3.0 METHODOLOGY

The purposes of this research are to determine the potential of *Ervatamia coronaria* and *Tinospora crispa* as antioxidant and antimicrobial as well as chemical investigation in these plants on treatment of sinusitis.

3.1 Experimental Material

Two species of plants samples i.e *Ervatamia coronaria* and *Tinospora crispa* were investigated in this study. *Ervatamia coronaria* was identified and collected at Selandar, Melaka whereas *Tinospora crispa* was collected at Jelebu, Negeri Sembilan. The plants were harvested at a specific time between 12 noon until 3 pm when photosynthesis activity is higher. Several plant parts were used (Table 3.1). The selected plants were ensured to be free from any diseases. The plants were washed and chopped into small pieces for efficient drying process. Each dried plant material was subsequently ground into fine powder.

Table 3.1: Plant materials for investigation

Plant species	Plant parts	Drying process
Ervatamia coronaria	Roots Stems Leaves	Air dried for 3-5 days followed by drying in the oven at 40ºC for 3 days
Tinospora crispa	Stems	Air dried for 2 days followed by drying in the oven at 40ºC for 3 days

3.2 Preparation of extracts

For the plants extraction, 30 g of plants samples powder were consecutively extracted using 4 solvents which are petroleum ether (b.p 40ºC - 60ºC), chloroform, methanol and finally distilled water.

Initially, 30 g of plant samples were defatted with 300 ml of petroleum ether in a conical flask. The mixtures were placed in the environmental shaker at room temperature, with the rotation speed of 220 rpm for 72 hours. After 72 hours, each mixture was filtered using Whatman filter paper. The filtered extracts were then evaporated to a concentrated crude extracts using a rotary evaporator at 40-45ºC. The concentrated crude extracts were then transferred into specimen tubes and wrapped with aluminium foil. The concentrated crude extracts were placed in desiccators and eventually kept at -20ºC until use.

The residues from the filtration procedure were then re-extracted with chloroform followed by methanol. The above procedures were repeated for both chloroform and methanol. The extraction procedure using distilled water as solvent was slightly different. After the concentrated crude extract was transferred into specimen bottle, it was freeze-dried.

The yield of crude extracts was calculated as follows:

Dried weight of plant sample = α gram

Empty specimen bottle = β gram

Specimen bottle + crude extract = γ gram

Weight of crude extract = (γ – β) gram

$$\text{Percentage of crude extract yield(gram)} = \frac{(\gamma - \beta)}{\alpha} \times 100\ \%$$

3.3 Screening for Phytochemical Components

Thin layer chromatography (TLC) is a chromatography technique used to separate mixtures. Thin layer chromatography is the cheapest and simplest method of detecting plant constituents.

3.3.1 Thin Layer Chromatography (TLC)

TLC silica gel 60 (Merck) was prepared. The plant extracts of *Ervatamia coronaria* (roots, stems and leaves) and *Tinospora crispa* (stems) were loaded separately on TLC plate as a band or spot using capillary tube. Before the spotting of crude extracts, a parallel line was drawn which served as an indicator for placement of sample and also as a reference point from which to measure the R_f values. This line which was drawn with pencil was about 1 cm from the bottom of the plate. Once the sample bands/spots have been placed, they were left to dry.

When sample bands/spots on the TLC plate were ready, the plate was placed in the chromatography tank filled with chloroform-methanol (9:1) solvent / dichloromethane solvent / acetone-chloroform (7:3) solvent. The solvent however was not allowed to touch the sample band/spot. The solvent advanced through the sample spots until the solvent front reached approximately 1 cm from the top of the plate. The developed chromatography was removed from the tank and dried in fume cupboard before being observed under visible light and UV-light (short-wave). The R_f value was calculated for each band/spot. After that, the developed chromatography was sprayed with chemical reagent such as dragendorff, vanillin-sulphuric acid, anysaldehyde-sulphuric acid or iodine vapour (The procedure to prepare the reagents are as detailed in Appendix A). The R_f value for each band/spot which appeared after sprayed with chemical reagent was observed again under visible light and UV-light. The R_f value for each band/spot was calculated.

