacteria-suspension onto MHA. Disc diffusion method utilized the MBC of the respective extract using 5 mm filter disc.

1.2 Objective

To evaluate broad spectrum-antimicrobial effects of *Tinospora Crispa*, *Anacardium Occidentale*, *Garcinia Atroviridis* and *Hibiscus Cannabinus* against Gram positive; *Staphylococcus aureus* ATCC 25923 and Gram negative bacteria; *Escherichia coli* ATCC 25922 by using Minimal inhibitory concentration (MIC), Minimal Bactericidal concentration method (MBC) and disc diffusion method.

CHAPTER 2

LITERATURE REVIEW

2.1 *Tinospora Crispa*

An ethnic Group in Sabah (Borneo), the Murut community, traditionally uses *Tinospora Crispa* to treat diabetes, hypertension, and lumbago (A.Fasihuddin, 2000). The antimalarial effect was confirmed in a study (Rahman et al., 1999). The previous study was done on hypoglycemic effect of *Tinospora Crispa* dry powder in outpatients with metabolic syndrome at King Chulalongkorn Memorial (Chutima Sriyapai, 2009). We can conclude that receiving 250 mg *Tinospora Crispa* twice a day dry powder that is 0.98% dry weight marker A; for 2 months can reduce fasting blood glucose significantly from baseline. Although 16.7% of the samples had increased aspartated aminotransferase and/or alanine aminotransferase more than 3 times the baseline levels but using *T. Crispa* dry powder with statin may cause risk.

Study on cardiac contractility of two triterpenes that are cycloeucalenol and cycloeucalenone isolated from *Tinospora Crispa* also was done (Kongkathip N et al.,, 2002). As a result, cycloeucalenol slightly elevated the right atrial force of contraction whereas it indicated an initial reduction followed by sustained reduction of about 10% on the left atria of the rat in vitro. Cycloeucalenone indicated slight change from the

control on the right and left atrial force. These results show that cycloeucalenol and cycloeucalenone produced mild cardiotoxic effects.

2.2 *Anacardium Occidentale*

Anacardium Occidentale is a plant that is useful as a folk remedy for diabetes mellitus (Longuefosse *et al.*, 1996, Kamtchouing *et al.*, 1998). The previous study on hypoglycemic effect of *Anacardium Occidentale* methanol extract and fractions on Streptozotocin-induced diabetic rats was done (S.D. Sokeng *et al.*, 2007). To confirm the diabetic state, effect of the methanol extract of *Anacardium Occidentale* on serum as glycosuria was determined. For this experiment, we need rats with fasting blood glucose values of at least 200 mg/kg and a positive urine glucose level. Oral administration with doses of 35, 175 and 250 mg/kg of the methanol extract showed a decrease in blood glucose levels of treated rats compared to control rats. Streptozotocin- induced diabetes provides a condition of insulinopenia. It has been described as a useful experimental model to evaluate the activity of hypoglycemic agents.

Another previous study was done is about evaluation of antiinflammatory activity of the leaves Of *Anacardium Occidentale*. Ethanolic crude extract of *Anacardium Occidentale* leaves and its five different crude fractions, petroleum ether, solvent ether, ethyl acetate, butanol and butanone were become to be preliminary qualitative chemical investigations (M.B Patil, 2003). The ethanolic extract and all other fractions were screened for antiinflammatory activity in albino rats (300 mg/kg). When

compared with control and standard drug diclofenac sodium (100 mg/kg), ethanolic extract and butanone fraction exhibited significant antiinflammatory act. All fractions of the extract exhibited varying levels of activity against the test organism except petroleum ether fraction, which showed no activity at all concentrations. Besides, ethyl extract was active against *E.coli* only at 1000 Lg/ml.

2.3 *Hibiscus Cannabinus*

The previous study was done on phytotoxic and fungitoxic activities of the essential oil of *Hibiscus Cannabinus* leaves and its composition (Mozaina Kobaisy et al., 2001). Gas chromatography-mass spectroscopy (GC-MS) was used to determine the chemical composition of the essential oil of *Hibiscus Cannabinus*. Fifty-eight components were grouped from *Hibiscus Cannabinus* with (E)-phytol (28.16%), (Z)-phytol (8.02%), n-nonanal (5.70%), benzene acetaldehyde (4.39%), (E)-2-hexenal (3.10%), and 5-methylfurfural (3.00%) as the major constituents. The oil was phytotoxic to lettuce and bentgrass and had antifungal activity against *Colletotrichum fragariae*, *Colletotrichum gloeosporioides*, and *Colletotrichum accutatum*. Besides, the oil exhibited little or no algicidal activity.

The alcoholic extract of *Hibiscus Cannabinus* was studied for antidiabetic activity in streptozotocin induced diabetic rats (T.Raj Kumar *et al.*,2010). This inducement was done by oral administration of extract 400 mg/kg body weight for 15 days. The effect was compared with oral dose of 0.5 mg/kg Glibenclamide and the

determination of blood glucose level was done by GOD-POD kit method. As a result, the alcoholic extract of *Hibiscus Cannabinus* leaves significantly lowered the blood glucose of hyperglycemic rats. Apart from that, from the toxicity study it was shown that methanolic extract of *Hibiscus Cannabinus* was nontoxic up to 5g/kg body weight. Besides, phytochemical study showed the presence of phytosterols, flavonoids and glycosides. In short, we can say that *Hibiscus Cannabinus* leaf extract has significant antidiabetic activity, which lowered the fasting blood glucose level in Streptozotocin induced diabetic rats.

2.4 *Garcinia Atroviridis*

It is noted that a decoction of the leaves of the parent tree has been traditionally used for the treatment of earache (Tisdale *et al.*, 2003). The previous study was done on the determination of effect of *Garcinia Atroviridis* on oxidative stress and atherosclerotic changes in experimental guinea pigs (Adel.A et al., 2010). As a result, a significant increase was showed in the percentage of DNA damage in the tail and in the tail moment (comet assay) in the group II compared to the group I. Besides, Group III showed pattern of reduction in the percentage of DNA damage by 42% and tail moment by 50% compared to the group II. For histological studies, compared to group II, in group III it showed a tendency in the reduction of the fat deposition in the aorta and number of foam cells. We can conclude that the supplementation with *Garcinia Atroviridis* decreased the DNA damage and deposition of lipids in the wall of the aorta in hyperglycemic guinea pigs.

Next, the previous study on this herb is about evaluation of antimicrobial, antioxidant, antitumour-promoting and cytotoxic activities of crude extracts (methanol) of various parts, such as the leaves, fruits, roots, stem and trunk bark, of *Garcinia Atroviridis* (Mackeen *et al.*,2000). They were screened for antimicrobial, cytotoxic, brine shrimp toxic, antitumour-promoting and antioxidant activities. The crude extracts indicated predominantly antibacterial activity with the root extract. From this result, the strongest inhibition against the test bacteria is at a minimum inhibitory dose (MID) of 15.6 µg/disc. Although all the extracts failed to inhibit the growth of most of the test fungi, significant antifungal activity against *Cladosporium herbarum* was shown by most notably the fruit (MID: 100 µg), and the leaf (MID: 400 µg) extracts. None of the extracts were significantly cytotoxic, and lethal towards brine shrimps. The root, leaf, trunk and stem bark extracts (except for the fruits) indicated strong antioxidant activity exceeding that of the standard antioxidant, α-tocopherol and also, the fruit, leaf, stem and trunk bark extracts showed antitumour-promoting activity (>95% inhibition).

2.5 *Staphylococcus aureus*

Staphylococci are Gram-positive spherical bacteria that occur in microscopic clusters showing grapes in shape. Bacteriological culture of the nose and skin of normal humans invariably yields staphylococci. About 20% of the human populations are long-term carriers of *S. aureus* (Kluytmans J *et al.*, 1997). *S. aureus* can cause a range of illnesses from minor skin infections, such as pimples, impetigo, and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, and sepsis. It is still one of the five most common causes of nosocomial infections. It also often causes postsurgical wound infections. by the surgeon Sir Alexander Ogston was one who discovered *S. aureus* in Aberdeen, Scotland in 1880, in pus from surgical abscesses (Ogston A., 1984).