3.3.2 Determining the R_f Value

The distance that a compound travels in a specific chromatography system, compared to how far the solvent has traveled is constant. The relationship between the distance traveled by the solvent front and the substance is usually expressed as the R_f value (Figure 3.1).

$$R_f \text{ value} = \frac{\text{Distance traveled by substance (B)}}{\text{Distance traveled by solvent front (A)}}$$

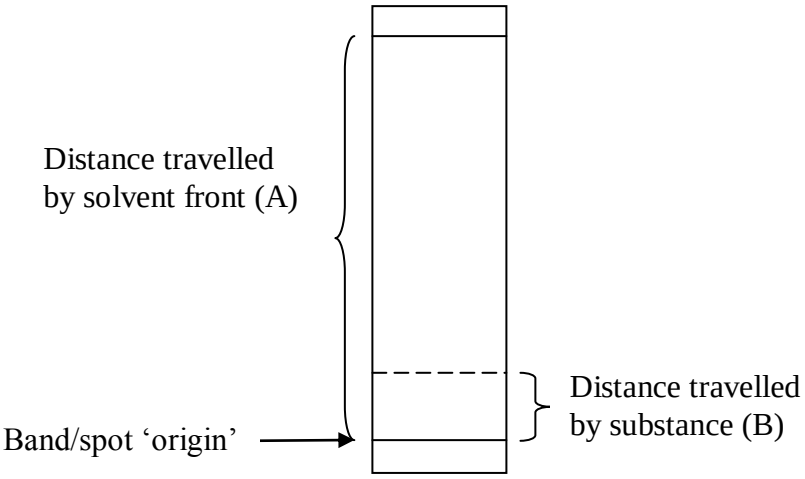

Figure 3.1: TLC plate showing distances traveled by the band/spot and the solvent after solvent front nearly reached the top of the TLC plate.

3.4 Total Phenolic Content

The Total Phenolic Content was measured using Folin-Ciocalteu method as described by Amin *et al*. (2004). The purpose of this assay is to calculate the content of phenolic compound in the sample based on the calibration curve of the standard.

3.4.1 Gallic acid as a Positive Reference Standard

A 0.05 mg/ml stock standard solution of gallic acid was prepared by dissolving gallic acid in distilled water. Working standards ranging from 0.01 mg/ml to 0.05 mg/ml were prepared by diluting the stock solution with distilled water. A 100 µl of gallic acid standard at different concentrations were transferred into test tube followed by 0.75 ml of Folin-Ciocalteu reagent (previously diluted 10 fold with distilled water) and the mixtures

were mixed. The mixtures were allowed to stand at room temperature for 5 minutes. After that, 0.75 ml of 6 % (w/v) sodium carbonate were added and mixed gently. After standing at room temperature for 90 minutes, the absorbance was read at 725 nm using UV/Vis spectrophotometer (Shimadzu UV-1700). Distilled water was used as blank. All tests were carried out in triplicates and the readings were averaged. The linear standard calibration curve of gallic acid at different concentrations was plotted to get the r^2 value and linear equation of gallic acid (GAE) using Microsoft Excel 2007.

3.4.2 Total Phenolic Content of Crude Extracts

The phenolic contents of 1 mg/ml each crude petroleum ether, chloroform, methanol and water extracts were evaluated using Total Phenolic Content assay as described in 3.4.1. All tests were carried out in triplicates and the readings were averaged. The Total Phenolic Content of each extract was measured using the gallic acid equation (GAE) based on calibration curve of the standard.

3.4.3 Statistical analysis

Experimental results were mean ± standard deviation (SD) of three parallel measurements. Statistical analysis was performed using SPSS 16.0 software. Significant differences between samples were analyzed using analysis of variance (ANOVA) with Banferonni Adjustment. Pearson's correlation was used to determine the correlation of data among DPPH free radical-scavenging activity (%), reducing power assay, metal chelating

assay, haemolysate catalytic assay and total phenolic content. The values for $P < 0.05$ were regarded as significant and $P < 0.01$ as highly significant.