For every year, some 500,000 patients in American hospitals contract a staphylococcal infection (Bowersox, John., 1999). *S. aureus* may occur as a commensally on skin and also occurs in the nose frequently (Whitt *et al.*, 2002) and the throat less commonly. The occurrence of *S. aureus* under these circumstances does not always indicate infection. Thus, it does not always require treatment. It is because the treatment may be ineffective and recolonisation may occur. It can survive on domesticated animals, such as dogs, cats, and horses. Furthermore, it can survive for hours to weeks, or even months, on dry environmental surfaces, depending on strain (Cimolai.,2008). *S. aureus* infection can cause a severe disease - staphylococcal scalded skin syndrome (SSSS) in infants (Curran JP, Al-Salihi FL., 1980).

2.6 *Escherichia coli*

In 1885, *E. coli* was discovered by German paediatrician and bacteriologist Theodor Escherich (Feng P *et al.*, 2002). *Escherichia coli* (commonly abbreviated *E. coli* and named after Theodor Escherich) is a Gram-negative rod-shaped bacterium. It is commonly found in the lower intestine of warm-blooded organisms (endotherms). Most *E. coli* strains are harmless, but some, such as serotype O157:H7, can cause serious food poisoning in humans, and also are occasionally responsible for product recalls (Vogt RL, Dippold L., 2005) The harmless strains are part of the normal flora of the gut, and can give advantage to their hosts by producing vitamin K2 (Bentley R, Meganathan R., 1982), and by preventing the production of pathogenic bacteria within the intestine (Hudault S *et al.*, 2001, Reid G *et al.*, 2001).

E. coli's ability to survive for certain periods outside the body makes them an good indicator organism to test environmental samples for fecal contamination.(Feng P *et al.*, 2002, Thompson, Andrea 2007). It is one of the best-studied prokaryotic model organisms. It also an important species in biotechnology and microbiology as the bacteria can be grown easily. Besides, its genetics are comparatively simple and easily manipulated or duplicated through a process of metagenics.

2.7 Antibiotic Sensitivity Test

Antibiotic sensitivity test is the phenotypic typing method that is useful in research laboratory. Typing system is based on the premise that similar closed to isolated share characteristics by which they can be differentiated from unrelated isolates. The usage of a particular characteristic for typing is depending on its stability within a strain and its diversity within the species (Arbeit, 1995).

There are two method two determined antibiotic sensitivities, which are Kirby-Bauer disk diffusion and Minimal Inhibitory/Bactericidal concentration (Bauer et al., 1996). to confirm resistance of microorganisms to an antimicrobial agent and also to control the activity of new antimicrobial agents, minimum inhibitory concentrations are imporant in diagnostic laboratories (Andrews 2001). Basically, MIC can be described as the most basic laboratory measurement of the activity of an antimicrobial agent against an organism (Turnidge JD *et al.*, 2003). Minimum Bactericidal Concentration (MBC) is the lowest concentration of antibiotic required to kill the microorganism. Bactericidal if the MBC is no more than four times the MIC (French GL, 2006). The minimum inhibitory concentration (MIC) values for the microorganisms were described as the sensitivity to the extracts by checkerboard assay method (Kumarasamy et al., 2002; Jeniffer et al., 1999).

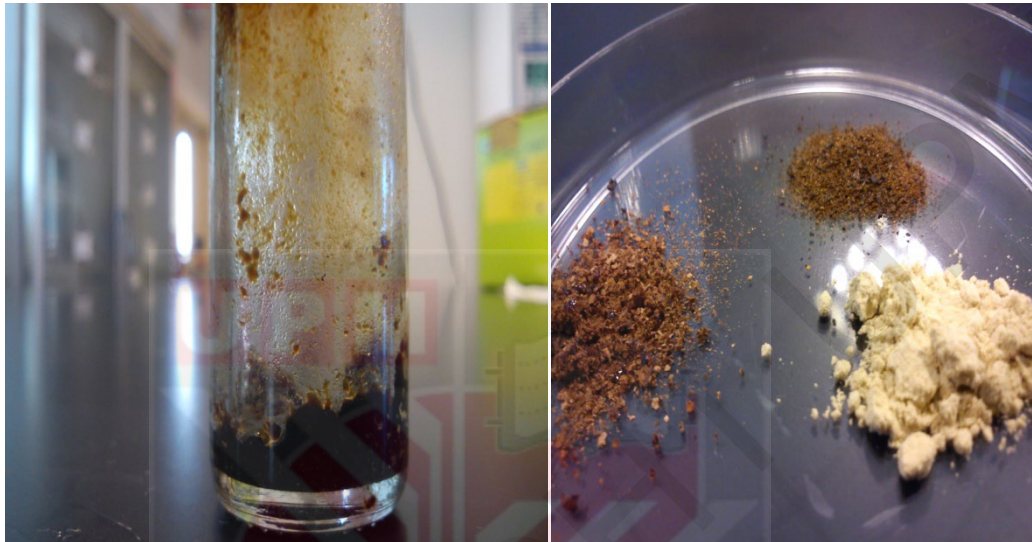
The quantitative data that is given by Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) is not obtainable with the Kirby-Bauer method. Because of determination of MIC can be costly and time consuming, disc diffusion method susceptibility testing is among the most widely adopted method. The Kirby-Bauer disc diffusion test is very useful in determining a therapeutic course against a particular infection and to test the efficacy of a new antimicrobial. Besides, it can also recognize whether an organism is susceptible or resistant to a selection of antimicrobial agent.

CHAPTER 3

METHODOLOGY

3.1 Preparation of plant extracts

The plant specimens were collected from FRIM for its leaves. First, the plant was weight and dried in the oven. Then, it was blended into powder and 100g of each type of these herbs were soaked into 1L of distilled water. Next, this soaked herb was stirred in water bath at 60⁰C for 6 hours and then filtered with Whatman 1 filter paper. After that, supernatant is moved into plain tube and was freezed at -80⁰C for 24 hours. Subsequently, it was sent transfer for freezing drying producing 10% crude extracts of herbs powder which was kept in air.



A

B

Figure 3.1 A: Aqueous extract of *Hibiscus Cannabinus*

B: Aqueous extracts of *Tinospora Crispa* (left) *Garcinia Atroviridis* (right) and *Anacardium Occidentale* (up)

These pictures show four types aqueous that have been prepared in aqueous one. For *Hibiscus cannabinus*, it can be safe in room temperature where as aqueous extracts of *Tinospora Crispa* *Garcinia Atroviridis* and *Anacardium Occidentale* should be kept in refrigerator in -20°C.

3.2 Microorganisms and maintenance

Total of 2 species were employed in the study. 1 American Type culture Collection (ATCC) of *Staphylococcus aureus* and 1 type ATCC of *Escherichia coli* were buy from supplier. Both of *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were cultured on Mueller Hilton Agar. There are several test of conformation that we have done on both of these bacteria so that we can make sure that bacteria are gram-positive bacteria *Staphylococcus aureus* and gram-negative bacteria *Escherichia coli*.

3.2.1 Gram Stain Test

A sample of a bacterial culture was placed on a microscope slide. A wooden stick was used to smear the bacterial culture over the microscope slide. Then, the slide containing the bacterial culture was dried by holding the slide over a small flame from a Bunsen burner. Crystal violet stain was poured over the bacterial specimen on the slide and let it for approximately 10 to 60 seconds depending on the thickness of the smear on the slide. The slide was rinsed with water. Gram's iodine solution was placed on the bacterial smear for another 10 to 60 seconds depending on smear thickness. Rinsed off it with more water. Several drops of a decolorizer were added to the bacterial smear on the slide such as acetone or alcohol. A typical time for this is approximately 5 seconds. A counterstain, such as basic fuchsin solution or safranin, was placed on the slide over the

bacterial smear for approximately 40 to 60 seconds and then rinse off the counterstain with water. The slide was let to air to dry. Microscope was used to examine the slide containing the bacterial smear. Gram-positive bacteria *Staphylococcus aureus* will be colored purple, and Gram-negative bacteria *Escherichia coli* will have a red or pink color.

3.2.2 Mannitol Salt Agar (MSA) Test

Culture that we want to test was inoculated on the MSA plate. Tjis was done by using the streak method. MSA contains of NaCl, mannitol and phenol red as pH indicator. It can be selective and differential; selective since it allows only the growth of high salt or saline loving organisms and differential since manitol fermenter organisms change the red pH indicator color (neutral) to yellow (acidic). Non-mannitol fermenters do not change the color of the media. As a result, mannitol fermenters include *Staphylococcus aureus*, and negative growth includes *Escherichia coli*.

3.3 Antibiotic Sensitivity Test

3.3.1 Minimal Inhibitory Concentration

3 or five colonies were moved to Mueller Hilton broth. Test tubes were incubated on a mechanical shaker at 35°C growth phase was confirmed by monitoring an organism growth curve in broth for 4-6 hours. Turbidity was adjusted with sterile Mueller Hilton broth to match OD 0.08-0.1 turbidity standard by using spectrophotometer at 625 nm. Serial tubes containing which is containing aqueous herbs of each extracts from 250 mg/ml to 0.98 mg/ml were prepared using ½ dilutions. 0.1 ml bacterial suspension was added to each serial tube by using spectrophotometer (OD standard 0.08- 0.1 at 625 nm). The serial tubes were incubated for 6-8 hours.

3.3.2 Minimal Bactericidal Concentration

After the serial tubes were incubated for 6-8 hours, each of these tubes was streaked on each MHA respectively by using sterile wire loop. Then, these plates of MHA were incubated for 24 hours at 35°C.

3.3.3 Agar Disc Diffusion Method

The tops of four to five colonies of bacteria from pure culture were picked up with a sterile loop. The colonies were suspended in 5 ml of sterile physiologic saline. The inoculum was standardized to equivalent of a 0.08-0.1 nm OD (Optical Density) standard by using spectrophotometer at 625 nm. The entire surface of a Mueller Hilton

Agar plate was inoculated using a sterile swab. Disc containing control and extracts were placed using a sterile forceps onto the agar surface and gently pressed down to ensure contact. Plates were incubated at 35°C for 6-8 hours. Subsequently, the diameter of the zone of inhibition around each disk was measured.



CHAPTER 4

RESULTS

4.1 Minimal Inhibitory/ Bactericidal Concentration

Upon 18 hours incubation at 37°C, MIC was visually determined by taking the suspension that had no trace of bacterial growth. MBC was determined by further sub-culturing the extract-bacteria-suspension onto MHA. The MIC and MBC of the respective extract for both tests organisms were similar; 227.27 mg/mL for *Tinospora Crispa* and *Anacardium Occidentale*, 113.64 mg/ml for *Hibiscus Cannabinus*, and 56.82 mg/mL for *Garcinia Atroviridis*.

	<i>Tinospora crispa</i>	<i>Anacardium occidentale</i>	<i>Hibiscus cannabinus</i>	<i>Garciniaatrovidis</i>
<i>Staphylococcus aureus</i> ATCC 25923	227.27mg/ml	227.27mg/ml	113.64 mg/ml	56.82 mg/mL
<i>Escherichia coli</i> ATCC 2592	227.27mg/ml	227.27mg/ml	113.64 mg/ml	56.82 mg/mL

Table 4.1: Table of MIC / MBC of Each types of Extracts for Both Test Organisms



Figure 4.1 The example of Minimal Inhibitory Concentration

Because of the color of the extract is different from each other and cover the eye to see the result of MIC, we further use Minimal Bactericidal Concentration which we can identify the lowest concentration of herbs required to kill the bacteria which is not as commonly seen as the Minimum Inhibitory Concentration (MIC).

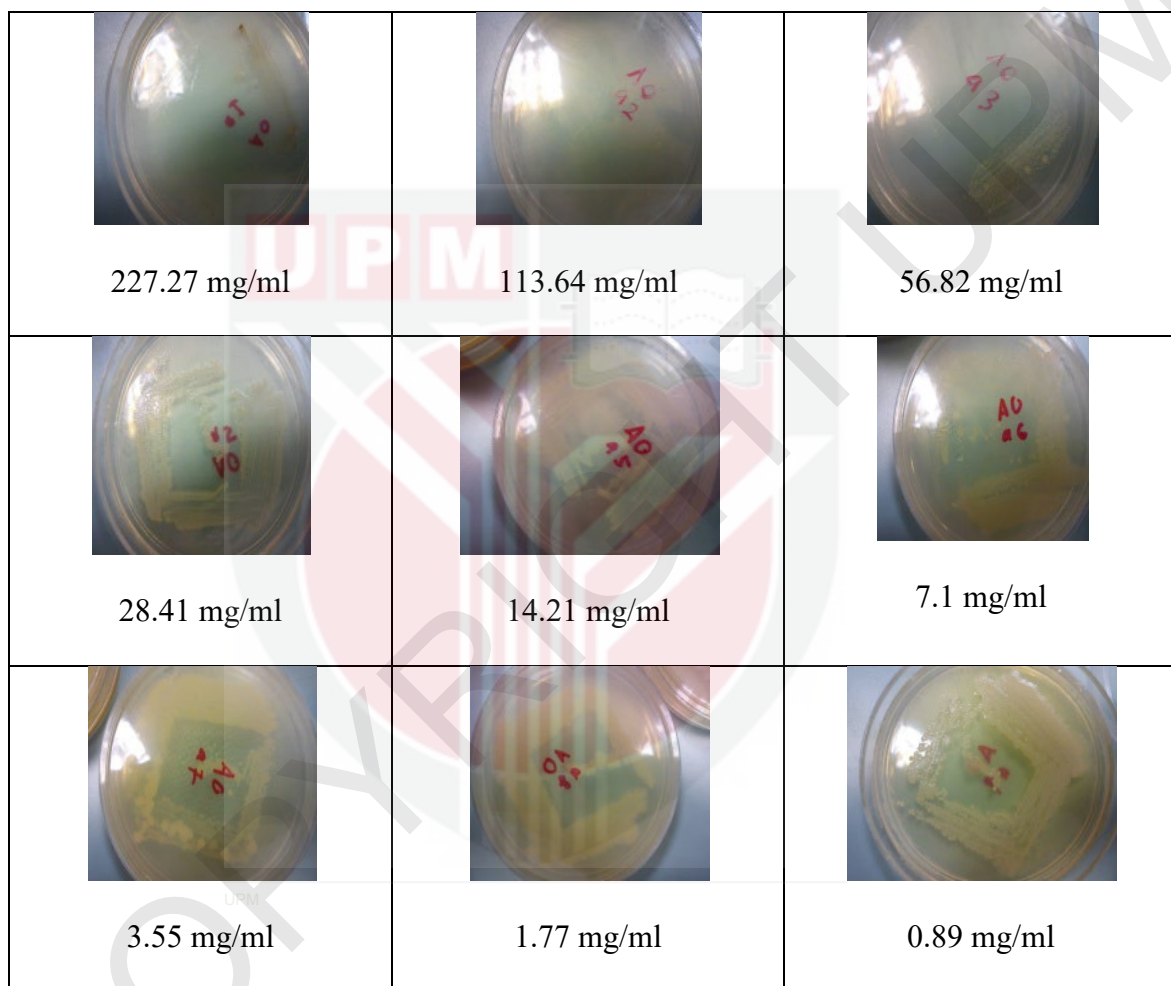


Figure 4. 2 Minimal Bactericidal Concentration for *Anacardium Occidentale* against *Staphylococcus aureus* ATCC 25923

The lowest concentration of *Anacardium Occidentale* required killing *Staphylococcus aureus* ATCC 25923 is 227.27 mg/ml.