3.5 Antioxidant Activity Assays

The antioxidant activity of each crude extracts from the selected *Ervatamia coronaria* and *Tinospora crispa* were evaluated using four different assays:

a) 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) Free Radical Scavenging Activity Assay
b) Reducing Power Assay
c) Metal Chelating Assay
d) Haemolysate Catalytic Assay

3.5.1 DPPH Free Radical Scavenging Activity Assay

The DPPH free radical scavenging activity was determined using the method of Blois (1958) with slight modification. The purpose of this assay is to evaluate the scavenging potential of *Ervatamia coronaria* and *Tinospora crispa* extracts against the stable 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical.

3.5.1 (i) Ascorbic Acid as a Positive References Standard

Ascorbic acid of different concentrations were used as the positive standard in the DPPH free radical scavenging assay. A stock solution of 1 mg/ml ascorbic acid and a stock of DPPH (Sigma) in methanol were prepared at concentration of 1mM. The reaction was initiated by the addition of DPPH radical to various concentrations of ascorbic acid. Reaction mixtures of ascorbic acid; DPPH and methanol were prepared based on Table 3.2.

Table 3.2: Reaction mixtures for the DPPH radical scavenging assay.

Concentration of Ascorbic Acid (mg/ml)	Volume of Methanol (µl)	Volume of Ascorbic Acid from stock (µl)	Volume of DPPH (µ)
0.0025	3990	10	500
0.0125	3950	50	500
0.0375	3850	150	500
0.125	3500	500	500
0.25	3000	1000	500
0.5	2000	2000	500
Control	4000	-	500

The reaction mixtures were incubated at room temperature in a dark room for 30 minutes to allow reactions to proceed. The absorbance was measured

spectrophotometrically at 517 nm with methanol as a blank. The DPPH without addition of ascorbic acid served as negative control. The degree of discoloration indicates the free radical scavenging efficiency of ascorbic acid. All tests were carried out in triplicates and the readings were averaged. The percentages at all ascorbic acid concentrations of inhibition were calculated. The dose-response graph was plotted to determine IC_{50} value.

3.5.1 (ii) DPPH Radical Scavenging Activity of Crude Extracts

Crude extracts at different concentrations (0.0025 mg/ml, 0.0125 mg/ml, 0.0375 mg/ml, 0.125 mg/ml, 0.25 mg/ml and 0.5 mg/ml) were tested for DPPH radical scavenging activity. Crude petroleum ether, chloroform and methanol extracts were dissolved in methanol while crude water extract was dissolved in distilled water. The similar procedure as the positive reference standard above was applied. All tests were carried out in triplicates and the readings were averaged. The percentage of inhibition was calculated. The graph was plotted to determine IC_{50} value.

3.5.1 (iii) Determination of Percentage of Inhibition

The percentage of inhibition of DPPH was calculated using the formula given below:

$$\left[\textit{Percentage of inhibition (\%)} = \frac{OD_{control} - OD_{sample}}{OD_{control}} X\ 100 \right]$$

Where:

$OD_{control}$ = absorbance of the control
OD_{sample} = absorbance in the presence of the samples of crude extract
The control only contains methanol and DPPH without ascorbic acid/crude extract

3.5.2 Metal Chelating Assay

The purpose of metal chelating assay is to evaluate the ability of *Ervatamia coronaria* and *Tinospora crispa* extracts to chelate ferrous ion and prevent the formation of ferrozine-Fe^{2+} complex. The chelate on ferrous ions by plant extracts was determined by the method of Dinis *et al*. (1994).