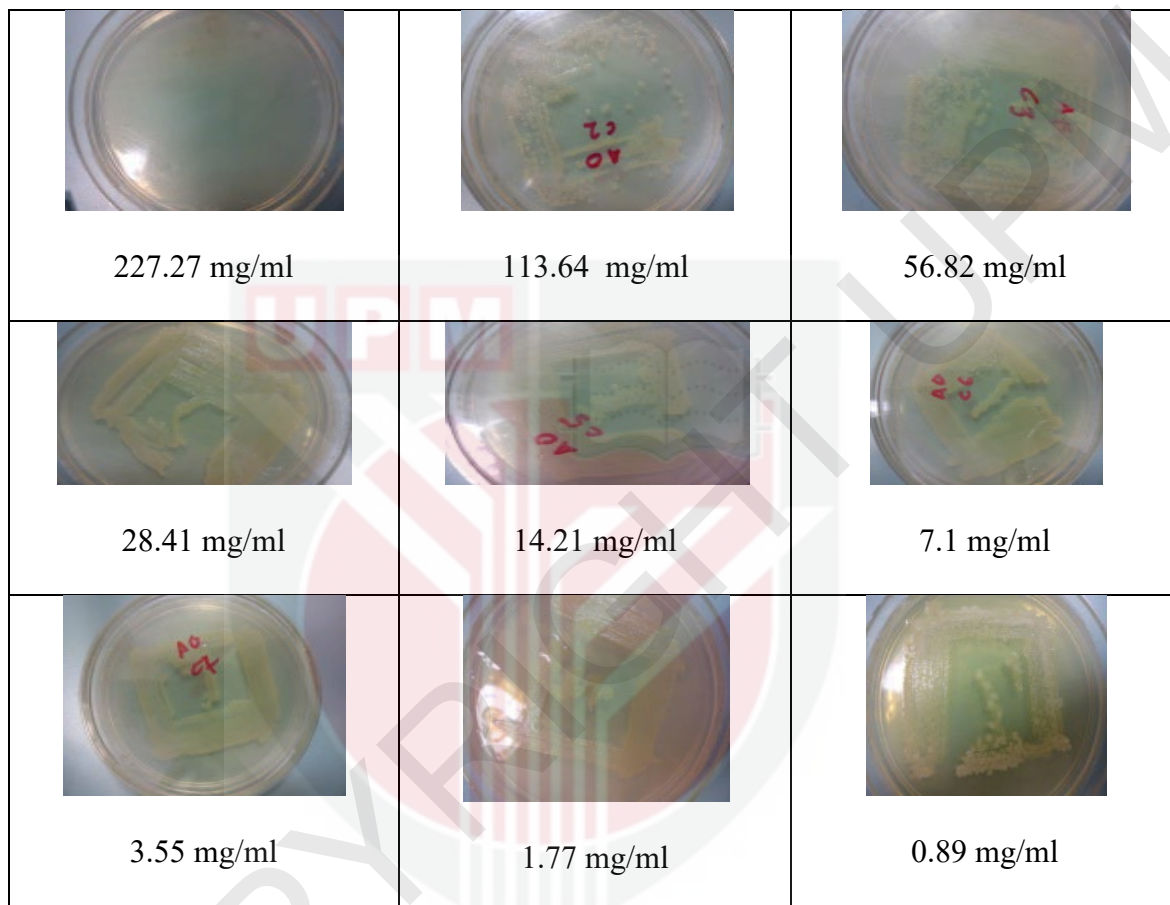


Figure 4. 3 Minimal Bactericidal Concentration for *Anacardium Occidentale* against *Escherichia coli* ATCC 25922

The lowest concentration of *Anacardium Occidentale* required killing *Escherichia coli* ATCC 25922 is 227.27 mg/ml.

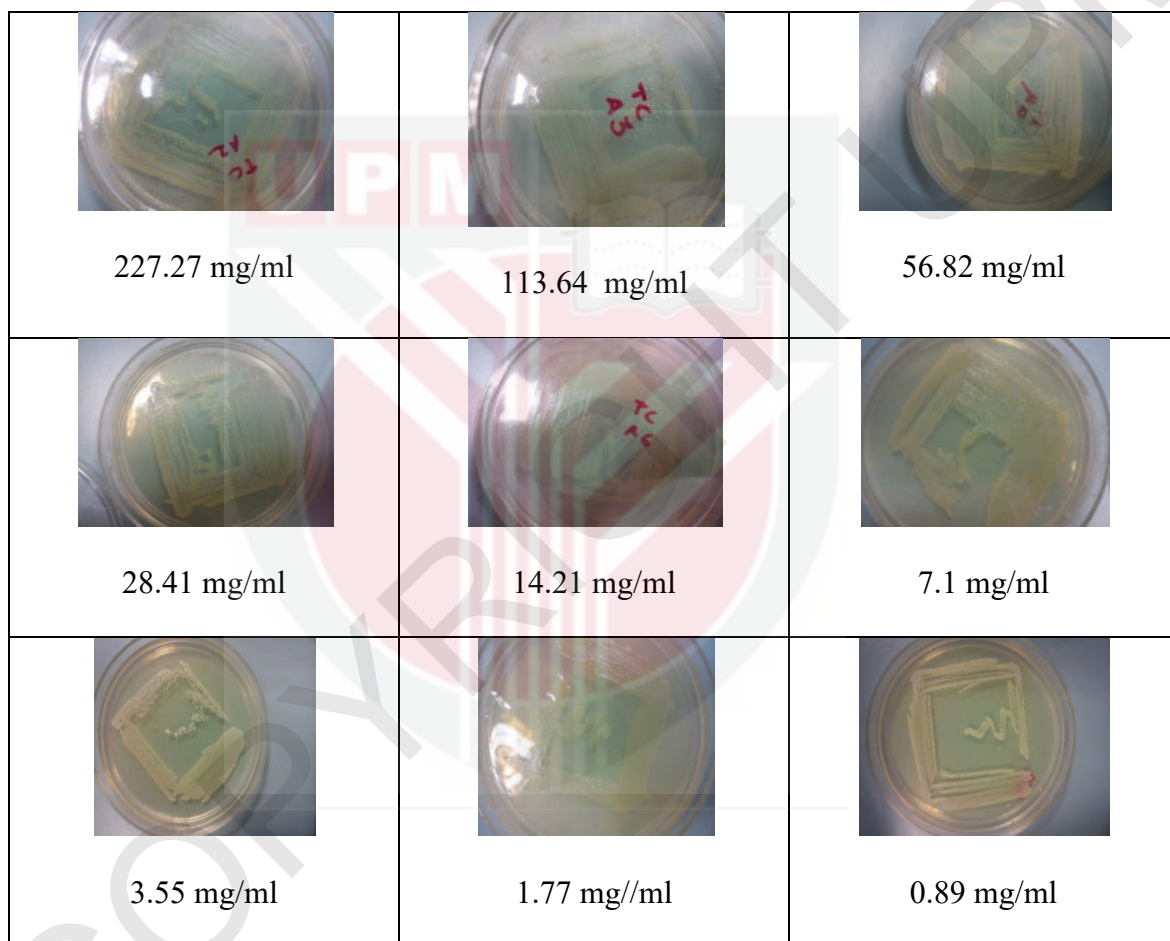


Figure 4. 4 Minimal Bactericidal Concentration for *Tinospora Crispa* against *Staphylococcus aureus* ATCC 25923

The lowest concentration of *Tinospora Crispa* required killing *Staphylococcus aureus* ATCC 25923 is 227.27 mg/ml.



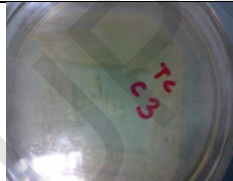
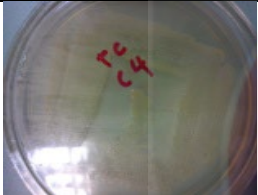
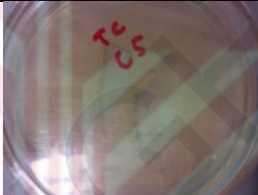

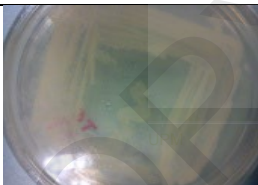
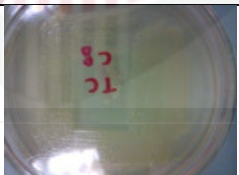

		
227.27 mg/ml	113.64 mg/ml	56.82 mg/ml
		
28.41 mg/ml	14.21 mg/ml	7.1 mg/ml
		
3.55 mg/ml	1.77 mg/ml	0.89 mg/ml

Figure 4. 5 Minimal Bactericidal Concentration for *Tinospora Crispa* against *Escherichia coli* ATCC 25922

The lowest concentration of *Tinospora Crispa* required killing *Escherichia coli* ATCC 25922 is 227.27 mg/ml.

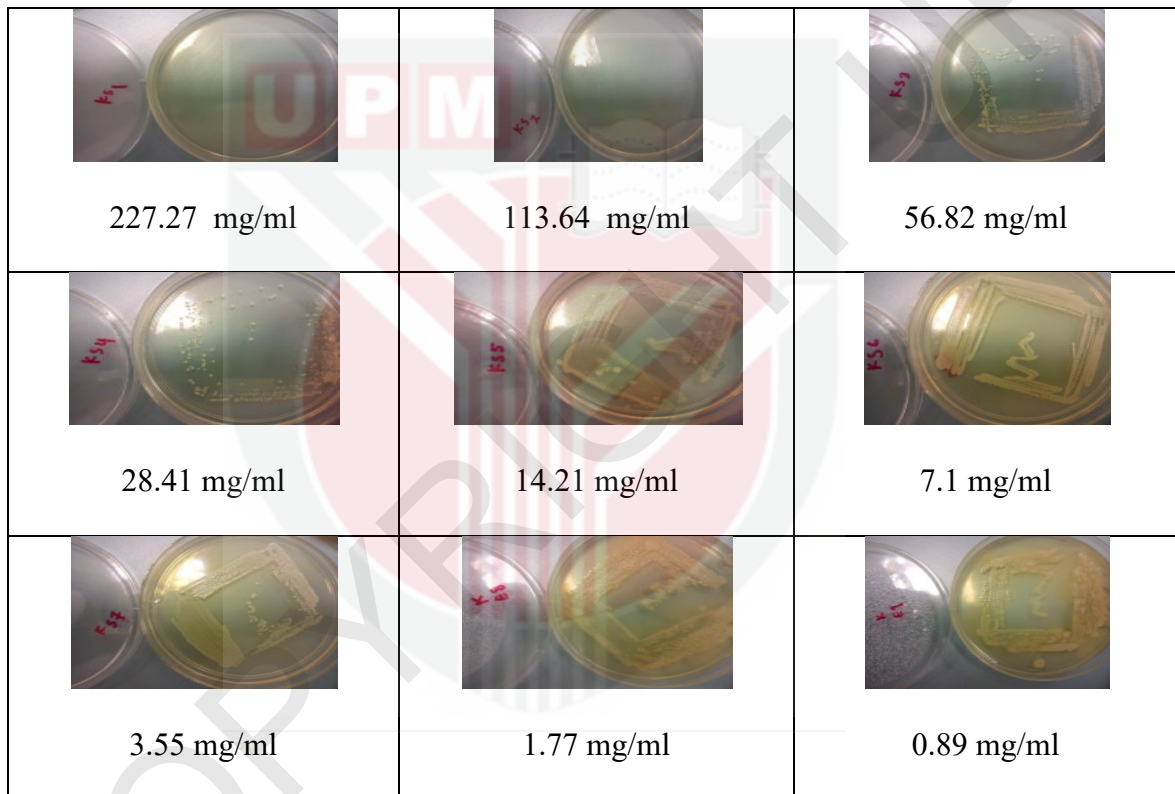


Figure 4.6 Minimal Bactericidal Concentration for *Hibiscus Cannibinus* against *Staphylococcus aureus* ATCC 25923

The lowest concentration of *T Hibiscus Cannibinus* required killing *Staphylococcus aureus* ATCC 25923 is 113.64 mg/ml.

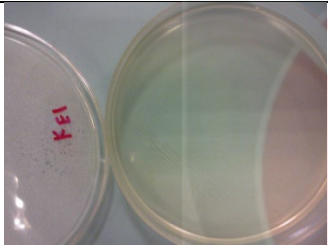
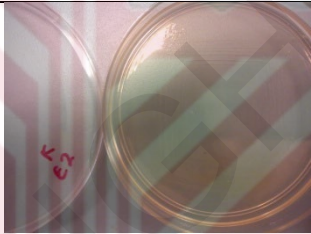
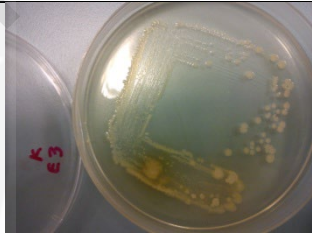


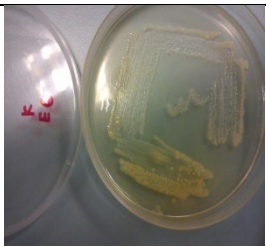

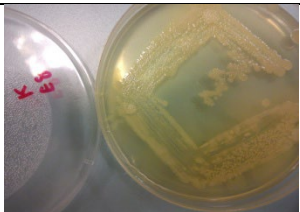

 <p>227.27 mg/ml</p>	 <p>113.64 mg/ml</p>	 <p>56.82 mg/ml</p>
 <p>28.41 mg/ml</p>	 <p>14.21 mg/ml</p>	 <p>7.1 mg/ml</p>
 <p>3.55 mg/ml</p>	 <p>1.77 mg/ml</p>	 <p>0.89 mg/ml</p>

Figure 4.7 Minimal Bactericidal Concentration for *Hibiscus Cannibinus* against *Escherichia coli* ATCC 25922

The lowest concentration of *Hibiscus Cannibinus* required killing *Escherichia coli* ATCC 25922 is 113.64 mg/ml.