3.5.2 (i) EDTA as Positive Reference Standard

EDTA (ethylenediaminetetraacetic acid) (Sigma) was used as positive reference standard in this assay. EDTA stock of 1 mg/ml was prepared by dissolving 0.01 g of EDTA in 4 ml deionized water. The pH was adjusted while stirring with NaOH solution until most of the EDTA has dissolved. Once the EDTA has dissolved, deionized water was added to make a total volume of 10 ml. The EDTA solution was further diluted to yield solutions of the various concentrations of 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 mg/ml and control solution (without EDTA) before added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml). The mixture was shaken vigorously and left standing at room temperature for 10 minutes. The optical density (OD) or absorbance of resulting solution was then measured at 562 nm. Deionized water was used as blank. All tests were carried out in triplicates and the readings were averaged. Percentages of inhibition of ferrozine-Fe^{2+} complex by EDTA at all concentrations tested were calculated as stated in 3.5.2(iii).

3.5.2 (ii) Metal Chelating for Crude Extracts

Crude extracts of *Ervatamia coronaria* and *Tinospora crispa* at different concentrations of 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 mg/ml were tested for metal chelating activity using the protocol described in 3.5.2 (i). The said concentrations were prepared by diluting the crude extract stock solutions. Crude extract stock of 1 mg/ml was prepared earlier by dissolving 0.01 g of crude extract in 10 ml methanol. The control consisted of reaction mixtures without the addition of plant crude extracts. All tests were carried out in triplicates and the readings were averaged. Percentages of inhibition of ferrozine-Fe^{2+} complex by all plant crude extracts at all concentrations tested were calculated.

3.5.2 (iii) Determination of Percentage of Inhibition

The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the formula given below:

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

Where :

$OD_{control}$ = absorbance of the control
OD_{sample} = absorbance in the presence of the samples of crude extract
The control only contains $FeCl_2$ and ferrozine without EDTA/crude extract

3.5.3 Reducing Power Assay

This assay is used to evaluate the ability of *Ervatamia coronaria* and *Tinospora crispa* extracts to reduce Fe^{3}+ to Fe^{2}+. The reducing power assay was determined according to a method by Oyaizu (1986) with slight modification.

3.5.3 (i) BHA as Positive Reference Standard

Butylated hydroxyanisole (BHA) was used as positive reference standard. BHA of different weights (1 mg, 0.5 mg, 0.25 mg, 0.125 mg and 0.0625 mg) was dissolved in 1.0 ml methanol and vortexed to mix. 1.0 ml of various concentrations of BHA prepared were then added with 2.5 ml of 2.0 M phosphate buffer (pH 6.6) and later with 2.5 ml of 1% (w/v) potassium ferricyanide (Sigma) in centrifuge tubes. The mixtures were incubated in water bath at 50ºC for 20 minutes.

Following incubations, 2.5 ml of 10% trichloroacetic acid (TCA) (Sigma) solution was added to each mixture and centrifuged at 1000 rpm for approximately 10 minutes. A 2.5 ml aliquot of the upper layer from the centrifuged solution was transferred into test tubes and added with 2.5 ml of distilled water and 0.5 ml of 0.1% (w/v) ferric chloride solution. The colour changes were observed. The mixtures were then transferred into cuvettes. Optical density (OD) or absorbance of the reaction mixtures were recorded using spectrophotometer (Shimadzu UV-1700) at 700 nm after 30 minutes incubation.

3.5.3 (ii) Reducing Power of Crude Extracts

Crude extracts of *Ervatamia coronaria* and *Tinospora crispa* at different concentrations (1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, and 0.0625 mg/ml) were tested for reducing power activity using the protocol described in 3.5.3 (i). All tests were carried out in triplicates and the readings were averaged. Mean values and standard deviation were calculated. Methanol was used as blank.

Table 3.3: Reaction mixtures for reducing power assay.

Concentration of crude extracts (mg/ml)	Phosphate Buffer 0.2M (mg/ml)	Potassium Ferricyanide (mg/ml)	Ferric Chloride (mg/ml)
1.0	2.5	2.5	2.5
0.5	2.5	2.5	2.5
0.25	2.5	2.5	2.5
0.125	2.5	2.5	2.5
0.0625	2.5	2.5	2.5

3.5.4 Haemolysate Catalytic Assay

The Haemolysate Catalytic Assay was determined using the method by Yusof (2006). The purpose of this assay is to assess the ability of the extracts to reduce hydrogen peroxide (H_2O_2) in synergism with haemolysate catalase. In the presence of H_2O_2 reductant which are catalase and plant extracts, the H_2O_2 molecule will be reduced into molecule oxygen and water. Decreased in the absorbance of H_2O_2 molecules at wavelength 240 nm is proportional to the degree of reduction of H_2O_2 molecules. Thus, the higher the reduction of H_2O_2, the lower the absorbance.

(a) Haemolysate Preparation

Human blood from antecubital vein was drawn into EDTA-vacutainer tube and stored at 4 ºC. The erythrocyte and supernatant were mixed by gently inverting the tube in up and down motion. The erythrocytes were hemolysed using sodium phosphate buffer pH 7.4 and the hemolysate was kept in ice.

(b) Stock Solutions Preparation

Stock solutions of 200 µg/ml of crude extracts and 1M H_2O_2 solution were prepared. Stock solutions of crude extracts were prepared by dissolving the crude petroleum ether, chloroform, methanol and water extracts of plant samples in sodium phosphate buffer pH 7.4. The plant extracts which have low solubility and cannot dissolve in the buffer were first added with a few drops of dimethyl sulfoxide (DMSO) before completely dissolved in

buffer. A 1M H_2O_2 solution was prepared by adding 35 % 11 M H_2O_2 with distilled water and kept in ice.

3.5.4 (i) Determination of the Haemoglobin Concentration in Haemolysate

A total of 1 ml of haemolysate stock was pipetted into the cuvette. The absorbance of haemolysate stock was measured using UV-Vis spectrophotometer (CARY 100 Bio) at 577 nm and the concentration of haemoglobin (Hb) was calculated using the extinction coefficient of 14.6 M^{-1} cm^{-1} for Hb according to the Beer-Lambert's Law. The total weight of haemoglobin in the haemolysate stock was determined from the concentration obtained by using molecular weight of 616.49 amu for haemoglobin (Silberberg, 2006). The general Beer-Lambert law is written as:

$$A = \varepsilon\, c l$$

Where: A = absorbance

ε = extinction coefficient

c = concentration of the substance

l = path length of cuvette (cm)

3.5.4 (ii) Determination of Standard Reduction of H_2O_2 by Catalase in Haemolysate

The consumption of H_2O_2 was measured using the UV-Vis spectrophotometer (CARY 100 Bio) at 240 nm. The exponential reduction of H_2O_2 was observed during the period of 1.5 minutes of reaction. The activity of the haemolysate catalase alone in the

reduction of H_2O_2 was used as positive reference standard in this assay. Sodium azide (1mM), the inhibitor of catalase was used to show the reducing activity of catalase H_2O_2. Reaction mixture containing the haemolysate and H_2O_2 were prepared as listed in Table 3.4. All tests were repeated in triplicates for accuracy.

3.5.4 (iii) Activity Measurement of Plant Extracts

The exponential reduction of H_2O_2 by different extracts in synergism with haemolysate catalase was observed and measured at 240 nm after the period of 1.5 minutes of reaction. The enhancement percentage of reducing activity was calculated using the formula given below:

$$\text{Enhancement percentage of reducing activity} = \left\{ \frac{\text{Abs (standard)} - \text{Abs (extract)}}{\text{Abs (standard)}} \times 100 \right\}$$

Where the standard is the haemolysate catalase alone without crude extract

All crude extracts were tested for their reducing activities at varying concentrations of 50 µg/ml, 100 µg/ml and 200 µg/ml. Reaction mixture containing crude extracts, haemolysate and H_2O_2 were prepared as listed in Table 3.4. All experiments were repeated in triplicates for accuracy.