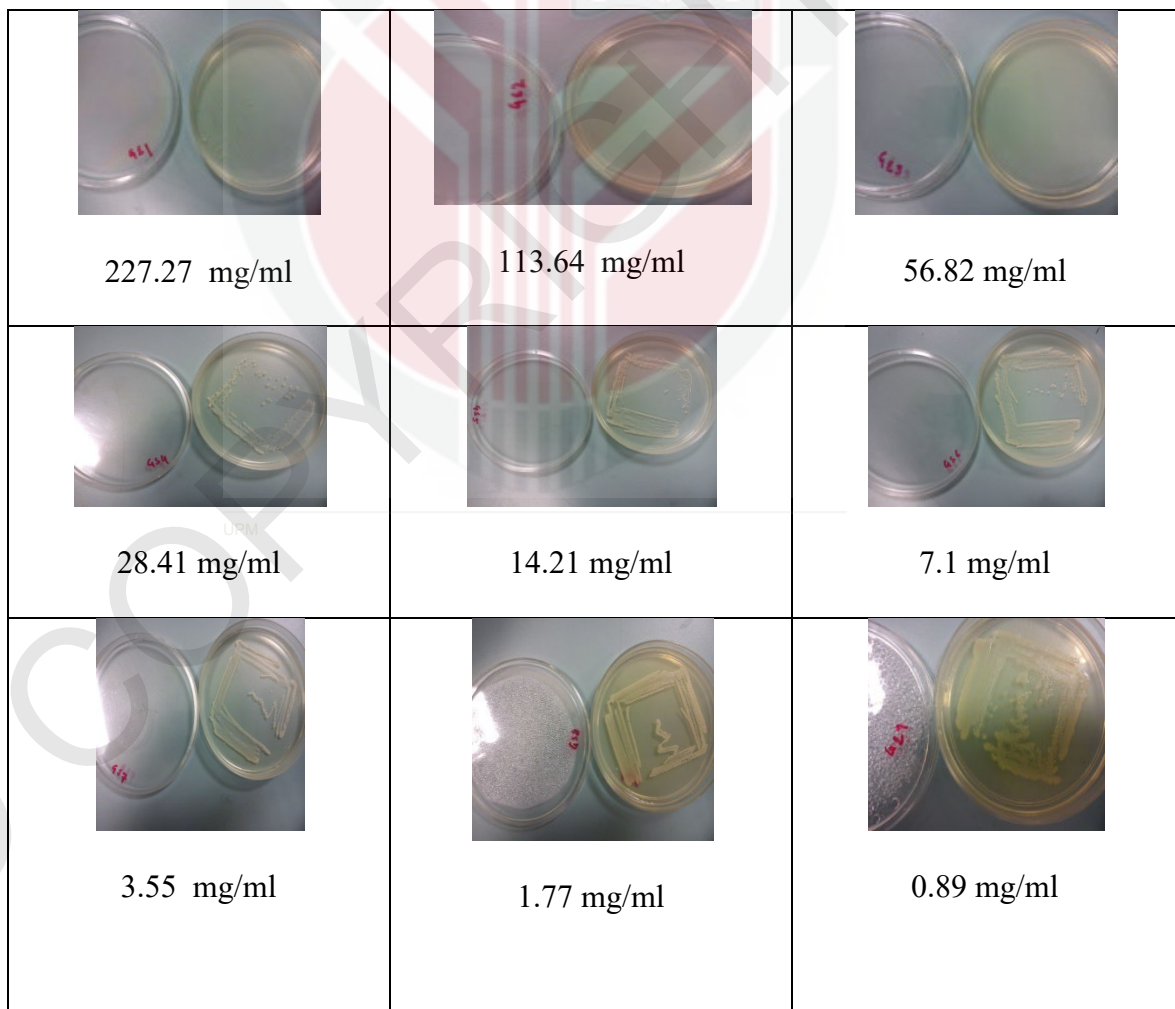
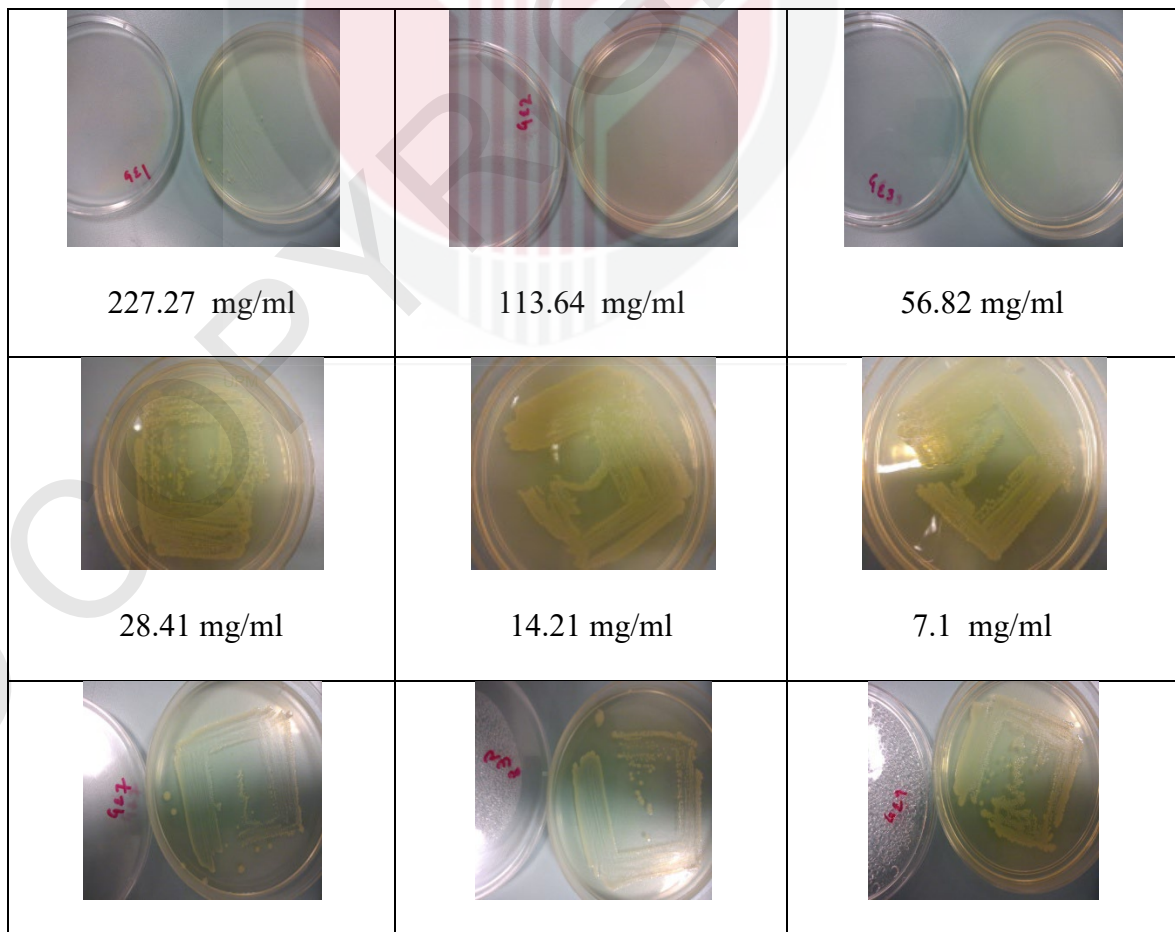


Figure 4.8 Minimal Bactericidal Concentration for *Garcinia Atroviridis* against *Staphylococcus aureus* ATCC 25923

The lowest concentration of *Garcinia Atroviridis* required killing *Staphylococcus aureus* ATCC 25923 is 56.82 mg/ml.



3.55 mg/ml	1.77 mg/ml	0.89 mg/ml
------------	------------	------------

Figure 4.9 Minimal Bactericidal Concentration for *Garcinia Atroviridis* against *Escherichia coli* ATCC 25922

The lowest concentration of *Garcinia Atroviridis* required killing *Escherichia coli* ATCC 25922 is 56.82 mg/ml.

4.2 Disc Diffusion Method

Disc diffusion method utilized the MBC of the respective extract using 5 mm filter disc. In disc diffusion method, all extracts showed a narrow range of inhibition zone ~(8.67-9.67).

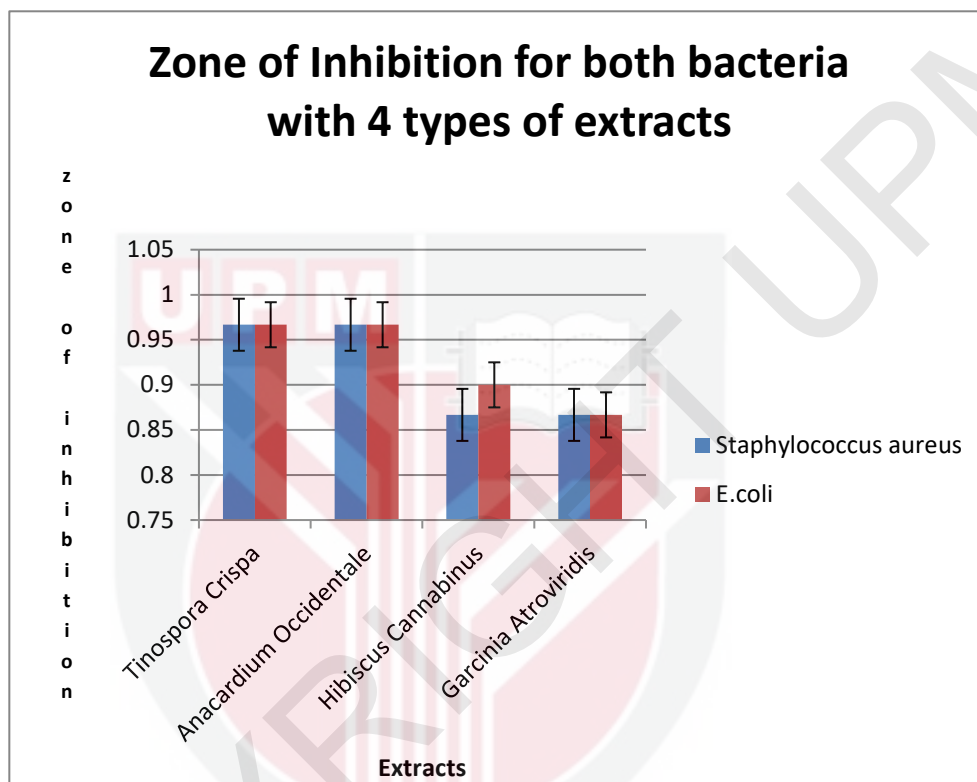


Figure 4.10: Zone of Inhibition for both bacteria with 4 types of extracts.