Table 3.4: Reaction mixtures for haemolysate catalytic assay

Concentration of crude extracts (µg/ml)	Volume of haemolysate stock (µl)	Volume of H_2O_2 solution (µl)	Volume of crude extract from stock (µl)
50	3000	75	7.5
100	3000	75	15
200	3000	75	30
Standard	3000	-	75

3.5.5 Statistical Analysis

Experimental results were presented as mean ± standard deviation (SD) of three parallel measurements. Statistical analysis was performed using SPSS software.

3.6 Antimicrobial Activity Assays

The purpose of this assay is to evaluate antimicrobial activity of *Ervatamia coronaria* and *Tinospora crispa* extracts against sinusitis-causing microorganisms.

3.6.1 Test Organisms

The antimicrobial screening was carried out against 9 bacteria and 1 fungus as test microorganisms. Some microorganisms were obtained from Microbiology Lab of Hospital Ampang, Pandan Indah while some were obtained from Department of Microbiology, Faculty Science, University Malaya. All the microorganisms were sinusitis-causing microorganisms (Table 3.5).

Table 3.5: Test microorganisms for antimicrobial activity study on the investigated plants

Group	Species	Location
Gram-positive bacteria	*Staphylococcus aureus* *Streptococcus faecalis* *Streptococcus pneumoniae (ATCC49619)*	Microbiology Lab, Hospital Ampang, Pandan Indah, Kuala Lumpur
Gram-negative bacteria	*Escherichia coli* *Pseudomonas aeruginosa* *Klebsiella pneumoniae* *Proteus mirabilis* *Haemophilus influenza (ATCC49247)* *Moraxella catarrhalis (ATCC23296)*	Department of Microbiology, Faculty Science, University Malaya Microbiology Lab, Hospital Ampang, Pandan Indah, Kuala Lumpur
Fungus	*Candida albicans (ATCC10281)*	

3.6.2 Media for Microbial Cultivation and Maintenance

Eight types of media (Nutrient Agar, Nutrient Broth, Mueller-Hinton Agar, Mueller Hinton Broth, Sabouraud Dextrose Agar, Sabouraud Dextrose Broth, Tryptone Soy Agar, and Tryptone Soy Broth) were prepared in accordance with the manufacturers' prescription and specification. Prepared media were sterilized using autoclave at 121° C for 15 minutes. Sterile fresh and warm agar media were poured into plastic, flat-bottomed petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. Alternatively, the media was left to solidify at a slanted position in "Universal" bottles. Freshly prepared plates or slants were used the same day or stored in the refrigerator at 2°C to 8°C for up to 2 weeks.

If plates were not used within 7 days of preparation, they were wrapped in plastic to minimize evaporation. Broth and liquid media were distributed into final containers before autoclaving. While 6 types of culture media (Mueller-Hinton+5% Defibrinated Blood Sheep Agar, Mueller-Hinton+5% Defibrinated Blood Horse Agar, Haemophilus Test

Medium, Chocolate Agar, Blood Agar, and MacConkey Agar) were bought ready-made from biomedia (BML) (Table 3.6)

Table 3.6: Culture media for microbial growth

Medium	**Purpose**
Nutrient Agar (Difco)	General cultivation and maintenance of many species of nonfastidious organisms
Nutrient Broth (Difco)	Cultivation of many species of nonfastidious microorganisms.
Mueller-Hinton Agar (Difco)	Susceptibility testing of non-fastidious bacteria
Mueller-Hinton Broth (Difco)	General purpose medium that may be used in the cultivation of a wide variety of fastidious and nonfastidious microorganisms.
Sabouraud Dextrose Agar (Difco)	General-purpose medium used in microbiology and mycology labs for the isolation, growth, and maintenance of fungi
Sabouraud Dextrose Broth (Difco)	Cultivation of fungi
Mueller-Hinton + 5% Defibrinated Blood Sheep Agar	Susceptibility testing of *Streptococcus pneumoniae*
Mueller-Hinton + 5% Defibrinated Horse Blood Agar	Susceptibility testing of *Moraxella Catarrhalis*
Haemophilus Test Medium	Susceptibility testing of *Haemophilus* sp
Chocolate Agar	Growing fastidious respiratory bacteria, such as *Haemophilus influenzae*
Blood Agar	Culture medium consisting of blood (usually sheep's blood) and nutrient agar, used in bacteriology to cultivate certain microorganisms, including *Streptococcus pneumoniae.*
Mac Conkey Agar	Culture medium designed to grow Gram-negative bacteria
Tryptone Soy Agar (Difco)	General purpose agar which will support the growth of a wide range of micro-organisms.
Tryptone Soy Broth (Difco)	Cultivation of a wide range of microorganisms.

3.6.3 Paper Disc Diffusion Method

3.6.3 (i) Inoculum Preparation - Direct Colony Suspension Method

Microorganisms were standardized according to a method by National Committee for Clinical Laboratory Standard (NCCLS, 2000). The inoculums were prepared by making a direct normal saline suspension of microorganism selected from 18 to 24 hour nutrient agar or mac conkey agar plate for non-fastidious organism/ chocolate agar or blood agar for fastidious microorganism and sabouraud dextrose agar for fungus. The suspensions were adjusted to match the 0.5 McFarland turbidity standard using saline and vortex mixer. This was done by comparing the tubes (suspension tube and McFarland tube) against a sheet of white paper on which sharp black lines were drawn (Figure 3.2). If the microorganism suspension did not appear to be the same density as the McFarland 0.5, the turbidity were reduced by adding sterile saline or increased by adding more microorganism growth.

Figure 3.2: Comparison of McFarland 0.5 with inoculum suspension. From left to right, the tubes are the McFarland 0.5 standard and bacterial suspensions

3.6.3 (ii) Inoculation of Test Plates

Within 15 minutes after adjusting the turbidity of the inoculums suspension, 50 µl standardized suspension of the organisms were used to seed each sterile agar media depending on the microorganisms to be tested (Table 3.7) using cotton swab by streaking the swab over the entire sterile agar surface for three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of inoculums. Finally, the edge of the agar surface was swabbed around.

Table 3.7: Medium for inoculation

Organisms	**Medium**
Non-fastidious bacteria:-	
a. Staphylococcus aureus *b. Streptococcus faecalis* *c. Escherichia coli* *d. Pseudomonas aeruginosa* *e. Klebsiella pneumoniae* *f. Proteus mirabilis*	Mueller-Hinton Agar
Fastidious bacteria:-	
a. Haemophilus influenzae	Haemophilus Test Medium (HTM)
b. Moraxella catarrhalis	Mueller-Hinton Agar+ 5% defibrinated horse blood
c. *Streptococcus pneumoniae*	Mueller-Hinton Agar+ 5% defibrinated sheep blood
Fungus	
Candida albicans	Sabaouraud Dextrose Agar

3.6.3 (iv) Application of Discs to Inoculated Agar Plates

A double dilution of each of the extracts was prepared to obtain the following concentrations; 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml and 3.12 mg/ml. 60 µl of

each concentration of the extracts was pipetted onto each sterile filter-paper disc (Whatman No. 1, 6 mm in diameter) and the discs were dried after each 10 µl application to evaporate the solvents [Figure 3.3]. The discs were transferred onto the surface of solidified agar. Each disc was pressed down to ensure complete contact with the agar surface and not relocated once it has come into contact with agar surface. Penicillin, chloramphenicol, imipinem and Chlorhexidine were included as positive control while filter-paper discs saturated with solvent were used as negative controls. The plates were then incubated at 37 ^{o}C in air for 16-20 hours with exception to fastidious organisms such as *Haemophilus influenzae, Moraxella catarrhalis and Streptococcus pneumoniae* which needed incubation in 4-6% CO_2. Each extract and control was employed in triplicates for each organism. Diameter of clear zone or inhibition zone diameter (IZD) produced around the discs were measured after the incubation time using ruler or sliding caliper.