	<i>Tinospora Crispa</i>	<i>Anacardium Occidentale</i>	<i>Hibiscus Cannabinus</i>	<i>Garcinia Atroviridis</i>
<i>Staphylococcus aureus</i>	0.9667 ±0.05774	0.9667 ±0.05774	0.8667 ±0.05774	0.8667 ±0.05774
<i>E.coli</i>	0.9667 ±0.05774	0.9667 ±0.05774	0.9 ± 0	0.8667 ±0.05774

Table 4.2: Result of Statistical Analysis for Zone of Inhibition for both bacteria with 4 types of extracts.

From this graph and table, for both bacteria, the result of zone of inhibition is almost same. We can determine that *T.Crispa* and *A.Occidentale* is no significant to each other, same case for *H.Cannabinus* and *G.Atroviridis* for both bacteria.

	inhibition
Chi-Square	5.900
Df	3
Asymp. Sig.	0.117

a. Kruskal Wallis Test
 b. Grouping Variable:
 species

Table 4.3: Statistical Analysis using Kruskal Wallis Test for Zone of Inhibition for *E.coli* against 4 extracts of herbs.

These values indicate that zone of inhibition for *E.coli* do not significantly different across 4 extracts of herbs $X^2(3, N=12) = 5.900$ $P = 0.117$, $P > 0.05$.

	inhibition
Chi-Square	5.989

Df	3
Asymp. Sig.	0.112

a. Kruskal Wallis Test

b. Grouping Variable:
species

Table 4.4: Statistical Analysis using Kruskal Wallis Test for Zone of Inhibition for *Staphylococcus aureus* against 4 extracts of herbs.

These values indicate that zone of inhibition for *Staphylococcus aureus* do not significantly different across 4 extracts of herbs $X^2 (3, N=12) = 5.989 P = 0.112, P > 0.05$.



Figure 4.11 Disc diffusion of *Tinospora Crispa* against *Staphylococcus aureus* ATCC

25923

For *Tinospora Crispa* against *Staphylococcus aureus* ATCC 25923, the mean of 3 disc diffusion on the MHA is 9.67 and the standard deviation is 0.5774.



Figure 4.12 Disc diffusion of *Anacardium Occidentale* against *Staphylococcus aureus* ATCC 25923

For *Anacardium Occidentale* against *Staphylococcus aureus* ATCC 25923, the mean of 3 disc diffusion on the MHA is 9.67 and the standard deviation is 0.5774.

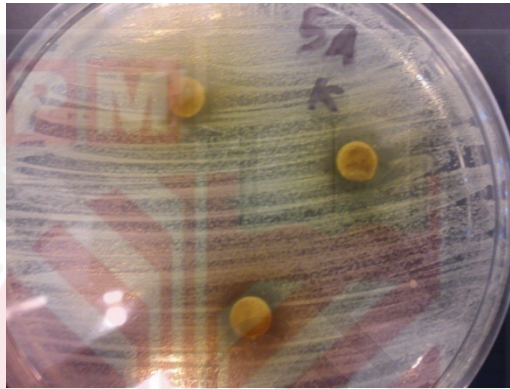


Figure 4.13 Disc diffusion of *Hibiscus Cannabinus* against *Staphylococcus aureus*
ATCC 25923

For *Hibiscus Cannabinus* against *Staphylococcus aureus* ATCC 25923, the mean of 3 disc diffusion on the MHA is 8.67 and the standard deviation is 0.5774.



Figure 4.14 Disc diffusion of *Garcinia Atroviridis* against *Staphylococcus aureus* ATCC 25923

For *Garcinia Atroviridis* against *Staphylococcus aureus* ATCC 25923, the mean of 3 disc diffusion on the MHA is 8.67 and the standard deviation is 0.5774.

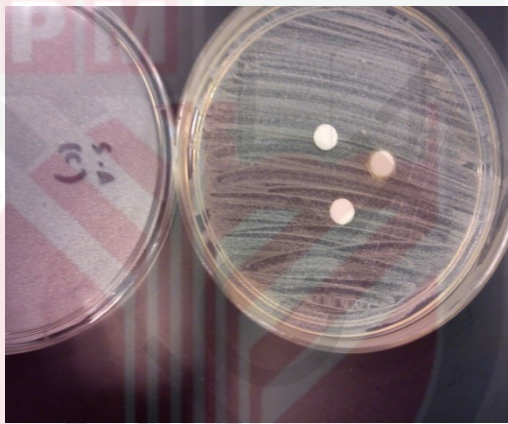


Figure 4.15 Disc diffusion of Control against *Staphylococcus aureus* ATCC 25923

For Control against *Staphylococcus aureus* ATCC 25923, the mean of 3 disc diffusion on the MHA is 5.00.

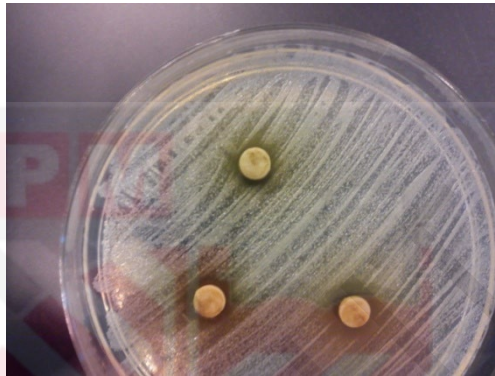


Figure 4.16 Disc diffusion of *Tinospora Crispa* against *Escherichia coli* ATCC 25922

For *Tinospora Crispa* against *Escherichia coli* ATCC 25922, the mean of 3 disc diffusion on the MHA is 9.67 and the standard deviation is 0.5774.



Figure 4.17 Disc diffusion of *Anacardium Occidentale* against *Escherichia coli* ATCC 25922

For *Anacardium Occidentale* against *Escherichia coli* ATCC 25922, the mean of 3 disc diffusion on the MHA is 9.67 and the standard deviation is 0.5774.

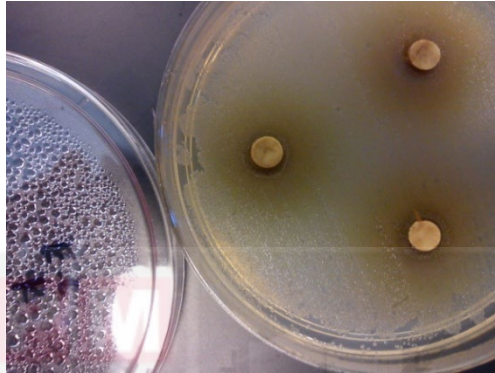


Figure 4.18 Disc diffusion of *Hibiscus Cannabinus* against *Escherichia coli* ATCC

25922

For *Hibiscus Cannabinus* against *Escherichia coli* ATCC 25922, the mean of 3 disc diffusion on the MHA is 9.0 and the standard deviation is 0.