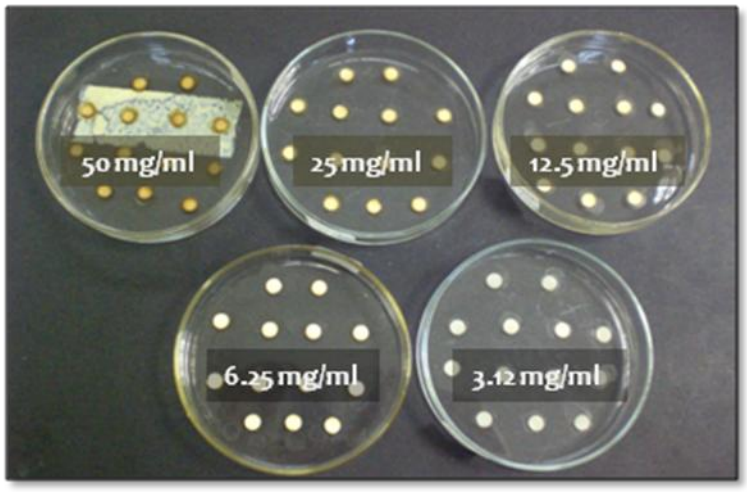

Figure 3.3: Five different concentration of disc impregnated with extract

3.6.4 Minimum Inhibition Concentration (MIC)

MIC is the lowest concentration of antimicrobial agent that inhibits microorganism growth. The MIC for the organisms were determined which is the lowest concentration at which no visible growth was observed in Inhibition Zone Diameter (IZD).

3.6.5 Minimum Bactericidal Concentration (MBC) / Minimum Fungicidal Concentration (MFC)

The MBC is the lowest concentration of the extracts at which there is no bacterial growth or the lowest concentration of the extract that can kill (cidal) the bacteria.

The MBC of the extracts were determined using the broth dilution method and eventual plating. The MBC is used to check the bactericidal effect of the plant extract on the bacteria. MBC was determined using the method by Waterworth (1978) with slight mofidications.

In broth dilution method, the nutrient broth was used and 5 ml of the broth media was introduced into different test tubes. 50µl of standardized suspension of the organisms (0.5 McFarland) were used to seed the broth. Now 100µl of each concentration of the extracts, which showed no visible growth during the determination of the MIC, were pipetted into the corresponding tubes. This was allowed to stand for 30 minutes before incubation at 37 ºC for 16-20 hours. After this a loopful of the culture was collected from the test tubes and streaked on nutrient agar plates. This was followed by incubation for 16-20 hours at 37 ºC. The MBC for the organisms were determined which is the lowest

concentration of the extracts that showed no bacterial growth. The same procedure of MBC was utilized for MFC.

3.7 Liquid Chromatography Mass Spectrometry (LCMS)

3.7.1 Preparation of Samples

Only methanol extracts for each sample were analyzed for Liquid Chromatography –Mass Spectrometry (LCMS). 1 ml concentrated sample of methanol extracts were diluted 5 times with methanol and filtered with 0.2 µM nylon filter prior to being analyzed.

3.7.2 Liquid Chromatography Mass Spectrometry (LCMS) System

After being filtered, 10µl samples were injected into Phenomenex Aqua C18 (50mm x 2.0mm x 5µM). The LCMS/MS of full scan with MS/MS data collection was applied. Biosystems 3200Q hybrid ion trap LCMS/MS coupled with ionization technique of athmospheric pressure electrospray ionization (APESI) where full scan mode of rapid screening at 15 min run time of positive and negative mode was used for the analysis. The following chromatographic conditions were applied: gradient system starting with 10% (Water with 0.1% formic acid and 5 mM ammonium formate) to 90% (Acetonitrile with 0.1% formic acid and 5 mM ammonium formate) from 0.01 min to 8.0 min with 0.25 ml/min flow rate and hold for 3 min with 0.4 ml/min flow rate then back to 10% A in 1.0 min and re-equilibrated for 4 min with 0.25 ml/min flow rate. The pre-run equilibration time was 1.0 min.