Figure 4.19 Disc diffusion of *Garcinia Atroviridis* against *Escherichia coli* ATCC 25922

For *Garcinia Atroviridis* against *Escherichia coli* ATCC 25922, the mean of 3 disc diffusion on the MHA is 8.67 and the standard deviation is 0.05774.



Figure 4.20 Disc diffusion of Control against *Escherichia coli* ATCC 25922

For negative control against *Escherichia coli* ATCC 25922, the mean of 3 disc diffusion on the MHA is 5.00.

CHAPTER 5

DISCUSSION

There were several assessments of its activity against a wide collection of pathogens. Besides, as the experiment was done before, it is proved that all of these herbs have its medicinal value. For instance, *Tinospora Crispa* is known as oral hypoglycemic agent as was reported by Noor H *et al.*, (1989). *Anacardium Occidentale* methanol and aqueous extract also can be hypoglycemic agent (S.D. Sokeng *et al.*, 2007) where as the methanolic extract of *Hibiscus Cannabinus* leaf exhibited significant hypoglycemic activity in streptozotocin induced diabetic rats as was reported by T. Raj Kumar *et al.*, (2011). The marked inhibitory all of these extract herbs against selected pathogens Gram positive; *Staphylococcus aureus* ATCC 25923 and Gram negative bacteria; *Escherichia coli* ATCC 25922 was confirmed. The other *Garcinia Atroviridis* extract exhibits strong antioxidant, antitumour and anti-inflammatory activities (Mackeen *et al.*, 2000).

Kirby-Bauer disk diffusion and Minimal Inhibitory/Bactericidal Concentration method are two methods that are determined as Antibiotic Sensitivity testing. (Bauer *et al.*, 1996). Minimum inhibitory concentrations are important in diagnostic laboratories to

confirm resistance of microorganisms to an antimicrobial agent and also to control the activity of new antimicrobial agents (Andrews 2001). As the result of MIC is cannot directly determined by observation, we further the investigation use Minimal Bactericidal Concentration which we can identify the lowest concentration of herbs required to kill the bacteria which is not as commonly seen as the Minimum Inhibitory Concentration (MIC). From the result, The MIC and MBC of the respective extract for both tests selected pathogens Gram positive; *Staphylococcus aureus* ATCC 25923 and Gram negative bacteria; *Escherichia coli* ATCC 25922 were similar; 227.27 mg/mL for *Tinospora Crispa* and *Anacardium Occidentale*, 113.64 mg/ml for *Hibiscus Cannabinus*, and 56.82 mg/mL for *Garcinia Atroviridis*. This result show that the most effective extract is *Garcinia Atroviridis* due to its lowest concentration, followed by *Hibiscus Cannabinus*, and then *Tinospora Crispa* and *Anacardium Occidentale*.

For agar disc diffusion method, as compared to control against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923, which is the mean of 3 disc diffusion of negative control on the MHA is 5.00, all extracts showed narrow range of mean in inhibition zone $\sim(8.67-9.67)$ with standard deviation 0.5774 except for *H.Cannabinus* for *E.coli* is 0. From this graph figure 4.10 and table 4.2, we can determine that *T.Crispa* and *A.Occidentale* is no significant to each other, same case for *H.Cannabinus* and *G.Atroviridis* for both bacteria. For Kruskas Wallis Test, we can say

that the values indicate that zone of inhibition for *E.coli* do not significantly different across 4 extracts of herbs $X^2 (3, N=12) = 5.900$ $P = 0.117$, $P > 0.05$. Also, the values indicate that zone of inhibition for *Staphylococcus aureus* do not significantly different across 4 extracts of herbs $X^2 (3, N=12) = 5.989$ $P = 0.112$, $P > 0.05$. This result shows that although the extract's concentration used to soak the disc differed accordingly for each extract, all showed a similar size of inhibition zone.

The result of present study is show that all four extract show the antimicrobial properties similar as be done in previous study. Results of N. T. Al- alusi et al., (2010) stated that the *S. Mahagoni* and *T. Crispa* mixture has potential antibacterial activity against (MRSA). This result also collaborates to the report of Kudi (1999) that stated that the plant extract of *Anacardium Occidentale* had a good in vitro antibacterial activity against *E. coli* and also to Bicalho et al. (2001) who reported that the bark extract of *Anacardium occidentale* exhibited antibacterial activity in vitro against 13 to 15 microorganisms tested. Besides as antimicrobial agent, *Hibiscus Cannabinus* of leaf extract can also kill earthworms better than the Standard Drug, Albendazole. (Kota Chaitanya Sravanti et al., 2011). Next, the report showed that extracts of *G. atroviridis* exhibit strong antimicrobial, antioxidant and antitumour-promoting activities (Mackeen et al., 1997; Mackeen et al., 2000) which support of this result.

CHAPTER 6

CONCLUSION

As conclusion, we can prove that *Tinospora Crispa*, *Anacardium Occidentale*, *Garcinia Atroviridis* and *Hibiscus Cannabinus* has antimicrobial properties against Gram positive; *Staphylococcus aureus* ATCC 25923 and Gram negative bacteria; *Escherichia coli* ATCC 25922. All of these extracts have the potential to be developed as an alternative antimicrobial. We should prefer to all of these plants extracts as the antimicrobial properties as plant-based antimicrobials have enormous therapeutic potential as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials as documented by Iwu *et al.*, in 1999. Furthermore, the screening of several medicinal plants for their potential antimicrobial activity was been developed because of the increasing failure of chemotherapeutics and antibiotic resistance showed by pathogenic microbial infectious agents.

A few recommendations in improving this evaluation of *Tinospora Crispa*, *Anacardium Occidentale*, *Garcinia Atroviridis* and *Hibiscus Cannabinus* as

antimicrobial properties are stated here. For agar disc diffusion technique, we can improve the quality of the experiment by using positive control.



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APPENDIX A

Kruskal-Wallis Test

		Ranks	
	Species	N	Mean Rank
inhibition	<i>T. Crispa</i>	3	8.83
	<i>A. Occidentale</i>	3	8.83
	<i>H. Cannabinus</i>	3	4.17
	<i>G. Atroviridis</i>	3	4.17
	Total	12	

		Ranks	
	Species	N	Mean Rank
inhibition	<i>T. Crispa</i>	3	8.67
	<i>A. Occidentale</i>	3	8.67
	<i>H. Cannabinus</i>	3	5.00
	<i>G. Atroviridis</i>	3	3.67
	Total	12	

Case Processing Summary

Cases

Included		Excluded		Total	
N	Percent	N	Percent	N	Percent

inhibition * species	12	100.0%	0	.0%	12	100.0%
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Case Processing Summary

	Cases				Total	
	Included		Excluded			
	N	Percent	N	Percent		
inhibition * species	12	100.0%	0	.0%	12	100.0%

Report

inhibition species	Mean	N	Std. Deviation
<i>T. Crispa</i>	.9667	3	.05774
<i>A. Occidentale.</i>	.9667	3	.05774
<i>H. Cannabinus</i>	.9000	3	.00000
<i>G. Atroviridis</i>	.8667	3	.05774
Total	.9250	12	.06216

Report

inhibition

species	Mean	N	Std. Deviation
<i>T. Crispa</i>	.9667	3	.05774
<i>A. Occidentale</i>	.9667	3	.05774
<i>H. Cannabinus</i>	.8667	3	.05774
<i>G. Atroviridis</i>	.8667	3	.05774
Total	.9167	12	.07177

Zone of Inhibition by Triplicate

	<i>T. Crispa</i>	<i>A. Occidentale</i>	<i>H. Cannabinus</i>	<i>G. Atroviridis</i>
<i>S.aureus</i>	1.0,1.0,0.9	1.0,1.0,0.9	0.9,0.8,0.9	0.9,0.8,0.9
<i>E.coli</i>	1.0,1.0,0.9	1.0,1.0,0.9	0.9,0.9,0.9	0.9,0.8,0.9



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